Ketosis Suppression and Ageing (KetoSAge): Suppression of ketosis negatively affects multiple biomarkers associated with chronic diseases and ageing

Cooper, Isabella

https://doi.org/10.34737/wzvw8

Ketosis Suppression and Ageing (KetoSAge): Suppression of ketosis negatively affects multiple biomarkers associated with chronic diseases and ageing

Isabella D. Cooper

A thesis submitted in partial fulfilment of the requirements of the University of Westminster for the degree of Doctor of Philosophy

Discipline: Pathophysiology, Molecular Biology and Biochemistry

Supervisors for the project:

Dr Bradley T. Elliott, Dr Sigrun Lange and Dr Stephen Getting

February 2025

Abstract

Metabolic dysfunctions are among the best documented hallmarks of ageing. Ageing is typically understood in its chronological context, as the length of time that has passed since a person's birth, whereas biological age is the measure of functional age, often measured in terms of physical and mental performance, as well as morbidities that decrease quality of life and youth-span. Cardiovascular disease, Alzheimer's disease, cancer, type 2 diabetes mellitus, metabolic-dysfunction-associated steatosis liver disease, and fragility fractures are diseases of hyperinsulinaemia that reduce life and healthspan. Hyperinsulinaemia pathologies present with increases in loss of function at all levels, from: the cellular, tissue, organ and ultimately systemic. Sustained ketosis with euglycaemia is a measurable metabolic phenotype to indicate absence of hyperinsulinaemia. This thesis investigates the metabolic phenotype of long-term sustained ketosis (euketonaemia) in healthy females with no prior metabolic diseases. Assessing their baseline biochemical profile (metabolic phenotype) and changes in biomarkers associated with ageing and age associated diseases after suppressing ketosis (SuK) for 21 days, followed by a further investigation after removing the intervention of suppressing ketosis, with the participants reverting back to their habitual lifestyle of being in a state of ketosis for 21 days, to further ascertain if the changes seen after the ketosis suppression phase, were likely due to the intervention. Over 50 biomarkers were tested, suppression of ketosis was found to negatively affect a host of markers most strongly associated with ageing and declining metabolic health. Strategies that can prevent and/or reduce hyperinsulinaemia may therefore improve cellular and vascular health, leading to decreased risk of developing chronic diseases that reduce quality of life, healthspan and lifespan.

Acknowledgements

It is said that it takes 10,000 hours to become a master at anything. My brother and I sat down and calculated the number of hours I spent during my undergraduate years studying everything I could about insulin, mitochondria and diseases of ageing. Suffice to say, even after 10,000 hours completed, I recognised and still see that we probably only know about 1%, and I myself 1% of that 1%, just enough to warrant earning this PhD. I would like to humbly thank the wonderful woman who dedicated themselves as participants, that allowed this research project to even exist. Their sacrifices, dedication and tenacity were magnificent and inspiring. I would like to thank my supervisor Dr Bradley T. Elliott, who put up with my absolute insistence to do what I believe needed to be done and challenged me to justify my points. I cannot thank enough the friendship, support and research assistance from Dr Yvoni Kyriakidou and Sandra Jacome. They made each day in the lab fun and time pass fast. Many thanks to Dr Paul Curley for his positive enthusiasm and encouraging comments on the progression of this research, and to Drs Sigrun Lange and Steve Getting for their super-fast and extremely helpful comments on the written works in this thesis. My biggest thanks to my parents James and Oi-Chun Cooper, my sister Elizabeth and brother Martin, and finally to Mathieu and my daughters Naja and Zarya, they have all endured and supported my obsession to complete this research, and their love is what has kept me going, I have lived with the security of knowing that they love me even when I fail, which gives me the strength to always keep trying, I am in debt to them.

Table of contents

Abbreviations

Acetoacetate (AcAc)
Acetyl-Co-enzyme-A (ACoA)
Acyl-Co-enzyme A (acyl-CoA)
Acetyl-CoA carboxylase (ACC)
Adenosine monophosphate (AMP)
Adenosine monophosphate (AMP) activated protein kinase (AMPK)
Adenosine-triphosphate (ATP)
Advanced glycation end products (AGES)
Ak mouse strain thymoma (AKT-1)/protein kinase B (PKB)
All-cause dementia (ACD)
Alzheimer's disease (AD)
Antioxidant responsive element (ARE)
Apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC)

Beta-hydroxybutyrate (BHB)
Bioelectrical impedance (BIA)
Biological ageing (BA)
Blood brain barrier (BBB)
Body Mass Index (BMI)
Brain derived neurotrophic factor (BDNF)

Calorie restriction (CR)
Carbon dioxide (CO_2)
Carboxylated osteocalcin (cOCN)
Cardiolipin (CL)
Cardiovascular disease (CVD)
Carnitine palmitoyltransferase-1 (CPT-1)
Confidence interval (CI)
Coronary artery calcification (CAC)
Coronavirus-19 (COVID-19)
Co-enzyme A (CoA)
C-reactive protein (CRP)
Cytochrome C oxidase (CcO)

Dauer formation-2 (*daf-2*)
DAF2(insulin)/IGF-1 (DAF2-IIS)
Diacylglycerol (DAG)
Disseminated intravascular coagulopathy (DIC)
Deoxyribonucleic acid (DNA)
DNA methyl transferase 1 (DNMT1),
Dynamin related protein-1 (Drp1)

Electron transport chain (ETC)
Endothelial progenitor cells (EPC)

Enzyme linked immunosorbent assay (ELISA)
Epidermal growth factor (EGF)
Epidermal growth factor receptor (EGFR)
ERK kinase (MEK)
Ethylenediaminetetraacetic acid (EDTA)
Extracellular-signal-regulated kinase (ERK)

Farnesyltransferase (FTase),
Fasting mimicking diets (FMD)
Fatty acid (FA)
Fatty acid ester of hydroxy fatty acids (FAHFA)
Forkhead transcription factors of the O class (FOXO)
FOXO transcription factors (TF)
Free radical theory of ageing (FRTA)
Free triiodothyronine (fT3)

Gamma-glutamyl transferase (GGT)
Gastroesophageal reflux disease (GERD)
GEF/SOS-Ras-Raf-Mitogen Activated Protein (MAP) Kinase (MAPK)
Glucagon like peptide-1 (GLP-1)
glucagon like peptide-1 receptor (GLP-1R)
Glucose ketone index (GKI)
Glucose stimulated insulin secretion (GSIS)
Glucose uptake transporters (GLUT)
Glutathione - reduced form (GSH)
Glutathione – oxidised form (GSSH)
Glutathione peroxidase (GPx)
G-protein coupled receptor (GPR109A)
Growth hormone-inhibiting hormone (GHIH)
Guanine nucleotide exchange factors (GEF)

Hepatocyte nuclear factor 4 (HNF4)
Heparan sulfate (HS)
Heparan sulphate–degrading endoglycosidase heparanase-1 (HPR1)
Heparan sulphate proteoglycans (HSPG)
High density lipoprotein (HDL)
HIF prolyl hydroxylase (PHD)
HIF-transcription factors (HIF-TF)
Histone acetyl-transferase 1 (HAT1)
Histone 2B (H2B)
Histone deacetylase (HDAC)
Homeostatic Model Assessment for Insulin Resistance (HOMA)
Hormone sensitive lipase (HSL)
Hydrogen peroxide (H_2O_2)
Hydroxyapatite (HA)
Hydroxycarboxylic acid receptor 2 (HCAR2) or HCAR2/GPR109A
Hydroxyls (^{-}OH)

3-hydroxy-3-methylgluytaryl-COA (HMG-CoA)
Hyperinsulinemia/IGF1 (H-IIS)
Hypoxia inducible factor-1a (HIF-1a)
Hypoxia inducible factor-1a (HIF-1b)

IGF1-binding proteins (IGF1BP)
Ileo-colonic (L)
Inhibitory protein inhibitor of KB (IkB)
Inner mitochondrial membrane (IMM)
Insulin receptor (InR)
Insulin/insulin-like growth factor-1 receptor signalling (IIS)
Insulin-like growth factor-1 (IGF-1)
Insulin-like growth factor binding protein 3 (IGFBP-3)
Insulin resistance (IR)
Insulin receptor substrate (IRS) ½ (IRS1/2)
1,3,5-inositol triphosphate (IP3)
Interferon-gamma (INF-**γ**)
Interleukin (IL)
Interleukin-1β (IL-1b)
Interleukin-6 (IL6)
Interleukin-18 (IL-18)
Intermittent fasting (IF)
Iodothyronine deiodinase enzymes (DIO)
Ischemic heart disease (IHD)
Isocitrate dehydrogenase (IDH2)

Ketogenic metabolic therapy (KMT)

Lactic acid dehydrogenase (LDH)
Lipoprotein receptor-related protein 1 (LRP1)
Lithium heparin (LH)
Low carbohydrate healthy fat (LCHF)
Low density lipoprotein (LDL)

Manganese superoxide dismutase (MnSOD2),
Mechanistic target of rapamycin pathway (mTOR)
Medium chain triglycerides (MCT)
Melatonin receptor 2 (Mt2)
Metabolic dysfunction-associated steatosis liver disease (MASLD)
Metabolic phenotype (MP)
Metabolic syndrome (MetS)
Mitochondrial (Mt)
Mitochondrial DNA (mtDNA)
Mitochondrial (mt) ROS (mtROS)
Mitochondrial gene transcription activator TF-A (Tfam)
Mitogen activated protein kinase (MAPK)
Mitofusin-2 (Mfn2)

Monocarboxylate transporter 1 (MCT1)
Monocyte chemotactic protein-1 (MCP-1)
Mouse endothelial fibroblasts (MEFs)

Niacin receptor 1 (NIACR1)
Nicotinamide adenine dinucleotide (NAD+)
Nicotinamide adenine dinucleotide (NAD) + hydrogen (H) (NADH)
Nicotinamide adenine dinucleotide phosphate (NADP)
Nicotinamide adenine dinucleotide phosphate (NADP) + hydrogen (H) (NADPH)
Non-alcoholic fatty liver disease (NAFLD)
NOD-like receptor protein 3 (NLRP3)
Nuclear respiratory factor (erythroid-derived 1)-like 2 (NRF1)
Nuclear respiratory factor (erythroid-derived 2)-like 2 (NRF2)
Nuclear respiratory factors 1 and 2 (NRFs 1 and 2)
Nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB)
Nutritional ketosis (NK)

Odds ratio (OR)
One meal a day (OMAD)
One-way analysis of variance (ANOVA)
Oral glucose tolerance tests (OGTT)
Osteocalcin (OCN)
Oxidative phosphorylation (OXPHOS)
Oxygen (O_2)

Parkinson's disease (PD)
Palmitic-acid-9-hydroxy-stearic-acid (9-PAHSA)
Pentose phosphate pathway (PPP)
Peroxiredoxin (PRDN)
Peroxisome proliferator activated receptor alpha (PPARa)
Peroxisome proliferator-activated receptor gamma coactivator 1-a (PGC-1a)
Phase 1 (P1)
Phase 2 (P2)
Phase 3 (P3)
Phosphoinositide-dependent kinase-1 (PDPK1)
Phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt)
Phosphatidylinositol 3-kinase/Akt/mTOR (PI3K)
Phosphatidylinositol (3,4,5)- triphosphate (PIP3)
Plasma membrane (PM)
Plasminogen activator inhibitor type 1 (PAI-1)
Platelet-derived growth factor (PDGF),
Poly-adenosine diphosphate-ribose polymerase (PARPs)
Post-translational modifications (PTM)
Protein Kinase B (PKB)
Protein Kinase C (PKC)
Pyruvate dehydrogenase (PDH)
Pyruvate dehydrogenase kinases (PDK)

Pyruvate dehydrogenase kinase-1 (PDK1)

Randomised control trial (RCT)
Ras/Raf/Mek/Erk (MAPK)
Reactive oxygen species (ROS)
Receptor for advanced glycation end-products (RAGE)
Receptor tyrosine kinases (RTK),
Red blood cells (RBC)
Respiratory quotient (RQ)
Retinoblastoma (RBBP7)

Saturated fatty acid (SFA)
Senescence associated secretory profile (SASP)
Severe acute respiratory syndrome corona virus 2 (SARS-CoV-2)
Sirtuin 2 (SIRT2)
Sirtuin3 (SIRT3)
Small intestinal bacterial overgrowth (SIBO)
Standard U.K. diet (SUK)
Subclinical atherosclerosis (SA)
Succinate dehydrogenase (SDH)
Suppressed ketosis (SuK)
Superoxide (O_2^-)
Superoxide dismutase (SOD)
Superoxide dismutase 2(SOD2)
Superoxide dismutase 3 (SOD3)

Time restricted feeding (TRF)
Tissue-type PA (tPA)
Thin on the outside fat on the inside (TOFI)
Thioredoxin (TXN)
Thioredoxin reductase (TRx)
Thyroid hormone (TH)
Thyroid stimulating hormone (TSH)
Thyroxine (T4)
Total body water (TBW)
Total osteocalcin (tOCN)
Transcription factor (TF)
Tricarboxylic acid cycle (TCA)
Triiodothyronine (T3)
Type 1 diabetes (T1DM)
Type 2 diabetes mellitus (T2DM)
Tumour necrosis factor-α (TNF-α)

Umbilical vein endothelial cells (Huvecs)
Uncarboxylated osteocalcin (unOCN)
Uncoupling proteins 1 and 2 (UCP1 and 2)
Urokinase-type PA (uPA)

Vascular endothelial growth factor (VEGF)
Very low carbohydrate high fat diet (VCHF)
Volume carbon dioxide (VCO_2)
Volume oxygen (VO_2)

Well-formulated ketogenic diets (WFKD)
White blood cells (WBC),
Winged helix/forkhead box transcription factor DNA binding domain (FOXO)
Winged helix/forkhead box O3 transcription factor (FOXO3a)

Authors declaration: I declare that all the material contained in this thesis is my own work

Chapter 1

1. Introduction

1.1 Hyperinsulinaemia drives a common intracellular phenotype across diverse cell types in different chronic diseases and ageing, with dysfunctional mitochondria at centre-stage

Metabolic diseases such as cardiovascular disease (CVD), hypertension, type 2 diabetes mellitus (T2DM), metabolic syndrome (MetS), cancer, neurodegenerative dementias' Alzheimer's (AD) and Parkinson's disease (PD), and chronic inflammation are traditionally considered "diseases of ageing" as prevalence increases with age. However, this correlation is not causation, as demonstrated by the increasing higher rate of occurrence of these chronic diseases in younger population groups (Menke et al., 2015; Araújo, Cai and Stevens, 2018). Furthermore, hyperinsulinaemia explains the increases in susceptibility to and poorer outcomes from, communicable diseases like respiratory infections, such as from severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) causing coronavirus-19 (COVID-19) (Cooper et al., 2020). A common denominator of these diseases and their presentations, chronic hyperinsulinaemia, provides a plausible mechanism of action underlying the pathogenesis of these morbidities and co-morbidities, where it can be seen they share common unifying features at the cellular and mitochondrial level (Crofts et al., 2015; Cooper et al., 2020).

Hyperglycaemia increases blood coagulability via increasing glycation damage producing advanced glycation end products (AGES), glycated haemoglobin and hepatic clotting factors, and decreasing heparan sulphate proteoglycans (HSPG) that aid in: anticoagulant function, cellular communication and intercellular transport mechanisms, via increasing heparanase expression (Stegenga et al., 2006; An et al., 2011). Concomitantly, hyperinsulinaemia inhibits fibrinolysis via the elevation of plasminogen activator inhibitor type 1 (PAI-1), and decreases HSPG levels via sulphate wastage (Bolt et al., 2004; Perkins et al., 2015). Together, hyperinsulinaemia and hyperglycaemia produce a combined and compounded effect of impairing vascular health and endothelial function (Stegenga et al., 2006; Perkins et al., 2015), a pervasive common feature of CVD, cancer, neurodegenerative disorders AD and PD, T2DM and hypertension, the leading causes of non-communicable

disease morbidity and mortality (Bots et al., 1998; Gupta et al., 2004; Stegenga et al., 2006; Oh et al., 2014; Perkins et al., 2015; Pan et al., 2018; ONS, 2023).

Chronic hyperinsulinaemia propagates an increase in intracellular reactive oxygen species (ROS) production, decreased endogenous antioxidant activity, and impaired mitochondrial health leading to dysregulated: energy metabolism, intracellular calcium trafficking, haem synthesis, vitamin D activation, and epigenetic alteration on cell cycle progression and functions that otherwise aid in cellular homeostasis (Bikle, 2017; Thakur and Chen, 2019; Cooper et al., 2020; Swenson et al., 2020; Cooper et al., 2021; Cooper, Kyriakidou, Petagine, et al., 2023). These pathological intracellular features are shared across a vast array of different cell types, in different tissues and organs, and represent a common feature of chronic disease hyperinsulinaemia-intracellular/mitochondrial phenotype (Wang et al., 2009, 2020; Ruderman et al., 2013; Smith et al., 2013; Hansen et al., 2014; Kobayashi et al., 2015; Li et al., 2015; Sampson et al., 2017; Gao et al., 2018; Gao, Chen and Zhu, 2018; Paradies et al., 2019; Agius, Ford and Chachra, 2020; Picca et al., 2020; Wu and Zou, 2020; Georgiadou and Rutter, 2020; Hong et al., 2020; Calvo-Rodriguez and Bacskai, 2021; Chen et al., 2021).

1.1.1 Calorie restriction, beta-hydroxybutyrate and longevity

Calorie restriction studies across many species from the single celled yeast, to the nematode worm *Caenorhabditis elegans*, mice through to primates, have been shown to extend lifespan with healthspan (Ristow and Zarse, 2010; Lee and Min, 2013; Newman and Verdin, 2014a; Fontana and Partridge, 2015; Veech et al., 2017). The mechanism of how this is achieved has not been fully resolved, however a number of key aspects of calorie restriction (CR) defines the metabolic changes that is induced within the organism and humans included, namely ketosis (Veech et al., 2017). This is the endogenous production of the small molecule D-beta-hydroxybutyrate (BHB), a ketone body synthesised primarily by hepatocytes from free fatty acids derived from adipocytes when fasted (Cahill et al., 1966; Rojas-Morales, Tapia and Pedraza-Chaverri, 2016; Miller, Villamena and Volek, 2018). CR and ketosis have been shown to mediate their effects via altered signalling of a number of critical pathways involved in ageing and lifespan, those signalling pathways include: insulin/insulin-like growth facto-1 receptor signalling (IIS), the mechanistic target

of rapamycin pathway (mTOR) and adenosine monophosphate activated protein kinase (AMPK) pathways, winged helix/forkhead box transcription factor DNA binding domain (FOXO) proteins signalling phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt) and mitogen activated protein kinase (MAPK) pathways and sirtuin activities (Cantley, 2002; Luong et al., 2006; Lee and Min, 2013; Dibble and Cantley, 2015; Saxton and Sabatini, 2017).

1.1.1.1 Animal data

Over a period of 4 years, McCay *et al.* (1935) demonstrated in rats an increase in average lifespan from 500 days to 820 days, through 30% to 50% CR (McCay, Crowell and Maynard, 1989). Anderson *et al.* (2008) demonstrated that CR without malnutrition in mice, resulted in lived longer mice than their non-calorie restricted counterparts, this was further shown to be inversely proportional to the degree of restriction (Anderson, Shanmuganayagam and Weindruch, 2009). This effect was achieved in adult C3B10F_1 and C57Bl/6 mice, where CR was implemented at 1 year of age, leading to an increase in mean and maximal lifespan by 10 to 20% respectively. Furthermore, spontaneous incidence of lymphoma cancer was inhibited by the nutrient-enriched CR intervention (Weindruch and Walford, 1982). The evidence that CR could increase healthspan and lifespan in adult mice paved the way for implementing nutritionally replete CR in rhesus monkeys, starting in young adulthood. CR of about 30% significantly improved all-cause and age-related survival (Colman et al., 2014). Metabolic profiles improved along with less oxidative stress, indicated by isoprostane levels in the plasma, in CR rhesus monkeys (Mattison et al., 2012).

Physiological or genetic mimicked CR in the unicellular yeast *Saccharomyces cerevisiae* produced a substantial life-span extension. This effect required the nicotinamide adenine dinucleotide (NAD+) dependent sirtuin 2 (SIRT2) pathway activity, as CR did not elicit these extension effects in mutant *SIR2,* encoding silencing protein Sir2p which mediates chromatin silencing, and *NPT1* a gene in NAD synthesis pathway, strains. This suggests increased longevity requires both NAD+ and SIRT2 activity (Lin, Defossez and Guarente, 2000). Lifespan extension in the fruit fly *Drosophila* was achieved via reducing mTOR activity which blocked activated-FOXO mediated insulin resistance (IR), lowered glucose

levels and increased conversion of lipids to BHB resulting in increased ketone body levels (Luong et al., 2006). mTOR is positively responsive to elevated insulin and insulin-like growth factor-1 (IGF-1) levels, and adjusts cellular growth in response to integrating responses to nutrients: lipid, glucose and amino acid levels, insulin and IGF1 levels, oxygen tension and mitochondrial metabolite signalling (Luong et al., 2006).

Genetic mutation *age-1* in *Caenorhabditis elegans* significantly increases lifespan. *Age-1* encodes the *C. elegan* AGE-1 PI3K catalytic subunit, that sits downstream of the IIS pathway (Johnson, 1987). Further mutagenesis studies resulted in the doubling of lifespan in *C. elegans* via gene mutations encoding the transmembrane dauer formation-2 (*daf-2*) a member of the insulin/IGF-1 receptor family of the IIS pathway, homologue to the mammalian IIS receptor (Kenyon et al., 1993; Kimura et al., 1997). This DAF2(insulin)/IGF-1 (DAF2-IIS) mutagenesis induced doubling of lifespan effect required the activity of the *daf-16* gene. *Daf-16* encodes forkhead box transcription factor DAF-16, *C. elegan* homologue to dFOXO and FOXO3a in *D. melanogaster* and mammalians species respectively (Veech et al., 2017). *Daf-2* and *daf-16* together regulate the formation of developmentally arrested larval form, termed the dauer state, that is resistant to desiccation (Gottlieb and Ruvkun, 1994). Activating the DAF-2/IIS-AGE-1/AKT-1/2/PKB signal cascade negatively regulates DAF-16/FOXO, which regulates longevity, metabolism and the expression of the dauer gene profile that induces the diapause state at the cellular and organismal level in *C. elegans* (Vowels and Thomas, 1992; Lin et al., 1997). Nuclear localised DAF-16/FOXO transcription factor regulators, enables the increased expression of more than 200 longevity associated genes, involved in antioxidant defence mechanisms, metabolism, autophagy and DNA repair (Lin et al., 1997). There are four human FOXO genes, extreme longevity is associated with a FOXO3a allele (Willcox et al., 2008).

1.1.2 The hypothesis: Hyperinsulinaemia is the root cause of major chronic diseases

The free radical theory of ageing (FRTA) was postulated by Harman in 1955 (Harman, 1955; Denham Harman, 1956, 1957), that progressively excessive cellular ROS production, with decreased capacity to neutralise or repair from ROS damage is fundamental to the mechanisms of ageing and chronic disease development (Denham Harman, 1957, 1991; Harman, 2006, 2009). On this continuum, “inflammageing” is postulated to be the primary

hallmark of ageing (Calder et al., 2017). As more research has progressed into elucidating mechanisms of ageing and chronic disease that limit lifespan, evidence showing an inverse relationship between mitochondrial ROS and lifespan has accumulated to support the FRTA (D. Harman, 1991; Harman, 2006; Veech et al., 2017). This theory is supported by the evidence of chronic intracellular inflammation as a common marker of metabolic diseases, contributing to chronic systemic inflammation (Hamanaka and Chandel, 2010; Pamukcu, Lip and Shantsila, 2011; Tschopp, 2011; Wang et al., 2015; Forrester et al., 2018; Fiordelisi et al., 2019; Jha et al., 2019; Picca et al., 2020).

The insulin/insulin-like growth factor-1 receptor signalling (IIS) pathway is upstream of and activates the PI3K and MAPK growth and division pathways (Zhang et al., 2011; Hopkins, Goncalves and Cantley, 2020). IIS receptor activation transduces a parallel cascade of signals either via insulin receptor substrate (IRS) proteins, or directly. IIS activates mammalian IRS1/2 which subsequently goes on to activate a class 1 phosphoinositide 3-kinase in the PI3K pathway, generating second messenger's phosphatidylinositol (3,4,5)-triphosphate (PIP3), resulting in an increase in plasma membrane (PM) PIP3 concentration. A high PIP3 concentration in the PM, results in the phosphorylation of FOXO transcription factors (TF) and subsequent cytosolic sequestration, by propagating the PI3K/Akt signal cascade via activating phosphoinositide-dependent kinase-1 (PDPK1) phosphorylation of Ak mouse strain thymoma (AKT-1)/protein kinase B (PKB) kinases. Phosphorylated AKT-1/PKB in turn phosphorylates and therefore negatively regulates FOXO transcription factors (Kapeller and Cantley, 1994; Toker and Cantley, 1997; Cantley, 2002; Fruman et al., 2017; Hopkins, Goncalves and Cantley, 2020). Hyperinsulinaemia suppresses hepatic autophagy via phosphorylation inhibition of mammalian FOXO1 and FOXO3 proteins, leading to decreased expression of autophagy genes (Liu et al., 2009). Reducing IIS signalling via CR and ketosis reduces PI3K, MAPK and mTOR signalling (Luong et al., 2006; Lee and Min, 2013; Veech et al., 2017). Furthermore, chronic hyperinsulinaemia increases oxidative stress by increased mtROS generation via increasing ceramide synthesis, decreasing NAD+ availability for antioxidant activity and diminish mitophagy cellular clearance programs via multiple ROS mediated intracellular signalling pathways, leading to increased overall ROS production that propagates pathological and ageing transformation. These pathways include adenosine monophosphate (AMP)-activated protein kinase

(AMPK), mTOR, sirtuins 1 and 3 (SIRT1 and SIRT3), peroxisome proliferator-activated receptor gamma coactivator 1-a (PGC-1a) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Greer et al., 2007; X Li et al., 2007; Cantó et al., 2009; Hill and Tumber, 2010; Qiu et al., 2010; Smith et al., 2013; Hansen et al., 2014; Jeon, 2016; Dikalova et al., 2017; Ghosh et al., 2017; Rabinovitch et al., 2017; Saxton and Sabatini, 2017; Kolb et al., 2020).

At the cellular level, nutrient sensing of glucose, fatty acids, proteins and/or D-beta-hydroxybutyrate (BHB), provide a signal to cells, indicating the nutrient and energy status of the whole organism. The metabolic phenotype of CR and fasting is ketosis, with decreased basal concentration in glucose and insulin, where detectable plasma BHB concentration begins to exceed > 0.3 mmol/L and < 10 mmol/L, termed nutritional ketosis (NK), in an individual who is not an insulin-dependent type 1 diabetic, where there is not enough or an absence of endogenous insulin production to regulate ketogenesis with hyperglycaemia (Cahill et al., 1966; Veech et al., 2017; Miller, Villamena and Volek, 2018). CR essentially mimics the fasted state and is categorised as a fasting mimicking diet (FMD). The fasting phenocopied state has a phenotype of healthy blood glucose (3.5 – 6.0 mmol/L), insulin (< 60 pmol/L) and ketones (< 7.0 mmol/L in healthy individuals) (Shimazu et al., 2013; Hallberg et al., 2018; Min et al., 2018).

1.1.2.1 Hyperinsulinaemia is a driver in the pathogenesis of a chronic disease hyperinsulinaemia-intracellular and mitochondrial phenotype

While great gains have been achieved in increasing median lifespan expectancy due to improved medical care, it is not anyone's desire to live longer whilst suffering a chronic disease as the price for extended ageing (White et al., 2014; Menke et al., 2015; Araújo, Cai and Stevens, 2018; Dorsey and Bloem, 2018; Taylor, Swerdlow and Sullivan, 2019; Siegel, Miller and Jemal, 2020; Versele et al., 2020). The ideal aim of the game in ageing-well and longevity, is to have an extended healthy lifespan "healthspan" with negligible senescence. This means the absence of biological aging, such as reducing functional decline in organs and whole-body fitness, delaying loss of reproductive capabilities, and delaying death risk with age progression. (McHugh and Gil, 2018; Cooper and Bell, 2024). What is ultimately desired, is to extend youth, not extend ageing, and if this is achieved, then we may begin to push the envelope on increasing lifespan.

There is an instinctive understanding of what ageing is. It is seen in the manifestation in changing appearance, such as loss of skin elasticity and greying hair, to changes in physiology with loss of function, such as decreased fertility in females with each passing decade until complete cessation of menses at menopause and increases in cognitive decline. Younger people rarely suffer hip fractures, unlike older people do as their bones become weaker, where osteoporosis and fragility fracture rates increase (Hernlund et al., 2013; Tu et al., 2018), and as we age, sarcopenia, the gradual loss of muscle mass, becomes a strong predictor of morbidity and mortality (D. Harman, 1991; Harman, 2006; Mercken et al., 2012; Konopka and Sreekumaran Nair, 2013).

1.1.2.2 Common cellular features

There are numerous common features shared between these hyperinsulinaemia chronic diseases at the intracellular level, in a vast array of cell types in different tissues and organs. Those features include but are not limited to, increased: mitochondrial (mt) ROS (mtROS) formation, with decreased commensurate anti-oxidant activity, pyrin domain-containing NOD-like receptor protein 3 (NLRP3)-inflammasome assembly and activity, nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) activity, mitochondrial fission via altered dynamin related protein-1 (Drp1) to mitofusin-2 (Mfn2) ratio (Smith et al., 2013; Chen et al., 2020), and cell cycle progression rate (Diebold and Chandel, 2016). Along with decreased: AMPK, SIRT 1 and 3, winged helix/forkhead box O3 transcription factor (FOXO3a), PGC-1a, manganese superoxide dismutase (MnSOD2), catalase, beta-oxidation capacity, glutathione reduction, autophagy, mitophagy, and mitochondrial biogenesis (Skurk et al., 2005; Civitarese et al., 2006; Luong et al., 2006; Willcox et al., 2008; Su et al., 2009; Liu et al., 2009, 2018; Ambrogini et al., 2010; Manolopoulos et al., 2010; Gonzalez et al., 2011; Cai et al., 2012; Santo et al., 2013; Moreno-Santos et al., 2016; Tan et al., 2016; Kadlec et al., 2016; O'Neill et al., 2019; Blasiak et al., 2020; Ma et al., 2020; Wu and Zou, 2020; Chen et al., 2021). These features promulgate cellular "ageing" phenotype activities (Veech et al., 2017).

Hyperinsulinaemia pathologies present with increases in loss of function at all levels, from: the cellular, tissue, organ and ultimately systemic (Russell and Kahn, 2007; White et al., 2014; Menke et al., 2015; Araújo, Cai and Stevens, 2018). Sustained ketosis with

euglycaemia is a measurable metabolic phenotype to indicate absence of hyperinsulinaemia (Rojas-Morales, Tapia and Pedraza-Chaverri, 2016; Hyde et al., 2017; Cooper et al., 2020). Strategies that can prevent and/or reduce hyperinsulinaemia may therefore improve cellular and vascular health, leading to decreased risk of developing a chronic disease that reduces quality of life, healthspan and lifespan.

This thesis investigates the metabolic phenotype of long-term sustained ketosis (euketonaemia) in healthy females with no prior metabolic diseases. Assessing their baseline biochemical profile (metabolic phenotype) and changes in biomarkers associated with ageing and age associated diseases after suppressing ketosis (SuK) for 21 days, followed by a further investigation after removing the intervention of suppressing ketosis, with the participants reverting back to their habitual lifestyle of being in ketosis for 21 days, to further learn if the changes seen after the ketosis suppression phase, were due to the intervention.

Chapter 2

2. The background scientific rational: how hyperinsulinaemia causes the same intracellular phenotype, in different cell types, of different chronic diseases.

2.1 Hyperinsulinaemia causes mitochondrial pathological transformation via increased ROS and decreased oxidative defence capacity, while ketosis decreases ROS and increases antioxidant capacity.

2.1.1 Cellular energy and nutrient sensing

The availability of nutrients to provide energy and materials for cells to function, dictate the health and lifespan of a cell. It is essential for cells to understand; what is the energy status of the whole organism and thus also for itself. When nutrient availability is low, it would be wasteful to build more cellular biomass materials in preparation to progress towards mitosis. Accelerated mitosis decreases each cell's limited number of replicative cycles, known as the Hayflick limit (Hayflick and Moorhead, 1961). Caloric restriction (CR) and fasting studies have so far been shown to have the greatest positive effect on lifespan extension (Veech et al., 2017). Cells are capable of sensing energy availability and type of nutrients, consequently triggering signalling cascades that either stimulate anabolic or catabolic activities, both being vital processes, in which the harmony of the two contribute to cellular health and longevity (Greer et al., 2007; Lage et al., 2008; Saxton and Sabatini, 2017; Senyilmaz-Tiebe et al., 2018).

2.1.2 Oxidative stress as a result of living

Energy is required for the work cells must perform to maintain order in the fight against ever increasing entropic disorder. Mitochondria are charged with producing the largest bulk of energy for most of our cells (Miller, Villamena and Volek, 2018). These dynamic organelles are both a source of life sustaining energy and a source of destruction, from the processes of capturing chemical energy, via the breakdown of nutrients with the use of oxygen, into the highly efficient small molecule adenosine-triphosphate (ATP) (Veech, 2004; Marchi et al., 2012; Nourshahi et al., 2012; Robb et al., 2018).

Mitochondria are involved with a vast number of cellular processes, including regulation of metabolic flux (Muoio, 2014). They are charged with the job of producing cellular energy in the form of ATP and performing anabolic processes to furnish cells with precursors for lipid, amino acid and nucleotide biosynthesis, haem synthesis, calcium trafficking, ketogenesis, and are key regulators of apoptosis (Frezza and Gottlieb, 2009; Stein and Imai, 2012; Georgiadou and Rutter, 2020; Swenson et al., 2020). Mitochondria constitute a significant proportion of most human cells, increasing in numbers in cells that are highly metabolically active.

Free radicals are produced during oxidative phosphorylation (OXPHOS), from oxygen in the process of ATP production via the inner mitochondrial membrane (IMM) electron transport chain (ETC), situated on the matrix side of the cristae (Paradies et al., 2014, 2019). A class of free radicals produced, include: superoxide (O_2^-) oxygen with an extra electron, hydroxyls (^-OH) or hydrogen peroxide (H_2O_2). Collectively these are termed ROS (Marchi et al., 2012; Nourshahi et al., 2012).

2.1.3 Reactive oxygen species and healthspan

It was postulated in the 1950's that ROS toxicity is a key player in the ageing process (Harman, 1955, 2006; Dehnam Harman, 1956, 1957; Denham Harman, 1956, 1957, 1991). As more research has progressed into elucidating mechanisms of ageing and chronic diseases that limit lifespan, substantial amounts of evidence has accumulated to support this theory, this includes showing an inverse relationship between mitochondrial ROS production and lifespan, where greater amounts of mitochondrial-ROS generation results in decreased healthspan contributing to reduced life-expectancy (Harman, 2006, 2009; Wright, Scism-Bacon and Glass, 2006; Kaneto et al., 2010; Manoharan et al., 2016; Sarkar et al., 2017; Volpe et al., 2018; Elfawy and Das, 2019; Liao, Chua and Tan, 2019; Kaludercic and Di Lisa, 2020). Whilst ROS are considered damaging to a cell's internal structures and components, they are also signalling molecules in their own right. Ironically, some amount of ROS is necessary for health and life-span extension, this is the element of hormesis, the goldilocks zone (Hill and Van Remmen, 2014; Margaritelis et al., 2016).

2.1.4 Mitochondrial hormesis

Mitochondria are the greatest source of intracellular free radical ROS production, in close proximity to mitochondrial DNA (mtDNA) and the IMM containing the ETC protein complexes. ROS are also largely detoxified within mitochondria (Milder and Patel, 2012). ROS may act as a form of signalling, enabling mitochondria to communicate with the cell's nucleus, a mitochondrial-nuclear cross-talk, otherwise known as mitochondrial-ROS-retrograde signalling (Ristow and Zarse, 2010; Hill and Van Remmen, 2014; Herst et al., 2017; Miller, Villamena and Volek, 2018). ROS, including H_2O_2 are functional signalling molecules. ROS modulate the activity of redox sensitive proteins and enzymes via altering the oxidative state of cysteine residues within them, from reduced thiols to oxidized disulphide bonds, thus generating ROS activated sulphur switches (Brandes, Schmitt and Jakob, 2009; Muoio, 2014; Schieber and Chandel, 2014).

Mitochondrial ROS signalling inductive effect on nuclear gene transcriptional regulation, leads to the question of who/what is driving cellular behaviour? The genes in the nucleus, or signals from outside and within the cell from the mitochondria, transmitted to the nucleus to activate/elicit adaptive responses, altering gene expression as an epi-phenomena and consequently cellular outcomes (Long et al., 2014; Kinnaird et al., 2016; Sonnenschein and Soto, 2016; Herst et al., 2017). Some of those responses leads to the increase in production of antioxidant enzymes such as MnSOD2 and catalase to counter mtROS (Newman and Verdin, 2014a; Miller, Villamena and Volek, 2018). Vitamin C having antioxidant properties, is an essential vitamin that humans must consume, however humans also possess the ability to synthesise powerful endogenous antioxidants. These endogenously derived anti-oxidants such as reduced glutathione (GSH) and glutathione peroxidase (GPx) are able to recycle vitamin C (Veech et al., 2017). Just the right amount of ROS, drives mitochondrial biogenesis, thus helping to decrease ROS production once again.

2.2 Hyperinsulinaemia increases mtROS production and decreases oxidative defence

2.2.1 NAD+

Hyperinsulinaemia enforces glucose fuelling via inhibition of beta-oxidation, ketolysis and ketogenesis (Veech, 2004) (**Figure 1**). This leads to greater depletion of cytosolic NAD+, due to ATP generation from glucose substrate. To produce two acetyl moieties, glucose oxidation consumes four NAD+. In contrast, fatty acid, beta-hydroxybutyrate (BHB), acetoacetate (AcAc) oxidation: consume two, one and zero NAD+ respectively (Newman and Verdin, 2014a; Veech et al., 2017). NAD+ is a critical coenzyme in redox reactions, and an essential sirtuin and poly-adenosine diphosphate-ribose polymerase (PARPs) enzyme co-substrate (Verdin, 2015). A decrease in NAD+ leads to an increase in aerobic fermentation/glycolysis, this leads to upregulated activity of the inflammatory mediators: NLRP3 inflammasome and NFkB activity (Luengo et al., 2021). Decreased NAD+ availability, results in decreased NAD+ dependent SIRT3 induction of MnSOD and catalase oxidative defence activity to counter ROS (Newman and Verdin, 2014a; Veech et al., 2017). Thus, hyperinsulinaemia increases intracellular ROS by decreasing protein/enzymatic counter-ROS management capacity. As a result, mitochondrial morphological changes ensue, resulting in a further feedforward increase in ROS, which leads to chronic disease pathogenesis (Hansen et al., 2014).

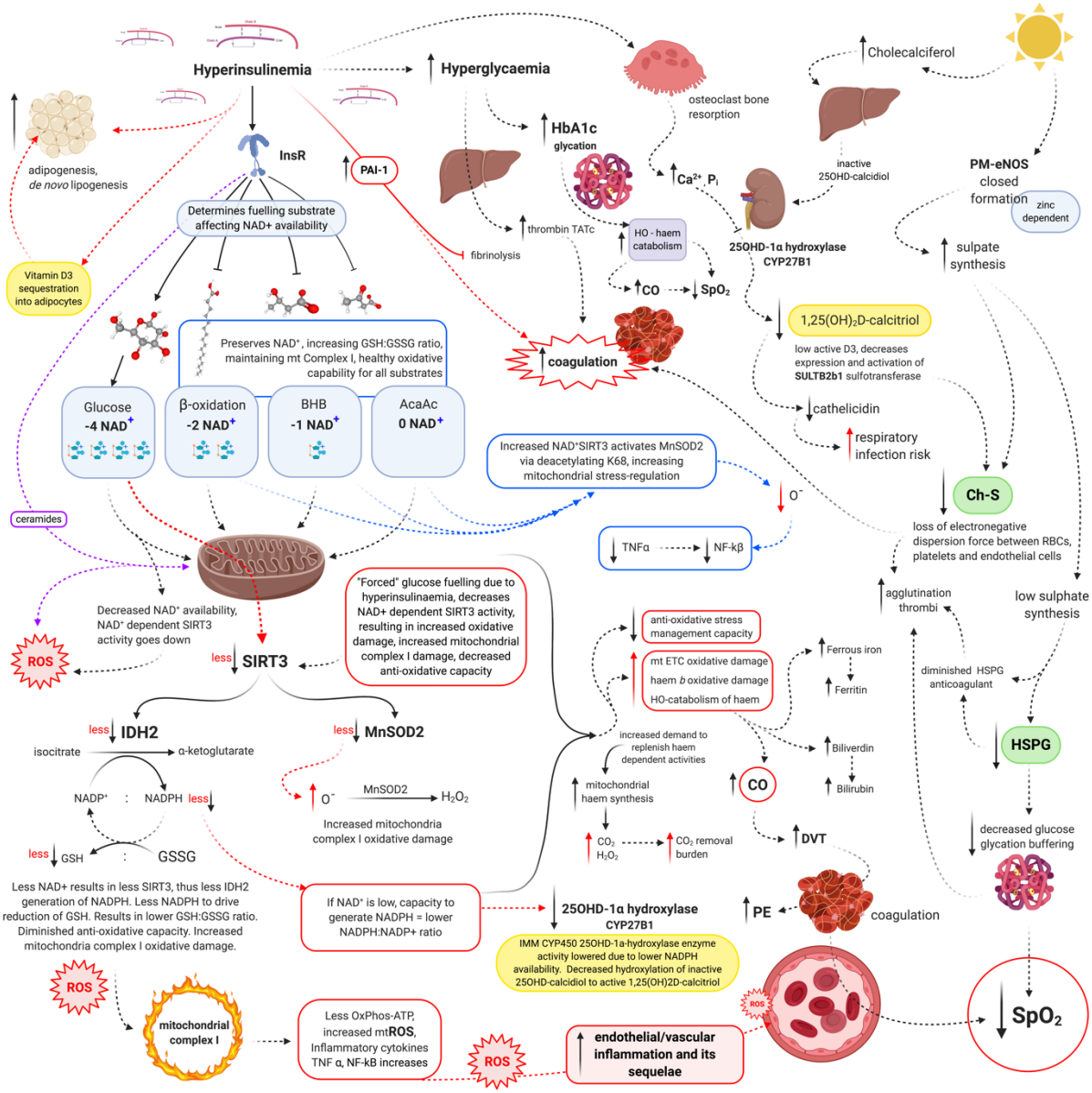

Figure 1. Schematic representation of the role of hyperinsulinaemia in endothelial/vascular inflammation, red blood cell (RBC) and platelet coagulation, sequestration and/or inhibition of vitamin D activation and its downstream consequences, such as decreased cholesterol sulfate (Ch-S), heparan sulfate proteoglycans (HSPG) and cathelicidin synthesis. Carbon dioxide (CO2), carbon monoxide (CO), deep vein thrombosis (DVT), endothelial nitric oxide synthase (eNOS), reduced glutathione (GSH), oxidised glutathione (GSSG), haemoglobin A1c (HbA1c), haem-oxygenase (HO), manganese superoxide dismutase 2 (MnSOD2), nicotinamide adenine dinucleotide (NAD+), plasma membrane (PM), plasminogen activator inhibitor type 1 (PAI-1), pulmonary embolism (PE), reactive oxygen species (ROS), oxygen saturation (SpO2), sirtuin 3 (SIRT3) and type 2 diabetes mellitus (T2DM).

2.2.2 Hyperinsulinaemia increases ROS leading to DNA damage, requiring PARP1 activity which depletes NAD+, leading to more ROS

Chronic oxidative stress, due to hyperinsulinaemia, induces DNA damage (Hinokio et al., 1999; Lee and Chan, 2015; Blasiak et al., 2020). T2DM is a condition of hyperinsulinaemia that increases risk of cancer, where damaged DNA is a marker and potentiator in cancer transformation (Sliwinska et al., 2008; Seyfried et al., 2019). Furthermore, T2DM presents with increased ageing, vascular inflammation and atherogenesis, with increased oxidative DNA damage (Dandona et al., 1996). Increased oxidative damage to DNA calls upon the NAD+ dependent DNA repair protein, PARP1 via NAD+ dependent type III deacetylase SIRT1. Activation of PARP1 catabolises the SIRT1 NAD+ cofactor, resulting in the precipitation of further oxidative stress (Scheibye-Knudsen et al., 2014). Increased NAD+ demands lead to mitochondrial compensation via reducing the IMM potential with subsequent reduction in OXPHOS derived ATP synthesis. This upregulates aerobic fermentation/glycolysis and effectively inhibits beta-oxidation, ketogenesis and ketolysis, in-order to facilitate replenishment of cytosolic NAD+ (Luengo et al., 2021).

Ketosis increases SIRT1 and SIRT3 activity, via sparing NAD+ availability. This leads to the increased expression of downstream genes such as PGC-1a, that increases mt biogenesis. SIRT1 increases autophagy, the cellular clearance program, removing damaged and defective organelles including compromised mitochondria. SIRT1 activates mt biogenesis and FOXO3a activity, which leads to the increased gene expression of MnSOD, catalase and melatonin receptor 2 (Mt2) (McCarty, DiNicolantonio and O'Keefe, 2015). Furthermore, ketosis, ketolysis and BHB mediated NAD+ sparing rescues premature ageing in Cockayne Syndrome, via increased provision for PARP1 DNA repair activity and rescued mitochondrial function (Scheibye-Knudsen et al., 2014).

2.3 AMPK, PGC-1a, SIRT1 and FOXO3a

5' Adenosine monophosphate (AMP) activated protein kinase (AMPK) is an intracellular fuel/nutrient-energy sensor enzyme, and critical modulator of mitochondrial gene expression and mitochondrial biogenesis (Steinberg and Kemp, 2009). Activated AMPK enhances cellular activities to manage oxidative stress and increase autophagy. AMPK mediates its effects via increasing cellular NAD+ levels, which enhances NAD+ dependent

type III deacetylase SIRT1 activity and consequently its downstream targets including PGC-1a and FOXO3a (Cantó et al., 2009). AMPK activity is diminished and dysregulated in hyperinsulinaemia metabolic conditions such as IR, T2DM, CVD, hypertension, neurodegenerative diseases (AD and PD), and cancer (Ruderman et al., 2013).

2.3.1 AMPK and epigenetics

AMPK is an epigenetic regulator, remodelling chromatin and increasing gene transcription by directly phosphorylating DNA methyl transferase 1 (DNMT1), histone acetyl-transferase 1 (HAT1) and retinoblastoma (RBBP7). AMPK mediated phosphorylation inhibits DNMT1 and via co-activation of RBBP7, this further inhibits DNMT1, forming a DNMT1-RBBP7 phosphorylated complex. As a result, there is a decrease in methylation of chromatin, which subsequently reduces nucleosome compaction. This enables transcription factor access to promotor regions, for mitochondrial biogenesis and function genes such as: PGC-1a, uncoupling proteins 1 and 2 (UCP1 and 2), nuclear respiratory (erythroid-derived 1/2) factors 1 and 2 (NRF 1 and 2) and mitochondrial gene transcription activator TF-A (Tfam). AMPK phosphorylation of HAT1 increases acetylation of chromatin (co-activated with RBBP7 as an active complex) in helix 1 of histone H4, relaxing it to a euchromatin state via HAT1-RBBP7 activated acetyltransferase complex acetylation. This results in the de-condensing of the chromatin structure. Co-mediation by cross-activation of AMPK phosphorylation of RBBP7, further enhances HAT1 activity as a histone chaperone complex, AMPK epigenetic regulation via modulating nucleosome remodelling, enables accessibility to regions of DNA, by transcription factor activating machinery and resultant gene transcription (Keck and Pemberton, 2012). Thus AMPK improves mtOXPHOS quality/capacity, by enhanced transcription of OXPHOS genes, as seen in transfected human umbilical vein endothelial cells (Huvecs) and mouse endothelial fibroblasts (MEFs) (Marin et al., 2017).

Activation of AMPK induces intracellular signalling cascades, altering epigenetic gene regulation, via phosphorylating and reducing the methylation of nuclear mitochondrial biogenesis promotor genes, such as PGC-1a, a master transcription factor (TF) that upregulates mitochondrial biogenesis (Irrcher, Ljubicic and Hood, 2009). PGC-1a regulates

mitochondrial biogenesis via transactivation of NRF1 and NRF2, consequently upregulating the gene expression of Tfam (Barbato et al., 2012). Nucleosome remodelling may be regulated via AMPK histone 2B (H2B) phosphorylation. Marin *et al,* demonstrated AMPK consensus phosphorylation sequences in the epigenetic factors, DNAMT1, HAT1 and RBBP7. The phosphorylation of this epigenetic regulatory network results in remodelling of the nucleosome, and methylation reduction with subsequent increase in the expression of PGC-1a, NRF1 and 2, and the uncoupling-proteins UCP2 and UCP3, driving the upregulation of mitochondrial biogenesis (Marin et al., 2017).

2.3.1.1 AMPK increases mitochondrial biogenesis

Upregulation of AMPK activity and its downstream targets increases; mitochondrial mass via mt biogenesis; mtDNA abundance; citrate synthase activity; increased activity of ETC complex 1 and 4; and ATP synthase; increased production of ATP with concomitant decreased basal ROS. AMPK mediates quality of function of mitochondria, being able to modulate mitochondrial membrane potential via transactivation of uncoupling proteins UCP2 and 3 that work to mitigate ROS production (Marin et al., 2017). AMPK nucleosome remodelling co-ordinates with AMPK activation (co-activated with SIRT1 deacetylation) of PGC-1a's binding to mitochondrial biogenesis nuclear promotors PGC-1a, NRF1 and 2 (Cantó et al., 2009). Transcription factors and coactivators PGC-1a, peroxisome proliferator activated receptor alpha (PPARa) and gluconeogenesis transcription factor hepatocyte nuclear factor 4 (HNF4), work cooperatively in adaptive upregulation of fatty acid (FA) oxidation capacity, ketolysis in peripheral tissues and hepatic gluconeogenesis (Lustig et al., 2011).

2.3.2 Hyperinsulinaemia inhibition of AMPK

Hyperinsulinaemia inhibits AMPK signalling, via Akt (also known as Protein Kinase B - PKB) activation (Hawley et al., 2014), which phosphorylates S487 of the AMPK-a1 subunit, and inhibition of upstream kinases that phosphorylate AMPK threonine residue 172 (phosphorylation would increase activity), results in subsequent inhibition of AMPK and it's downstream effectors (Ruderman et al., 2013; Hawley et al., 2014; Jeon, 2016). Akt/PKB is a downstream serine/threonine kinase target of the Pi3K signalling pathway. The cellular

energy/nutrient sensor AMPK and its downstream pathway is activated when the intracellular [AMP] is greater than the [ATP], and [ADP] > [ATP]. When there is an increase in AMP:ATP, this results in cellular regulatory feedback systems to indicate intracellular energy status (Lage et al., 2008; Grabacka et al., 2016).

2.3.2.1 AMPK activity is reduced in hyperinsulinaemia chronic diseases

The role of AMPK in regulating mitochondrial health and function indicates that chronic decreased AMPK activity may logically contribute to increases in impaired mitochondria accumulation (Wu and Zou, 2020), which would consequently result in increased dysregulated intracellular ROS, poor OXPHOS ATP synthesis capacity, and further consolidation in transition towards dysregulated mitochondria, with increased fission and crystolysis, a common intracellular phenotype of hyperinsulinaemia pathologies (Ruderman et al., 2013; Li et al., 2015; Gao, Chen and Zhu, 2018; Agius, Ford and Chachra, 2020; Wu and Zou, 2020; Chen et al., 2021). Reduced AMPK activity, or the therapeutic effects of activating AMPK in hyperinsulinaemia diseases include: T2DM (Ruderman et al., 2013; Agius, Ford and Chachra, 2020; Chen et al., 2021), CVD (Li et al., 2015; Gao, Chen and Zhu, 2018; Wu and Zou, 2020), AD (Chen et al., 2021), PD (Curry et al., 2018; Hang et al., 2019), and cancer (Hawley et al., 2014; Jeon, 2016). Where AMPK activity is shown to be highly implicated in mitochondrial function (Rabinovitch et al., 2017; Wu and Zou, 2020).

2.3.3 Glycogen inhibits AMPK

Insulin activates glycogen synthesis. *de novo* glycogenesis negatively regulates AMPK by decreasing its activity via binding to the β regulatory subunit. Exercise induced skeletal muscle glycogen depletion decreases this inhibition via glycogen binding, whilst increasing AMP and ADP binding to the alpha subunits increasing AMPK activity (Ruderman et al., 2013). Increase in glucose levels, increases malonyl-CoA, which allosterically inhibits carnitine palmitoyltransferase-1 (CPT-1) (Muoio, 2014), thus inhibiting beta-oxidation (Denis McGarry, 2002). CPT-1 is situated on the mitochondrial outer membrane, enzymatically converting long-chain fatty acyl-Co-enzyme A (acyl-CoA) into long-chain acylcarnitine's, in order to facilitate provision of fatty acids for mitochondrial beta-oxidation (Casals et al., 1992).

Insulin signalling, especially hyperinsulinaemia, enforces cellular fuelling from glucose and thus upregulates glycolysis. A rise in pyruvate from glycolysis, inhibits pyruvate dehydrogenase kinases (PDK), releasing inhibition via phosphorylation of pyruvate dehydrogenase (PDH), leading to increased coupling of glycolysis to oxidative phosphorylation of glucose within the mitochondria. Shifting fuelling substrate into "gasoline" glucose based, verses "electric" beta-oxidation. As a result of upregulated PDH activity, tricarboxylic acid cycle (TCA) anapleurosis increase of citrate leads to citrate "spill-out"/transport out of mitochondria into the cytoplasm, where ATP-citrate lyase cleavage produces one acetyl-CoA and one oxaloacetate moiety. Furthermore, cytosolic increase in citrate positively upregulates acetyl-CoA carboxylase (ACC) activity to carboxylate cytoplasmic acetyl-CoA, thus producing malonyl-CoA, inhibiting CPT-1 with resultant inhibition of beta-oxidation (Muoio, 2014; Aguiló, 2015).

2.3.4 Fasting increases AMPK activation

Fasting, which induces ketogenesis, decreases IIS receptor signalling, mTOR pathway activation and increases AMPK activation, leading to increasing longevity gene expression thus increasing housekeeping gene expression profiles, such as stimulating mitophagy and mitochondrial biogenesis (Veech et al., 2017). Fasting mimicking diets (FMD) enable the pleiotropic effects of fueling less from glucose and more off ketones, resulting in mimicking the lifespan inducing metabolic profile (Longo and Panda, 2016). Exercise whilst fasted has also been shown to have a greater impact on increasing insulin sensitivity, decreasing/normalising blood plasma glucose, increasing beta oxidation and more importantly has been shown to increase mitochondrial biogenesis, due to the amplification of AMPK activity as a result of exercise in a fasted and thus ketosis state, leading to increased glycogen depletion, whilst ramping up beta-oxidation (Steinberg and Kemp, 2009).

2.3.4.1 Ketosis increases AMPK activation

Ketosis, where glucose levels are normal or low, insulin is low and ketones are in therapeutic levels (>0.5 mmol/L) with euglycaemia and low insulin, increases AMPK activity

(Bae et al., 2016). Upregulation of AMPK inactivates ACC via phosphorylation which terminates beta-oxidation inhibition via decreasing ACC carboxylation and production of malonyl-CoA, simultaneously increasing beta-oxidation via upregulating activity of CPT-1 (Muoio, 2014).

2.4 FOXO3a

Transcription factor regulator forkhead box O3 (FOXO3a) upregulates gene transcription of over 200 genes that are known as the Dauer effect genes, this is the "stasis" of childhood (Webb, Kundaje and Brunet, 2016). Effectively "arrested" (paused) development, and in whole animal studies, increased expression and activity has resulted in extension of lifespan with healthspan (Birnbaum et al., 2019). Increased activation of FOXO3a leads to increased expression of the oxidative stress defence genes and proteins: GSH, superoxide dismutase (SOD2) and catalase (Gross, Van Den Heuvel and Birnbaum, 2008). Evidence from *in vitro* and *in vivo* research, show longer lived organisms have more FOXO3a expression, with more localisation within the nucleus (Webb, Kundaje and Brunet, 2016).

2.4.1 Hyperinsulinaemia negatively regulates FOXO3a

Hyperinsulinaemia and IIS, inhibits FOXO3a, via Akt mediated AMPK inhibition, leading to impaired expression of energy metabolism and oxidative stress defence genes (Greer et al., 2007; Webb, Kundaje and Brunet, 2016). In-order to be active, FOXO3a must be unphosphorylated, enabling nuclear localisation (Monsalve and Olmos, 2011). Hyperinsulinaemia signalling results in FOXO protein nuclear exclusion (Wolfrum et al., 2003). Insulin directed PI3K-Akt phosphorylation, sequesters FOXO3a in the cytosol, rendering FOXO3a unable to engage and upregulate the transcription of over 200 longevity associated genes (Morris et al., 2015).

2.4.1.1 Hyperinsulinaemia, ROS, microRNA and FOXO3a

Hyperinsulinaemia increases mtROS production leading to increased miRNA155 expression and NFkB nuclear localisation via K-Ras activation (Goalstone et al., 1997; M Goalstone et al., 1998; Golovchenko et al., 2000; Draznin, 2011). Nuclear localised NFkB enables direct binding to the promotor region of miRNA21 gene, and increasing transcription. miRNA155

inhibits FOXO3a, while miRNA21 leads to direct inhibition and downregulation of SOD2 and 3 dismutation activity, and catalase (Babu and Tay, 2019). Of note, miRNA21 targets SOD3 mRNA but not SOD2 mRNA, therefore miRNA21 downregulates SOD3 transcription but only directly targets MnSOD2 protein activity. In short, hyperinsulinaemia increases mtROS, leading to increased miRNA21 expression via NFkB nuclear localisation, resulting in decreased MnSOD2 antioxidant activity which results in further increased ROS levels. Insulin inhibits autophagy, by phosphorylating Akt at Ser473, leading to inhibition of autophagy (Saiki et al., 2011; Hopkins, Goncalves and Cantley, 2020). Hyperinsulinaemia propagates chronic elevated ROS, leading to increased cell cycle progression via promotion of G1 to S phase transition, this and inhibition of autophagy are hallmarks of tumourigenesis (Diebold and Chandel, 2016; Babu and Tay, 2019).

2.4.2 Ketosis a condition of avoidance of excessive insulin signalling, increases FOXO3a

2.4.2.1 BHB and FOXO3a

Modalities that enhance the metabolic state of ketosis, such as exercise, fasting, time restricted feeding (TRF), low carbohydrate healthy fat (LCHF) and well-formulated ketogenic diets (WFKD), and CR, reduce insulin demand and thus secretion, and consequently leads to a reduction in basal and bolus insulin signalling. This results in enabling the increase in beta oxidation and endogenous BHB synthesis (ketogenesis) and utilisation (ketolysis). With increased ketosis, this increases the expression of FOXO3a, catalase and MnSOD2 via AMPK signalling in a dose dependant manner (Shimazu et al., 2013; Veech et al., 2017). BHB signalling induces altered gene expression via the FOXO3a network, promoting improved management towards oxidative stress responses and the cell survival/regulated apoptosis process (Shimazu et al., 2013; Grabacka et al., 2016).

2.4.2.2 HDAC inhibition, regulates expression of oxidative detoxification genes

BHB inhibits histone deacetylase (HDAC) I and II. HDAC I and II inhibition increases the transcription of detoxification genes, such as mt MnSOD, metallothionein 2, and catalase, enabling a greater capacity to counter oxidative stress (Shimazu et al., 2013; Newman and Verdin, 2014a). Histone deacetylases repress gene transcription via lysine residue deacetylation of histone proteins, BHB inhibition of HDACs, results in hyperacetylation,

upregulating transcription of target genes coupled with co-activators and co-repressors associated with HDACs. Exhaustive exercise, fasting and KDs are able to produce physiologically safe nutritional ketosis (0.5-3 mmol/L), and thus induce HDAC inhibition (Cahill et al., 1966; Zupkovitz et al., 2006). Ketosis increases uncoupling protein expression in-order to combat dysregulated mtROS, this enables H+ protons to flow down its concentration gradient, uncoupled to ATP synthesis into the mt matrix. H+ protons may be considered as part of the antioxidant defence mechanism via mt-uncoupling, allowing the translocation of H+ into the mt matrix not through ATP synthase, where they are then able to neutralise mtROS (Mookerjee et al., 2010). Class I and IIa HDACs repress FOXO3a gene transcription, therefore BHB inhibition of HDAC3 results in increasing FOXO3a gene transcription, with this translating into a potential for FOXO3a to then be able to be activated in upregulating its downstream targets (Newman and Verdin, 2017).

2.5 Insulin/IGF1-signallng activates the PI3K/Akt/mTOR and MAPK signal transduction pathway

When nutrient availability is abundant, pancreatic beta-cells secret insulin in response to elevated blood glucose (Cahill et al., 1966; Lizcano and Alessi, 2002). IIS receptor signalling upregulates cellular growth and division pathway activities by activating the phosphatidylinositol 3-kinase/Akt/mTOR (PI3K) pathway via the PI3K phospholipase enzyme, generating second messenger's diacylglycerol (DAG) and 1,3,5-inositol triphosphate (IP3), and Ras/Raf/Mek/Erk (MAPK) signal transduction pathways. IIS activation of the PI3K and MAPK pathways mediate increased: intracellular inflammasome NLRP3 formation and mTOR activation (Cantley, 2002; Dibble and Cantley, 2015; Janus et al., 2016; Fruman et al., 2017; Hopkins, Goncalves and Cantley, 2020). Increasing mTOR activity mediates cell survival mechanisms and decreases autophagy (Yaktapour et al., 2013; Dibble and Cantley, 2015). Chronic mTOR activation is implicated in cancer neoplasia, and chronic elevated insulin signalling, hyperinsulinaemia, is implicated in the pathogenesis of cardiovascular diseases, type 2 diabetes and Alzheimer's disease (Draznin, 2010; Crofts et al., 2015). However, it is essential to recognise that insulin is mammalian life-essential, as a nutrient signalling molecule and growth factor regulator.

The mTOR complex integrates energy and nutrient signals from these nutrient and growth factor metabolic sensing pathways, decreased mTOR activity increases healthspan and lifespan (Saxton and Sabatini, 2017). Hyperinsulinemia/IGF1 (H-IIS) mediated chronic activation of the PI3K and MAPK pathways, drives cellular responses to increase: cellular growth and division, aerobic glycolysis, avoidance of apoptosis, with increased basal ROS production (Hamanaka and Chandel, 2010), at the same time, decreasing: autophagy, mitophagy and mitochondrial biogenesis (Liu et al., 2009; Seyfried, 2015; Janus et al., 2016; Fruman et al., 2017). These propel cells towards rapid replication, decreasing their Hayflick limit of replicative capabilities, and yet unable to trigger apoptosis, due to MAPK activated apoptosis avoidance mechanisms. Chronic IIS mediates vascular remodelling, increased cell-division and proliferation, and prevention of apoptosis and metastasis; these are key hallmarks of cancer (Hanahan and Weinberg, 2011). Furthermore, hyperinsulinemia precedes hyperglycaemia up to 24 years, where chronic IIS induces proliferation of vascular smooth muscle cells, and hyperglycaemia increases blood coagulability, these are prominent features of atherosclerosis and cardiovascular/cerebrovascular diseases (Stegenga et al., 2006; Gunter et al., 2009; Hanahan and Weinberg, 2011; Perkins et al., 2015; Cooper et al., 2020). The culminating effect is the development of more cells with an increased hyperinsulinaemia associated secretory phenotype producing and releasing inflammatory cytokines, leading to increased pathological transformation towards chronic disease manifestations (Wallace, 2005; Harman, 2006; Calder et al., 2017; Veech et al., 2017).

2.5.1 Insulin and insulin-like growth factor receptor signalling

The greatest increase in lifespan with healthspan, in model organism/animal model studies, has been through decreased insulin and insulin-like growth factor receptor signalling (IIS). These studies utilised knock-out, knock-down and FMD/ketosis methodologies. The mutagenesis lifespan extensions were further increased with dose dependent increases in BHB due to FMD or exogenous BHB addition (Narasimhan, Yen and Tissenbaum, 2009; Newman and Verdin, 2014b; Veech et al., 2017). Dysregulated, chronic insulin secretion and signalling, is driven primarily due to dietary derived carbohydrate ingestion that increases blood glucose (Lizcano and Alessi, 2002; Paoli et al., 2013; Muoio,

2014; Crofts et al., 2015). Chronic repeated stimulus results in hyperinsulinemia and IR which instigates a feed forward loop, over time increasing hyperglycaemia, further signalling pancreatic insulin secretion, and thus driving an increased insulin "set point" to be able to maintain euglycemia (Crofts et al., 2015; Hopkins, Goncalves and Cantley, 2020). Insulin signalling negatively regulates ketogenesis (Newman and Verdin, 2014b; Pinto et al., 2018), and increases IGF1 bio-availability via decreasing IGF1-binding proteins (IGF1BP). Furthermore, insulin potentiates IGF1 signalling via insulin receptor (InR) activation of the enzyme farnesyltransferase (FTase), farnesylating the RAS protein, enabling translocation into the plasma membrane, potentiating IGF1 and other growth factor signalling (Draznin, 2010, 2011).

2.5.1.1 Insulin potentiates growth factor signalling via RAS farnesylation

Insulin potentiates the mitogenic signals from growth factors such as IGF1, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and insulin itself, via modulating the physical state of intracellular Ras proteins which are encoded by the H-RAS, K-RAS and N-RAS genes (Shah et al., 2019). These small GTPase Ras proteins operate as intracellular molecular switches in the GEF/SOS-Ras-Raf-Mitogen Activated Protein (MAP) Kinase (MAPK) growth factor mitogenic signal transduction pathway (Krishna and Narang, 2008). Growth factors bind to the extracellular PM side of transmembrane receptor tyrosine kinases (RTK), inducing intracellular autophosphorylation, subsequently eliciting intracellular mitogenic effects via activating MAPK signalling cascade, which is dependent on the state of Ras proteins (Bos, 1995).

When growth factors, insulin inclusive, bind with their cognate receptors, this causes the intracellular side of their receptors to activate guanine nucleotide exchange factors (GEF) to mediate the replacement of GDP that is bound to Ras proteins, with GTP, causing activation of the Ras proteins (Leitner et al., 1997). In order for this GEF-Ras activation to be able to happen (GDP exchange to GTP-Ras activation), the Ras proteins must be bound/anchored to the cytosolic side of the plasma membrane. Ras proteins can only be anchored into the PM when they have a 15-carbon farnesyl moiety attached to them (Draznin, 2010, 2011; Cerqueira et al., 2016).

The farnesylation of Ras is exclusively insulin dependent (Goalstone et al., 1997); insulin binding to specific InRs causes the phosphorylation and activation of the alpha subunit of the FTase enzyme, triggering enzymatic farnesylation of a Ras cysteine residue (Goalstone et al., 1997). Once farnesylated, the Ras proteins are translocated into the PM, and are subsequently able to be further activated as an essential upstream cog within the MAPK signal transduction pathway (Draznin, 2010, 2011). The IIS stimulated mitogenic MAPK is not downregulated nor disturbed in the insulin resistant state, conversely it may be perversely upregulated (Leitner et al., 1997), leading to hyper-farnesyltransferase activity, resulting in increased prenylated/farnesylated membrane bound Ras, further enabling enhanced growth factor mitogenic responsiveness at lower plasma concentrations.

Hyperinsulinaemia independently, but even more so with insulin resistance, upregulates FTase activity, thus increasing the amount of farnesylated membrane bound Ras available, this increases growth factor signal transduction capacity and subsequent potentiation of pathological intracellular mitogenic activated pathways. Hyperinsulinaemia potentiates the cellular mitogenic response magnitude to growth factor stimulation, as insulin mediated Ras farnesylation is a major determinant (Leitner et al., 1997). Experimental studies in mice have supported this finding, where hyperinsulinaemia was induced in foetuses via either direct insulin infusion, or direct glucose infusion, a greater amount of farnesylated Ras proteins and activated farnesyltransferase were found in foetal organs including: liver, fat, skeletal muscles, aorta, white blood cells (WBC), human WBC, and other studies in vascular smooth muscle, endothelial, fibroblasts and breast cancer cells (Stephens et al., 2001). To demonstrate insulin mediated this increase, hyperinsulinaemic mice foetuses were additionally infused with growth hormone-inhibiting hormone (GHIH) also known as somatostatin, results showed complete prevention of the hyperinsulinaemia driven hyper-farnesylated Ras and upregulated FTase activity. These same results were also shown in mice and dogs, indicating a preserved mechanism across species (Stephens et al., 2001; Montagnani et al., 2002; Draznin, 2011).

2.5.1.1.1 The importance of why it matters if cells are overly responsive to mitogenic signals

The MAPK pathway regulates cell cycle rate, proliferation, differentiation and cell death (Krishna and Narang, 2008; Morrison, 2012). Cells need time to perform functional duties in their tissue specific capacity, to organ specific roles, in-addition to having the requisite time needed to grow, synthesise new DNA, repair damaged proteins and DNA before progressing into the mitotic phase. When regulatory signals to halt progression to the next phase are overridden, DNA damage repair activity is decreased, autophagy of damaged proteins are not sufficiently performed, leading to increased transmission of incorrect DNA copying into daughter cells once cells are committed to mitosis. Progression to and through mitosis in this context increases genomic/chromosomal/DNA mutations as a downstream phenomenon of dysregulated/upregulated cell cycle progression, due to hyperinsulinaemia augmenting proliferative events via potentiating growth factor stimulating MAPK responsiveness (Lineweaver, Davies and Vincent, 2014; Seyfried, 2015; Sonnenschein and Soto, 2016).

2.6 Cardiolipin and ROS

2.6.1 ROS mediated lipid peroxidation of cardiolipin

Phospholipid cardiolipin (CL) is biosynthesised by the mitochondria and is almost exclusively found in the cristae of the IMM. CL plays an essential role in the structural integrity of supporting respiratory chain super-complexes, stabilising tertiary and quaternary ETC complexes, substrate carrier proteins, mitochondrial permeability transition pores, and ATP-synthase. CL is an integral component in energy transducing membranes, whilst its unique dimeric-cross-linked phospholipid structure and inherent anisotropy enables the high curvature formation found in cristae (Sedlák and Robinson, 1999). CL is required for the trans-membrane protein-phospholipid complex IV cytochrome C oxidase (CcO). CcO is composed of 13 subunits, subunits I, II and III form the catalytic core of the enzyme and are encoded by mitochondrial DNA. CcO functionally requires CL, stabilising its structural integrity, a necessary requirement for the transport of electrons and translocation of protons (Paradies et al., 2014). Electron transport activity is diminished by 50% with the removal of CL, due to the dissociation of the respiratory chain complexes (Sedlák and Robinson, 1999). CL stabilises and acts as an allosteric ligand for

succinate dehydrogenase (SDH) and is essential for the curtailment of ROS production at this location.

CL is an essential phospholipid required for the catalytic activity of the ETC enzymes (Paradies et al., 2014). Increased lipid peroxidation, results in changes in CL fatty acid profile and total concentration of CL within the IMM. As a result, ETC efficiency reduces, increasing ROS production, concomitantly reducing OXPHOS ATP production capacity. CL is required for the maintenance of the highly invaginated mt cristae and ATP synthasome assembly. The superoxide O_2^- anion is most frequently generated at the ETC complex I nicotinamide adenine dinucleotide (NAD) + hydrogen (H) (NADH) CoQ oxidoreductase when mitochondrial matrix NADH/NAD^+ ratio is high, and especially when CL is oxidised by ROS. ROS induced CL peroxidation induces a vicious positive feed forward cycle (Paradies et al., 2019). If sustained chronically, this positive compounding cycle results in the accumulation of pathological mitochondria that dominate the hierarchy of intracellular signalling. This is seen in the pathogenesis of cellular tumourigenic transformation, with the upregulation of aerobic glycolysis/fermentation via ROS mediated direct mechanisms such as stabilising hypoxia inducible factor-1a (HIF-1a) and upregulating NLRP3 inflammasome assembly, or indirectly via histone deacetylase activity altering gene expression and transcription, leading to substantial changes in cellular phenotype. Consequently these chronic pathological signals dictate a direction towards greater entropic disorder resulting in cellular, tissue and organ system functional decline and disease (Marchi et al., 2012; Newman and Verdin, 2014a; Miller, Villamena and Volek, 2018).

Cells are unable to respire effectively when they have an oxidised CL composition or decreased content of CL. Furthermore, no tumour cells have yet to be shown to have normal content and composition of cardiolipin (Schild et al., 2012). Oxidised CL results in increased ROS, positively feeding forward producing further oxidative damage. ROS diffuse to the cytosol, inhibiting HIF prolyl hydroxylase (PHD) enzymes, resulting in the stabilising of the heterodimeric transcription factor sub-unit, HIF-1a, which is then able to interact with HIF-b, signalling hypoxia. This consequently activates inducible HIF-transcription factors (HIF-TF) to initiate pseudo/hypoxia programmes/signal cascades. Further effects

are in the modulation of gene expression to activate hypoxia mediated responses, such as enhanced gene expression for increased angiogenesis, erythropoiesis, cell cycle and survival mechanisms (Hamanaka and Chandel, 2010). In addition to upregulated glycolysis and glucose uptake and its phosphorylation, this is enabled by HIF driven increase of the glucose transporter GLUT1 (Semenza, 2009). CL is tightly bound to complex V of the ETC, where it is found to be critical in oligomerisation and ATP synthase assembly, depletion of CL correlates to abnormal mitochondrial morphology and subsequent OXPHOS capabilities, and increased ROS production (Paradies et al., 2014).

2.6.2 HIF-1α and ROS

Increasing protein stability of HIF-1a leads to upregulated hypoxia signalling, ROS directly mediates this by oxidation of HIF-1a amino acid residue Cys533 (F Li et al., 2007). Furthermore, indirect upregulation of HIF-1a by ROS is mediated via downregulation of the deacetylase sirtuin enzyme, SIRT1, resulting in the maintenance in acetylation of HIF-1a amino acid residue Lys647, adding and aiding in the stabilising of HIF-1a (Lim et al., 2010). HIF activation also inhibits pyruvate dehydrogenase kinase-1 (PDK1), which in turn inhibits pyruvate dehydrogenase, inhibiting the oxidation of pyruvate to acetyl-Co-enzyme-A (ACoA) in the mitochondria, whilst activating lactic acid dehydrogenase (LDH), resulting in increased aerobic fermentation of glucose (Frezza and Gottlieb, 2009). These ROS induced processes culminate in a dramatic increase in glycolysis and glucose shunting to the pentose phosphate pathway (PPP). ROS signal driven aerobic fermentation of glucose to lactate acid is a metabolic hallmark phenotype of cancer. Furthermore, mitochondrial ROS production may also be induced by hypoxia, activating ROS signalling pathways to promote adaptive transcription programs (Hamanaka and Chandel, 2010).

Chronic hyperinsulinaemia and hyperglycaemia increases haemoglobin glycation damage, decreasing oxygen saturation capacity, contributing to pernicious hypoxia (ACCORD et al., 2008; Cooper et al., 2020; Wang, Du and Zhu, 2020). Hypoxia is a near universal and distinguishing hallmark of cancer growth (Fruehauf and Meyskens, 2007). The epigenome and transcriptome are sensitive to the cellular metabolic state. Chronic hypoxia, coupled with increased ROS instigate inflammation and glycolytic enzyme upregulation signalling

pathways, leading to nuclear gene expression of the tumour phenotype (Carrer and Wellen, 2015; Thakur and Chen, 2019). Perpetuated over time this causes irreversible nuclear and mitochondrial genomic damage, and oncogenic transformation, a downstream epiphenomenon of impaired cellular respiration (Seyfried et al., 2014).

2.7 Dysregulated mitochondria, structure and pathology

The structure of mitochondria is intimately linked to their function, the ultrastructure of mitochondria in: AD, PD, T2D, CVD, tumour and beta-cells are often found to be significantly different from the ultrastructure of normal cell mitochondria (Anello et al., 2005; Yu, Robotham and Yoon, 2006; Arismendi-Morillo and Castellano-Ramirez, 2008; Zhang et al., 2016; Joshi et al., 2018; Chen et al., 2020). Tumour cells often have less mitochondria, with more oxidised CL and less total CL, and many have structurally abnormal morphology, with less or completely absent cristae, the site of OXPHOS (Seyfried, 2015; Paradies et al., 2019). This would support cellular need to upregulate glycolysis and glucose consumption in-order to satisfy cellular energy requirement, and for the PPP $NADP^+$-dependent reduction of cytosolic oxidised glutathione (GSSH) to reduced glutathione (GSH), in-order to counter elevated ROS to enable tumorous cells to survive their increasingly acidic intracellular environment. The degree of decreased mitochondrial numbers and abnormal morphology, is shown to be directly correlated with breast tumour degree of malignancy (Elliott, Jiang and Head, 2012).

2.7.1 Ceramide, ROS and mitochondrial fission, Drp1 and Mfn2

Hyperinsulinaemia increases mtROS production via: ceramide synthesis (Hansen et al., 2014), inhibition of AMPK-SIRT1-PGC-1a-FOXO3a oxidative defence pathways, and decreased NAD+ dependent anti-oxidant activities due to enforced glucose oxidation ATP synthesis. Excess ROS without commensurate antioxidative capacity, leads to greater oxidative damage than rate of repair, such as lipid peroxidation of ROS sensitive phospholipid CL (Paradies et al., 2019). This leads to increased mitochondrial fission, mediated by Drp-1. Furthermore, increased ceramide synthesis leads to stabilisation and increased activity of the inflammation mediators: NLRP3 inflammasome and the NFkB

transcription factor (Smith et al., 2013; Hansen et al., 2014). These pathways are found to be upregulated in a large majority of the leading chronic diseases of ageing.

2.7.1.1 ROS, Drp1, Mfn2, mitochondrial fission and fusion

Insulin mediated mitochondrial ceramide synthesis, directly increasing mitochondrial ROS leads to an increased Drp1 relative to Mfn2 ratio (Hansen et al., 2014). Drp1 promotes mitochondrial fission and Mfn2 promotes mitochondrial fusion (Giacomello et al., 2020). An increase in Drp1 to Mfn2 results in increased mitochondrial fission, leading to impaired energy production and cellular pathology, as seen in osteoporosis, cancer, beta-cell failure and neurodegenerative AD and PD (Manczak, Calkins and Reddy, 2011; Li et al., 2015; Joshi et al., 2018). Interestingly, endothelial dysfunction is ameliorated when AMPK is activated, as AMPK inhibits mitochondrial fission by inhibiting Drp1, this alleviates endothelial endoplasmic reticulum stress (Li et al., 2015). Clearly, healthy mitochondria, as indicated by structure which dictates function, are required for optimal cellular health.

Through multiple compensatory mechanisms that alter nuclear gene expression, chronic elevated ROS may ultimately result in permanent change in mitochondrial superstructure and function, thus induce heavier dependency on glycolysis and further altered intracellular epigenetic modulation. Concurrently, extracellular stimulus may also drive metabolic transformation. Metabolic syndrome (MetS) and T2DM are typified by a state of hyperinsulinemia. Hyperinsulinemia, considered a risk factor for cancer is a phenotype of MetS (Fine et al., 2012). Hyperinsulinemia, hyperglycaemia and inflammation, are suffered by type 2 diabetics, this may be a link as to why diabetics have an increased risk of cancer, cardiovascular disease, and dementia (Tsujimoto, Kajio and Sugiyama, 2017).

2.8 Sirtuins, MnSOD2 and ROS

Mitochondrial ROS damage without commensurate counter ROS management, can increase cellular need for glucose due to impaired and diminished OXPHOS capacity. Excess and enforced glucose fuelling subsequently depletes cytosolic NAD+, further impairing counter ROS management programs due to diminished NAD+ dependent SIRT1 and SIRT3 capacity (Lim et al., 2010; Bause and Haigis, 2013; Brenmoehl and Hoeflich, 2013; Cooper

et al., 2020). Furthermore, OXPHOS ATP synthesis is reduced when cellular need for NAD+ replenishment demand is high, thus further driving aerobic glycolysis activity and demand in a vicious circle (Scheibye-Knudsen et al., 2014; Luengo et al., 2021).

Sirtuins 3, 4 and 5 (SIRT3/4/5) are situated within the mitochondrial matrix. These are NAD+ dependent deacylases, deacetylating specific reversible post-translational modifications (PTM) of acyl-lysine residues that affect ROS damage control. NAD+ SIRT3 activation, deacylates the amino acid residues: lysine 53, 68 and 89 (K53, K68, K89) on the mitochondrial localised antioxidant metalloenzyme, MnSOD2, activating MnSOD2 to catalyse the dismutation of superoxide O2- into H_2O_2 and molecular oxygen (O_2), in the process NAD+ is consumed (Qiu et al., 2010; Chen et al., 2011; Newman and Verdin, 2014a, 2017; Dikalova et al., 2017; van de Ven, Santos and Haigis, 2017). SIRT3 expression has been shown to decrease with increasing biological and chronological age, and by 65 years of age is clinically shown to be reduced by 40%. This is in parallel to increased rates of hyperinsulinaemia metabolic syndromes and hypertension, conditions that reduce SIRT3 activity, have decreased SOD2 antioxidant detoxification activity and consequently increased levels of ROS (Dikalova et al., 2017).

Healthy mtROS management requires functional MnSOD2 activity. Depletion of NAD+ decreases MnSOD2 activity due to diminished SIRT3 activity which is NAD+ dependent. Therefore, metabolic signalling and demand, such as from hyperinsulinaemia enforced glucose fuelling, which drives greater NAD+ consumption relative to ketosis, results in increased mitochondrial ROS production. Chronic cellular glucose fuelling at the expense of beta-oxidation, decreases the NAD+ pool, and thus constrains SIRT3 activity, whilst concomitantly increases acyl donors, thus inhibiting ROS stress management and enhancing ROS signalling and damage (Muoio, 2014; Newman and Verdin, 2014a; Veech et al., 2017). A lack of SIRT3 activity and its downstream affects, decreases metabolic flexibility (Muoio, 2014), and increases mitochondrial ROS without concomitant activated antioxidant management enzymes. This is a cellular phenotype that appears to drive metabolic dysregulation and pathologies associated with ageing, and these pathologies are increasingly manifesting in younger populations with often undetected subclinical hyperinsulinaemia.

Regeneration of the endogenous antioxidant glutathione from its oxidized form (GSSG) to its reduced form (GSH) requires the production of reduced form nicotinamide adenine dinucleotide phosphate (NADP) + hydrogen (H) (NADPH) via isocitrate dehydrogenase (IDH2) which is dependent on NADH provision. Furthermore, glutathione reductase, thioredoxin (TXN) and peroxiredoxin (PRDN) are required to facilitate the redox recycling process (Molavian et al., 2015; Veech et al., 2017). These reactions are limited by the degree of SIRT3 activity, which is further limited by NAD+ availability. Compromised NAD+ and SIRT3 activity is a shared intracellular feature among the chronic hyperinsulinaemia diseases. Ketosis spares NAD+, which logically enables further provision towards sirtuin and PARP activities, leading to greater ROS regulation and DNA repair.

2.9 NRLP3 and NFkB

Hyperinsulinaemia drives increased NFkB and NLRP3 inflammasome complex activation (Golovchenko et al., 2000). NFkB is a transcription factor that regulates the expression of genes involved in the immune and inflammation response, and cytokine signalling, regulating formation of pro-inflammatory mediators tumour necrosis factor-α (TNF-α), and the cytokines interleukin-1β (IL-1b) and IL-6. NFkB is ubiquitously expressed and regulated by the inhibitory protein inhibitor of KB (IkB), causing cytoplasmic localisation (Liu et al., 2017). Dysregulate and upregulated NFkB activity is common in metabolic chronic diseases, such as CVD, neurodegenerative diseases AD and PD, T2DM, that are typified by vascular, tissue and cellular inflammation (Pamukcu, Lip and Shantsila, 2011; Fiordelisi et al., 2019). Elevated levels of TNF-α and IL-1β are detected in AD patient brain, indicating that NFkB activation and cellular inflammation play a functional role in AD pathogenesis (Ju Hwang et al., 2017; Jha et al., 2019). NFkB dysregulation is implicated in PD, in a post-mortem study of PD patient brains (n = 7) versus control (n = 5), there was a greater than 70-fold proportion of dopaminergic neurons of the substantia nigra with increase in NFkB nuclei activation (Hunot et al., 1997; Flood et al., 2011; Bellucci et al., 2020).

BHB ameliorates oxidative stress via AMPK activation which leads to inhibiting the assembly of the NLRP3 inflammasome complex, composed of NLRP3, caspase-1, and apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), and pro-caspase-1. The oligomerisation of the inflammasome complex

triggers autoactivation of caspase-1 and a consequent cascade of cleavage reactions of cytosolic targets, resulting in the activation of the proinflammatory interleukins IL-1β and IL-18 from their pro-inactivated forms (Bae et al., 2016). The anti-inflammatory effects of ketosis is also mediated by inhibiting NFkB, while BHB interacts with the inflammasome in immune cells to reduce inflammatory cytokine production (Achanta and Rae, 2017).

2.10 Beta-hydroxybutyrate (BHB)

The energy and nutrient status signalling metabolite beta-hydroxybutyrate (BHB), is synthesised by hepatocytes when insulin is low enough to no longer inhibit ketogenesis (**Figure 2**). BHB is released into the bloodstream to be used in extrahepatic tissues, to generate the necessary ATP a cell needs in-order to survive and function when glucose is not provided exogenously (Cahill et al., 1966). BHB is produced as a result of fasting, carbohydrate restriction or restricted energy provision to Animalia organisms (Rojas-Morales, Tapia and Pedraza-Chaverri, 2016). When plasma BHB levels are elevated, it acts as a signalling molecule from hepatocytes to the systemic system, altering cellular gene expression and behaviour to adapt to the signal that is telling them "the organism is not receiving much food/nutrients if any at all" and is utilising internal energy stores from fatty acids converted to BHB via ketogenesis. BHB signalling co-ordinates a systemic shift in cellular systems phenotype towards conservation (slowing down the cell cycle/decreasing mitotic rate) and increasing recycling of cellular materials (autophagy and mitophagy), culminating in greater cellular health and survival in the face of starvation (Newman and Verdin, 2014a; Rojas-Morales, Tapia and Pedraza-Chaverri, 2016).

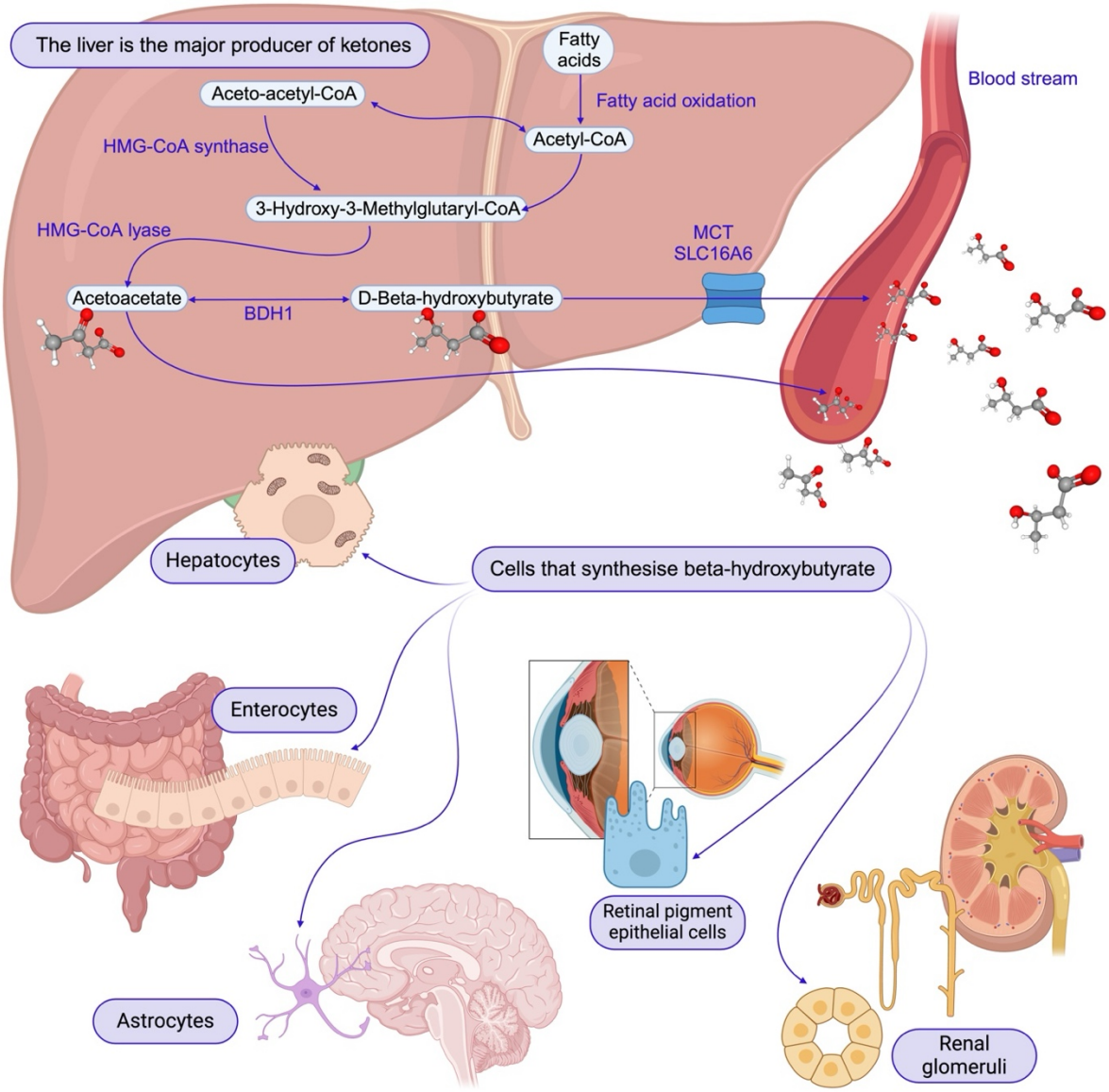

Figure 2. Production of ketone bodies beta-hydroxybutyrate and acetoacetate in the liver and cells in other organs that are also able to perform ketogenesis. Abbreviations: Beta-hydroxybutyrate (BHB); beta-hydroxybutyrate dehydrogenase-1 (BDH1); coenzyme A (CoA); 3-hydroxy-3-methylglutaryl (HMG); monocarboxylic acid transporter (MCT).

Created in BioRender. Cooper, I. (2025) https://BioRender.com/n40n524

2.10.1 Ketosis, a metabolic phenotype that mimics the fasted state

Additional FMDs that do not intentionally restrict calories include; TRF, where food is consumed in a narrow window of time within a 24 hour cycle, such as the 19:5 hour ratio, reflecting an eating window of 5 hours in 24 hours; intermittent fasting (IF), typified by fasting for 2 days out of 7, and WFKD which restrict carbohydrates to within an individuals' tolerance, that does not stimulate enough insulin to inhibit ketogenesis, which is typically < 50 grams of carbohydrates per day, however this varies depending on age, amount of physical activity, degree of metabolic health and presence of a chronic disease associated with hyperinsulinemia which includes CVD, atherosclerosis, cancer, neurodegenerative diseases (AD and PD), T2DM and hypertension (Crofts et al., 2015; Volek et al., 2015; Augustin et al., 2018).

Ketones are synthesised via ketogenesis from fatty acids derived either from adipocytes or from fat within a meal, made by hepatocytes (enterocytes, renal cells, retinal epithelial cells and astrocytes are also known to perform ketogenesis) (Auestad et al., 1991; Blázquez et al., 2002; Adijanto et al., 2014; Grabacka et al., 2016; Reyes-Reveles et al., 2017) (**Figure 3**), when the hormone insulin is sufficiently low enough to no longer inhibit the body's ability to produce ketones (Veech, 2004; Newman and Verdin, 2014a; Veech et al., 2017). Ketones are not only a source of fuel for most cells in the body, they also act as signalling molecules, effectively telling the intracellular organelles, the mitochondria and nuclei, what is going on at the systems level and how best to adapt and respond (Shimazu et al., 2013; Newman and Verdin, 2014a). Fasting mimicking diets such as eating in a narrow time frame within a 24-hour window, or very low carbohydrate-healthy fat with moderate protein diets, known as ketogenic diets, are also able to induce ketosis in individuals, without the conscious effort of calorie restriction (Newman and Verdin, 2014a). The result is decreased dietary induced stimulus of insulin secretion from the pancreas (**Figure 3**), due to decreased glucose nutrient sensing, the primary driver of insulin secretion, which is predominantly due to dietary starchy carbohydrates found in bre ad, pasta, rice, flour, corn, fruit and sugar. Ketosis, results in lower concentrations in the bloodstream of the hormone insulin, lower/well-regulated blood glucose and increased plasma ketone beta-hydroxybutyrate. If sustained, over time this induces a series of adaptive changes within cells, to shift their intracellular machinery to upregulate

their ability to utilise fat and ketones instead of glucose for fuel, however this is only the tip of the iceberg in changes induced by the presence of ketones, as well as the effects due to the decrease in chronic excess insulin signalling on cells (Volek et al., 2015; Miller, Villamena and Volek, 2018).

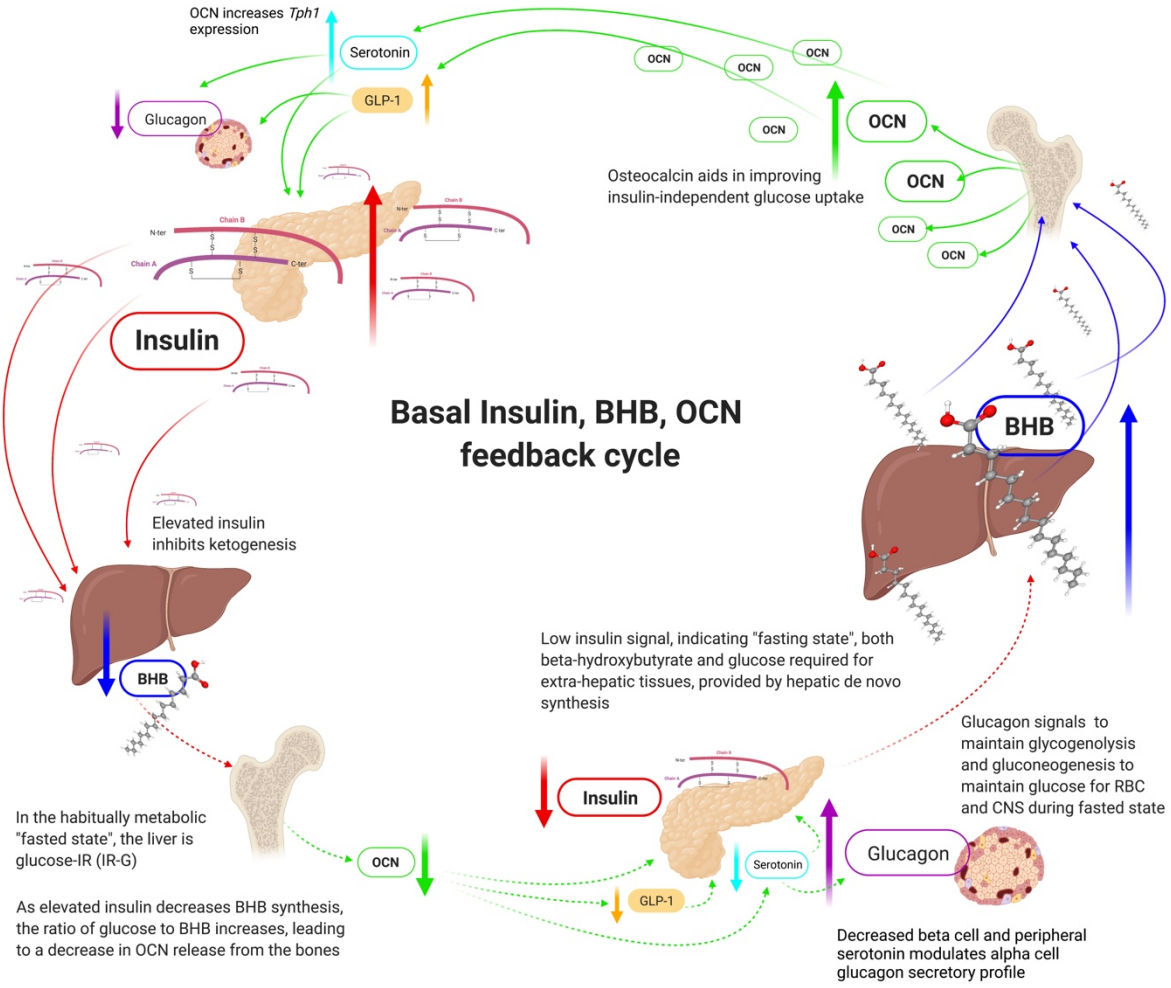

Figure 3. Proposed basal insulin, beta-hydroxybutyrate, osteocalcin feedback cycle in phenotype-1 individuals. Beta-hydroxybutyrate (BHB), central nervous system (CNS), glucagon-like peptide-1 (GLP-1), insulin resistance (IR), glucose-insulin resistance (IR-G), osteocalcin (OCN), red blood cells (RBC), tryptophan hydroxylase (Tph). [Figure from (Cooper, Brookler and Crofts, 2021)].

2.10.2 HCAR2, PI3K and mTOR. Glucose sparing

BHB activates the G-protein coupled receptor, hydroxycarboxylic acid receptor 2 (HCAR2) also known as niacin receptor 1 (NIACR1) and GPR109A. HCAR2 is present on adipocytes, neutrophils, tissue macrophages and in the brain (Rahman et al., 2014). BHB-HCAR2 activation leads to decreasing PI3K activity, resulting in decreased mTOR activity (Newman and Verdin, 2014a). mTOR is an intracellular nutrient and growth factor sensor and mediator of cellular adaptive responses. Decreased mTOR activity increases autophagy and mitophagy, preserving protein status and thus decrease muscle loss in times of fasting (Saxton and Sabatini, 2017). If during times of fasting muscle cells continued to utilise glucose for fuel, instead of fatty acids, this would decrease glucose availability for glucose dependent red blood cells (RBC) and the brain. Thus, hepatocyte BHB signalling decreases myocyte glucose uptake whilst increasing transcription of mitochondrial OXPHOS protein complex genes, in-order to increase beta-oxidation capacity. Concomitantly increasing gene transcription for antioxidative enzymes, improving ROS management, via histone deacetylase inhibition, upregulation of NAD+ sirtuin activity and directly modulating chromatin via beta-hydroxybutyrylation, resulting in decreased basal ROS levels, increasing cellular resilience and survival capacity (Newman and Verdin, 2014a). BHB signalling in the physiological context of metabolic phenotype 1 ketosis (euketonaemia; (Cooper et al., 2021)), co-ordinates cellular function in a multicellular organism into an integrated dynamic physiological system.

2.10.2.1 HCAR2 and the brain neural inflammation.

Ketone bodies area able to cross the blood brain barrier (BBB) via the endothelial proton linked membrane carrier monocarboxylate transporter 1 (MCT1) (Pierre and Pellerin, 2005), and are able to compensate the common deterioration in brain metabolism found in neurodegenerative conditions such as AD and PD, where hyperinsulinaemia is often indicated via insulin resistance and increasing glucose availability being unable to rescue regional brain glucose hypometabolism (Cunnane et al., 2016; Yang et al., 2019). BHB mediates its neuroprotective effects via binding to and activating HCAR2/GPR109A, lowering neuroinflammation (Rahman et al., 2014). Rahman et al. demonstrated in a mouse stroke model, BHB via ketosis, induced neuroprotective effects via altering the

phenotype of brain infiltrated bone marrow-derived macrophages resulting in improved outcomes (Rahman et al., 2014). Furthermore, BHB activation of HCAR2 on macrophages and microglia inhibits NFkB via increasing brain derived neurotrophic factor (BDNF). This leads to a reduction in intracellular pro-inflammatory cytokine production (Rahman et al., 2014; Fu et al., 2015). Elevated NFkB and intracellular cytokines TNF-α, IL-1β and IL-6 are found to be a common pathological intracellular phenotype in neurodegenerative disease and T2DM, conditions that are increased risk factors for poorer outcomes in infectious diseases such as COVID-19, where dysregulated cytokine production is also implicated (Granic et al., 2009; Jones and Kounatidis, 2017; Kandimalla, Thirumala and Reddy, 2017; Kuga et al., 2017; Yang et al., 2019; Cooper et al., 2020).

2.10.3 Nrf2 activations upregulates oxidative stress management

Ketosis, resulting in elevated BHB, low insulin and normoglycaemia, leads to an anti-inflammatory status. Leading to reducing mtROS production while improving OXPHOS efficiency by modulating uncoupling proteins, and able to bypass mtETC complex-1 dysfunction. Complex-1 dysfunction is a key feature in the neurodegenerative diseases AD and PD (Tieu et al., 2003; Achanta and Rae, 2017). BHB activates Nrf2 causing an upregulation in transcription and activity of the endogenous intracellular antioxidant system. NRF2 is a sensor of cellular redox status, BHB signalling results in Nrf2 nuclear localisation. Consequently, NRF2 binds to the antioxidant responsive element (ARE) located on the promotor regions of endogenous antioxidant genes that encode for the cytoprotective proteins and detoxifications enzymes, these include: superoxide dismutases (SOD), peroxiredoxin (PRDN), GSH and thioredoxin (TXN) (Rushmore, Morton and Pickett, 1991; Ma, 2013). Furthermore, activated Nrf2 improves the recycling of endogenous antioxidants via the induction of enzymes that regenerate oxidised antioxidants back into their active forms, these include the peroxiredoxin, glutathione reductase and thioredoxin enzymes. Increased transcription and activity of these anti-oxidant regenerative enzymes improve cellular ROS oxidative stress management and mitochondrial function, strong factors in longevity studies (Dinkova-Kostova and Abramov, 2015; Gureev, Shaforostova and Popov, 2019). These may also provide clues in the role of ketosis on the prevention in development of chronic disease risk and/or amelioration of existing disease.

2.10.4 ATP, electron backflow and superoxide

BHB improves ATP synthesis efficiency via increasing the redox span between the redox couples: nicotinamide adenine dinucleotide (NAD+/NADH) and ubiquinone/ubiquinol (dihydroxyquinone; Q/QH2). BHB metabolism decreases (reduces) the NAD+/NADH ratio while increasing (oxidising) the Q/QH2 ratio, leading to an increased redox span, which results in an increased proton translocation capacity from the mt matrix into the mt inter membrane space via the IMM cristae, as electrons are passed down from NADH to Q. This subsequently increases chemiosmosis ATP generation capacity. The increased redox span also decreases electron backflow via reverse electron transport, which prevents the formation of superoxide $O_2^{\cdot -}$ at complex-1, the most prevalent location of superoxide anion formation in neurodegenerative diseases AD, PD and other hyperinsulinaemia pathologies including T2DM and CVD (Sato et al., 1995).

Increased ROS generation via insulin mediated ceramide synthesis and decreased NAD+ availability due to enforced glucose fuelling, results in a lower redox span, as a result, QH2 electrons are shuttled backwards (reverse electron transport) to complex-1 to molecular oxygen forming the highly reactive superoxide $O_2^{\cdot -}$ anion, a reduced form of oxygen, further driving mitochondrial pathological transformation and increasing cytoplasmic inflammatory pathway activation (Sato et al., 1995; Veech, 2004).

2.10.5 Metabolic phenotypes

It is arguable that to best understand insulin's physiological functions, and effects, it would be to consider and study insulin dynamics in humans that are healthy and have never suffered a hyperinsulinaemia metabolic disease. This healthy representative human would be akin to the historical human metabolic phenotype context. This human metabolic phenotype 1 (**Figure 4**) would most likely be represented as follows: often not eating for 15 to 16 hour stretches and therefore TRF is in practice, only having access to high fibrous low fructose fruits during a short season in the year, and likely to have long periods with no access to carbohydrates and/or a regular significantly low carbohydrate provision in

their diet. This would result in a metabolic blood biomarker phenotype showing: well-regulated healthy glucose levels (euglycaemia), low insulin and presence of the ketone AcAc and ketone body BHB (euglycaemic-euketonaemia). This metabolic phenotype 1 biomarker profile, may also be described as nutritional ketosis (NK) (Miller, Villamena and Volek, 2018; Cooper et al., 2020, 2021).

It is essential to study and elucidate insulin dynamics in the context of healthy physiological function to better understand pathological transformation, function and consequences. This then aids in informing choice in clinical test strategies that provide better resolution in diagnostics and the development of more effective clinical and non-clinical intervention management algorithms.

The current research conducted in healthy human controls, are typically in those with the metabolic phenotype 2 (**Figure 4**). In phenotype 2, insulin's basal hierarchical role has been shifted into primarily managing glycaemia. Over time this downregulates ketogenesis and ketolytic gene expression, and alters many other signalling pathways (Somogyi, 1941; Casals et al., 1992; Nadal, Marrero and Haro, 2002; Grabacka et al., 2016; Newman and Verdin, 2017). This increased burden placed on pancreatic beta-cells and basal insulin function, to maintain normo-glycaemia, has cellular consequences above and beyond plasma glucose regulation (Ebeling, Koistinen and Koivisto, 1998; Draznin, 2010; Hansen et al., 2014; Hopkins, Goncalves and Cantley, 2020). Such as, tissues and cells are exposed to a higher basal insulin level, that affects cells irrespective of glucose uptake. This increase in basal insulin secretion results in increased cellular nutrient sensing, signalling nutrient abundance, which reduces AMPK activity (Hawley et al., 2014; Jeon, 2016), increases activation of the phosphatidylinositol 3-kinase/Akt/mTOR (PI3K) and MAPK growth and division signal transduction pathways respectively. Increased levels of basal insulin signalling alters cellular sensitivity and responsiveness to growth factors such as to IGF1 via Ras prenylation/farnesylation (Cantley, 2002; Draznin, 2010; Fruman et al., 2017). Furthermore, the increased reliance on insulin to regulate plasma glucose uptake, results in inhibition of hepatic ketogenesis, beta-oxidation and glycogenolysis, whilst driving up *de novo* lipogenesis and glycogen synthesis. Each of these responses to the increased basal insulin, result in further homeostatic responses that ultimately shift the set points on many

hormones and other molecules within cells and in the extracellular environments, in order to maintain adaptive homeostasis (Rojas-Morales, Tapia and Pedraza-Chaverri, 2016).

The commonly investigated healthy human metabolic phenotype, phenotype 2, is subtly different to phenotype 1's metabolic parameters (**Figure 4**) (Cooper et al., 2021), and consequently the metabolic hormones and metabolites hierarchy in function and their signalling dynamics are likely to differ (Cahill et al., 1966; Robinson and Williamson, 1980; Rojas-Morales, Tapia and Pedraza-Chaverri, 2016). Most human research with healthy controls, are conducted in phenotype 2 humans, with a common metabolic phenotype of fasting insulin < 200 pmol/L, fasting glucose > 3.5 – 6.0 mmol/L, fasting BHB < 0.3 mmol/L, with a Kraft pattern I insulin dynamic response pattern and osteocalcin levels greater than in those with hyperinsulinaemia conditions T2DM and CVD (Saleem, Mosley and Kullo, 2010; Crofts et al., 2016; Razny et al., 2017; Song, 2017; Crofts et al., 2019; Guney et al., 2019a; Riquelme-Gallego et al., 2020), and assumes this is the natural optimal state of healthy humans. Therefore, the ranges, clinical investigations and researchers may find and conclude for hormones, metabolites, cytokines, vitamins, minerals, intracellular and extracellular factors, are representative of phenotype 2's ideal physiological roles and ranges. Furthermore, due to the difficulty in diagnosing hyperinsulinaemia in its earliest stage, much research with "healthy human controls/phenotype 2" often include participants who appear to have healthy glucose and HbA1c levels, however the glucose and HbA1c levels may likely have been maintained by pernicious chronic higher than ideal levels of insulin (phenotype 3-stage-1), where there is no overt hyperglycaemia (see figure 2, metabolic phenotypes schematic proposal) (Crofts et al., 2016, 2019; Cooper et al., 2020).

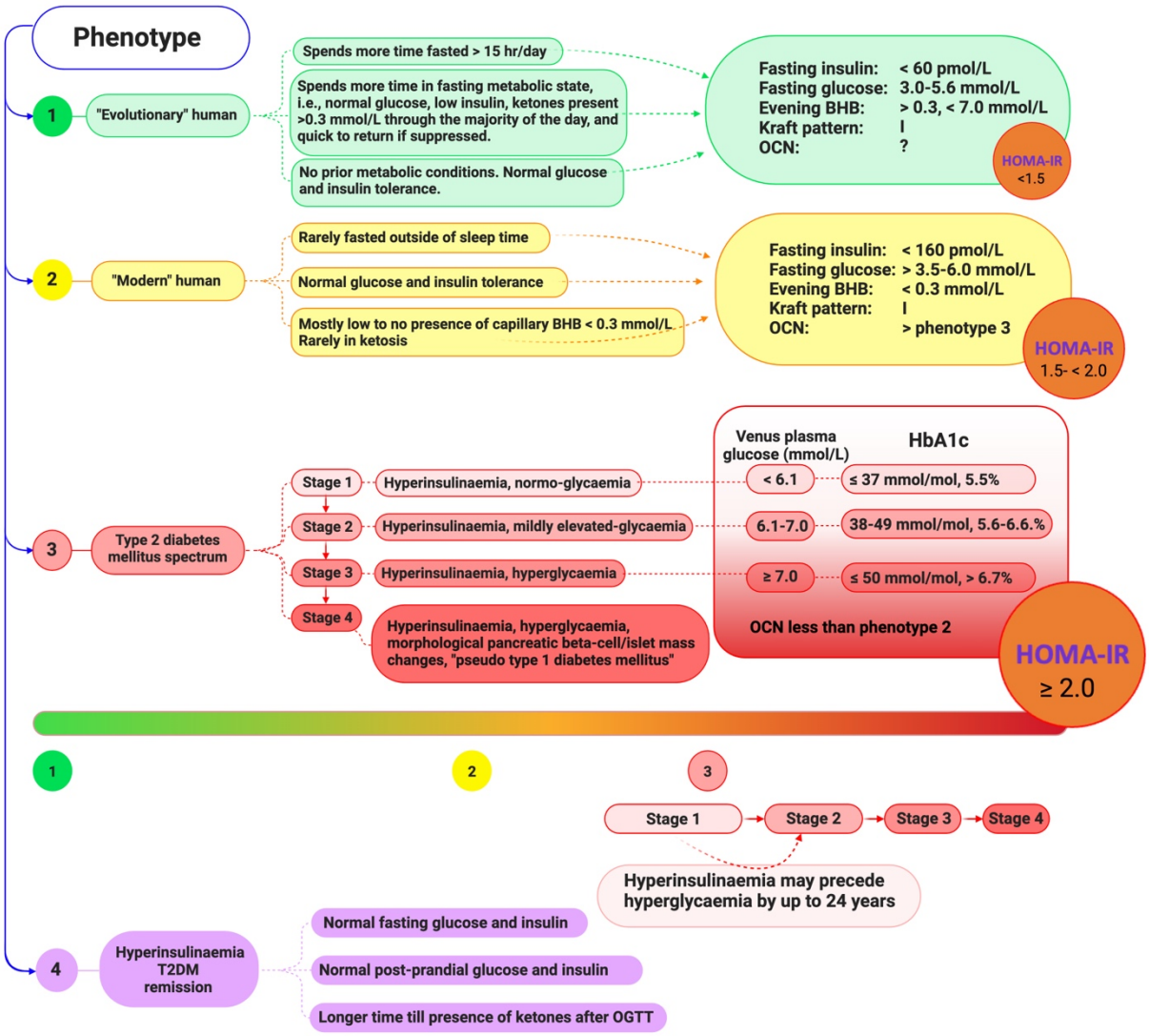

Figure 4. Classification of metabolic phenotypes. Beta-Hydroxybutyrate (BHB); haemoglobin A1c (HbA1c); oral glucose tolerance test (OGTT); osteocalcin (OCN); type 2 diabetes mellitus (T2DM). [Figure adapted from (Cooper, Brookler and Crofts, 2021)].

The pooling of phenotype 2 and phenotype 3-stage-1 (T2DM spectrum-stage-1, fasting plasma glucose < 6.1 mmol/L, HbA1c ≤ 37 mmol/mol or 5.5%, see figure 1) participants together due to not picking up on the pernicious low-grade higher levels but within range insulin concentration, as the healthy controls in studies, may further contribute to increased noise in investigative studies. In addition to potentially setting the foundation of generating what would appear to be correct interpretation of experimental results, leading to drawing conclusions based on faulty premises. An example to illustrate this: research on pancreatic beta-cell mass in type 2 diabetics. End stage chronic T2DM post mortem analysis of pancreatic beta-cells, show a loss in beta-cell mass, this has led to the literature published and the research community concluding that T2DM is a condition of insulin insufficiency due to loss in beta-cell mass (Chen et al., 2017). This description is more akin to type 1 diabetes (T1DM), the defining difference between T1DM and T2DM is insulin insufficiency in the former results in ketoacidosis with decreased serum bicarbonate. This is not the case for T2DM, where there are higher levels of insulin than phenotype 1 or 2 individuals (Savage et al., 2010; Seetho and Wilding, 2014; Cooper et al., 2020; Gosmanov and Nematollahi, 2020). Research conducted in obese (hyperinsulinaemic) individuals, provide evidence that in the earlier stages of hyperinsulinaemia, beta-cell mass is increased (Ogilvie, 1933; Cho et al., 2011). However, due to incorrect grouping of metabolic phenotype peoples in studies, this often causes the cancelling out of data and consequent contradictory or unclear results. Such that when participants with phenotype 2 and phenotype 3-stages-1 and stage-2 are pooled (normal beta-cell mass and increased beta-cell mass respectively) and then compared to phenotype 3-stage 3 plus stage 4 (T2DM spectrum-stage-4, hyperinsulinaemia with hyperglycaemia, morphological pancreatic beta cell/islet mass change, pseudo-type-1 diabetes) patients (increased beta-cell mass mixed with decreased beta-cell mass), this scenario increases the likelihood of not capturing the increase in beta-cell mass in the earlier longer lasting phase of hyperinsulinaemia, phenotype 3-stages 1-2.

Determining a more refined approach to ascertain the metabolic phenotype of the individual, especially for investigative studies and patient screening/treatment, will enable gaining better resolution in elucidation of what are the physiological roles and ranges for hormones and metabolites within each health-stage context.

2.10.6 Insulin's primary (hierarchical) function is the regulation of ketogenesis in phenotype 1 individuals. These individuals may serve as a better baseline comparator, in normative values.

With decreased demand on basal insulin levels to function in glucose homeostasis, insulins primary function may be better understood. In phenotype 1, insulin's primary function appears to be: regulation of endogenous *de novo* ketogenesis, aid in nutrient sensing, growth factor signalling/sensitivity and intracellular ROS hormesis regulation (Draznin, 2010, 2011; Hyde et al., 2017). In this context, it is hypothesised that presence of plasma BHB in non T1 diabetics, at nutritional ketosis levels > 0.3 mmol/L and < 10 mmol/L, serves as a proxy-marker that an individual's basal insulin is likely to be in a range reflective of their ideal-healthy level. In other words, when there is presence of BHB (in non T1 diabetics), an individuals' insulin levels is likely to be at a level that has a lower negative effect on blood biomarker metabolic phenotype changes, where higher insulin levels may induce a biomarker trend in the directions associated with pathology and ageing. In addition, those parameters drive the intracellular changes that reflect the hyperinsulinaemia intracellular/mitochondrial phenotype that drives morbidity pathogenesis at the cellular level (Veech et al., 2017; Miller, Villamena and Volek, 2018).

A fasting-insulin test does not always identify hyperinsulinaemia. Fasting glucose and insulin can often appear healthy, especially in the earlier stages of hyperinsulinaemia (phenotype-3, stage-1) and HbA1c can often be maintained in-range by an insulin concentration within levels seen in non-diabetics (Crofts et al., 2019; Cooper et al., 2020). An illustration to demonstrates this point, without any clear reference range for fasting insulin nor a quick clear ability to determine individual ideal values, using a hypothetical fasting insulin reference range of 21–153 pmol/L (3.5–25.5 µU/mL), where 1 µU/mL = 6pmol/L (Knopp, Holder-Pearson and Chase, 2019): a patient's fasting blood test results may show, glucose 5mmol/L with insulin 144 pmol/L (24 µU/mL). In this example, this patient may have low grade pernicious chronically elevated than ideal insulin concentration for that individual. This enables the maintenance of a healthy glucose concentration. With these blood results, the patient would most likely not be considered hyperinsulinaemic, and consequently be missed for early interventions, or pooled in a study with the "healthy control" group (Crofts et al., 2016, 2019; Cooper et al., 2020).

In contrast to the first example, a fasting blood test sample from a true non-hyperinsulinaemic individual, may show a fasting blood glucose at 5 mmol/L with much lower insulin levels at 24 pmol/L (4 µU/mL). This individual does not maintain a healthy fasting glucose via a higher basal insulin level, and instead achieves healthy glucose homeostasis by maintaining either a low carbohydrate intake and/or lower frequency of carbohydrate intake, or practices: TRF, consumes one meal a day (OMAD), IF, and/or CR. These are essentially lifestyle methods that result in generating a FMD metabolic phenotype, which is akin to what would be seen in the phenotype 1 individuals' profile. This maintains healthy blood glucose concentration via the upstream input level, while also not frequently stimulating insulin secretion, especially not frequently calling upon pancreatic beta-cell first phase bolus glucose stimulated insulin secretion (GSIS). Thus preventing, both hyperinsulinaemia and hyperglycaemia development over time, through one action, the removal of external inputs (lifestyle) that place increased demand on insulin to regulate glucose homeostasis, outside of hepatic gluconeogenesis (Crofts et al., 2016, 2019; Cooper et al., 2020).

In both example cases provided, glucose and insulin appear to be healthy. The only way to know if someone is truly hyperinsulinaemic (excluding those who have overt hyperglycaemia and/or overtly elevated insulin levels) is by oral glucose tolerance and insulin sensitivity postprandial dynamic testing. In the aforementioned examples, if both patients were tested 2 hours postprandially, the first patient's glucose may have returned to 6.5 mmol/L, yet their insulin may still be elevated at >1000pmol/L (166 µU/mL). In the second example, this patient will more likely have a blood glucose reading of 5.3 mmol/L, with a markedly lower insulin concentration of 144pmol/L (24 µU/mL). These examples demonstrate the limitations of first and fasting readings, if dynamic response tests are not performed. As a result, this may lead a clinician/researcher to think a patient/participant is neither hyperglycaemic nor hyperinsulinaemic.

2.10.7 Suppressing ketosis in phenotype 1 individuals likely causes a shift in metabolic biomarkers associated with ageing and chronic diseases, towards the negative trend

Postprandial dynamic testing is required to truly ascertain hyperinsulinaemia (Crofts et al., 2016, 2019). There is a need for investigation in phenotype 1 individuals. It is likely that phenotype 1 individuals will present with plasma BHB levels > 0.3 mmol/L throughout the day, including in the evening. The evening reading may be the more reliable marker as a proxy to indicate the individual's basal insulin is within their "ideal" level, as this avoids the effect of an overnight fast. Given phenotype 1 individual's metabolic profile indicates predominant reliance on ketones and fatty acids for fuel, while preserving glucose for glucose dependent cells such as mature red blood cells (RBC), it is also likely that BHB will not be suppressed for greater than 3 hours after an oral glucose tolerance test (OGTT) challenge. However, this is likely to be lost if the phenotype 1 individual were to suppress ketosis for a consistent duration of time. This would result in a similar dynamic post prandial test response, however, BHB will be continually suppressed due to basal insulin having been kept at a level for a duration of time, that result in a significant shift in metabolic phenotype, from phenotype 1 to 2, as seen via presence or absence of a BHB response curve in an OGTT and/or in the evening, or 3 hours post prandial (Crofts et al., 2016, 2019; Rojas-Morales, Tapia and Pedraza-Chaverri, 2016).

An increasing body of literature is showing the metabolic phenotype of nutritional ketosis as seen in phenotype 1, as the opposite of hyperinsulinaemia. Ketosis provides an explanation in the retention of health, as observed in caloric restricted modules such as C. elegans, mice and primate studies (Weindruch and Walford, 1982; Mattison et al., 2012; O'Flanagan et al., 2017; Roberts et al., 2017; Veech et al., 2017), through maintenance of organismal level and cellular level homeostasis, resulting in the prevention of chronic disease pathogenesis. Furthermore, ketosis has increasingly been shown to aid in cellular repair of hyperinsulinaemia cellular damage, as indicated in phenotype 4 individuals (Paoli et al., 2013; Hyde et al., 2017; Sampson et al., 2017; Veech et al., 2017; Athinarayanan et al., 2019).

While suppressing ketosis, as an intervention, in phenotype 1 individuals for three weeks, may not constitute chronic suppression, the increasing demand placed on insulin to regulate glucose homeostasis leads to suppression of BHB synthesis for a duration that results in a lack of return of ketones 3 hours post-prandially, due to insulin mediated down regulation of ketogenesis and ketolytic enzymes (Puchalska and Crawford, 2017). This potentially results in a combined negative trend in biomarkers indicating intracellular oxidative resilience/stress and progression towards intracellular phenotypes often detected in chronic metabolic diseases (Hansen et al., 2014; Crofts et al., 2015; Sampson et al., 2017; Veech et al., 2017; Parker et al., 2018; Norwitz, Hu and Clarke, 2019). Although these intracellular changes between phenotype 1 and phenotype 2 are not expected to be extreme in difference, as both phenotypes are considered healthy, it is potentially arguable that chronic sustained phenotype 2, without much time spent in phenotype 1, may be the greatest silent part in the development of hyperinsulinaemia (Crofts et al., 2015, 2016, 2019), called sub-clinical hyperinsulinaemia.

This hypothesis may be tested in an *ex vivo/in vitro* stimulation model, using plasma from phenotype 1 individuals, in their baseline natural habitual state, and from after a period of 3 weeks of ketosis-suppression. It is hypothesised that cells cultured (SH-SY5Y and C2C12 cells, used in cell-model investigations in neurodegeneration dementias AD and PD, and T2DM and sarcopenia respectively),(Hansen et al., 2014; Hong et al., 2020) with phenotype 1 plasma may display an intracellular phenotype that is trended towards an associated healthier configuration. These markers include levels of ROS generation, anti-oxidative capacity, the NAD+/NADH ratio, and mitochondrial fission levels as indicated by the ratio of Drp1 to Mfn2, the fission and fusion mediating proteins respectively (Manczak, Calkins and Reddy, 2011; Reddy et al., 2011; Smith et al., 2013; Li et al., 2015; Joshi et al., 2018).

2.10.7.1 Hyperinsulinaemia in its earliest stages is “invisible”

It is currently difficult to diagnose hyperinsulinaemia in its earlier stages. There is no clear ideal reference range for fasting insulin, insulin has a pulsatile, ultradian and circadian rhythm, making it further difficult to capture the peak diagnostic reading, as a result fasting insulin does not always capture hyperinsulinaemia (Crofts et al., 2016). Dynamic insulin

response curves via OGTT have been shown to provide the most reliable method of determining an individual's metabolic phenotype and hyperinsulinaemia status, however this method is time intensive and costly (Crofts et al., 2016; DiNicolantonio et al., 2017). Additionally, with the current approximation reference ranges for fasting insulin, an individual's fasting insulin may be within range and yet be perniciously higher than what is ideal for their own cellular health and subsequent chronic health trajectory (Crofts et al., 2016, 2019; Cooper et al., 2020). While classical understanding of insulins primary role is considered to be the regulation of plasma glucose levels; in the fasted state where insulin is very low, glucose dependent cells do not rely on insulin action for glucose uptake, and insulins manifold functions pre-dominate (Cahill et al., 1966; Marliss et al., 1970; Goalstone et al., 1997; M Goalstone et al., 1998; Golovchenko et al., 2000; Ahrén and Thorsson, 2003; Draznin, 2011; Hansen et al., 2014; Hyde et al., 2017; İyikesici et al., 2017; Poff et al., 2019; Hopkins, Goncalves and Cantley, 2020).

2.10.7.2 Has there been a misplaced demonisation of ketones?

Before the discovery and manufacturing of insulin, dangerously high levels of BHB >10 mmol/L (with concomitant hyperglycaemia >13.8 mmol/L (250 mg/dL), and decreased bicarbonate pH <15-18), were a sign and symptom of T1DM. This is now known as the triad of diabetic ketoacidosis (Savage et al., 2010; Seetho and Wilding, 2014; Gosmanov and Nematollahi, 2020). As a result, BHB was considered a toxic dangerous waste product, and consequently, used as a determinant for dysregulated metabolic health. If investigation of BHB had been conducted in longstanding metabolically flexible habitual ketosis individuals (phenotype 1, see figure 1) during this time, it would have been found that BHB functions not only as an energy metabolite, but also as an anti-oxidant capable of scavenging free radicals, a cell signalling molecule and an epigenetic gene expression regulator via histone modification (Cahill and Veech, 2003; Veech, 2004; Grabacka et al., 2016; Puchalska and Crawford, 2017; Veech et al., 2017; Miller, Villamena and Volek, 2018). The absence of BHB in phenotype 2 individuals enabled the misattribution of BHB as a mediator and marker of pathology.

2.10.8 Osteocalcin is a metabolic hormone that may aid in further enhancement in diagnosing hyperinsulinaemia as part of a metabolic panel

Patients with T2DM and insulin resistance have significantly lower levels of circulating Osteocalcin (OCN) than healthy controls (Saleem, Mosley and Kullo, 2010; Razny et al., 2017; Guney et al., 2019b; Riquelme-Gallego et al., 2020). OCN is a non-collagenous protein, synthesized by osteoblasts and osteocytes that retain their expression of OCN (Wei and Karsenty, 2015), and levels serve as a marker of osteoblast and osteocyte health (**Figure 5**, schematic representation showing the dynamic role of osteocytes in the regulation of healthy and dysregulated bone and OCN involvement). Serum OCN levels positively correlate with: dynamic bone remodelling, decreased insulin resistance (IR), and reduced T2DM and CVD risk (Hill et al., 2014; Popko et al., 2018; Moser and van der Eerden, 2019; Riquelme-Gallego et al., 2020). Much published research show OCN increases insulin secretion, with conclusions stating decreased OCN levels would result in decreased insulin synthesis and secretion, and result in impaired glucose homeostasis. However, when assessed in humans, hyperinsulinaemia tracks with low levels of OCN (Aguayo-Ruiz et al., 2020; Riquelme-Gallego et al., 2020). Thus, providing evidence that production of high levels of insulin does not require high levels of OCN.

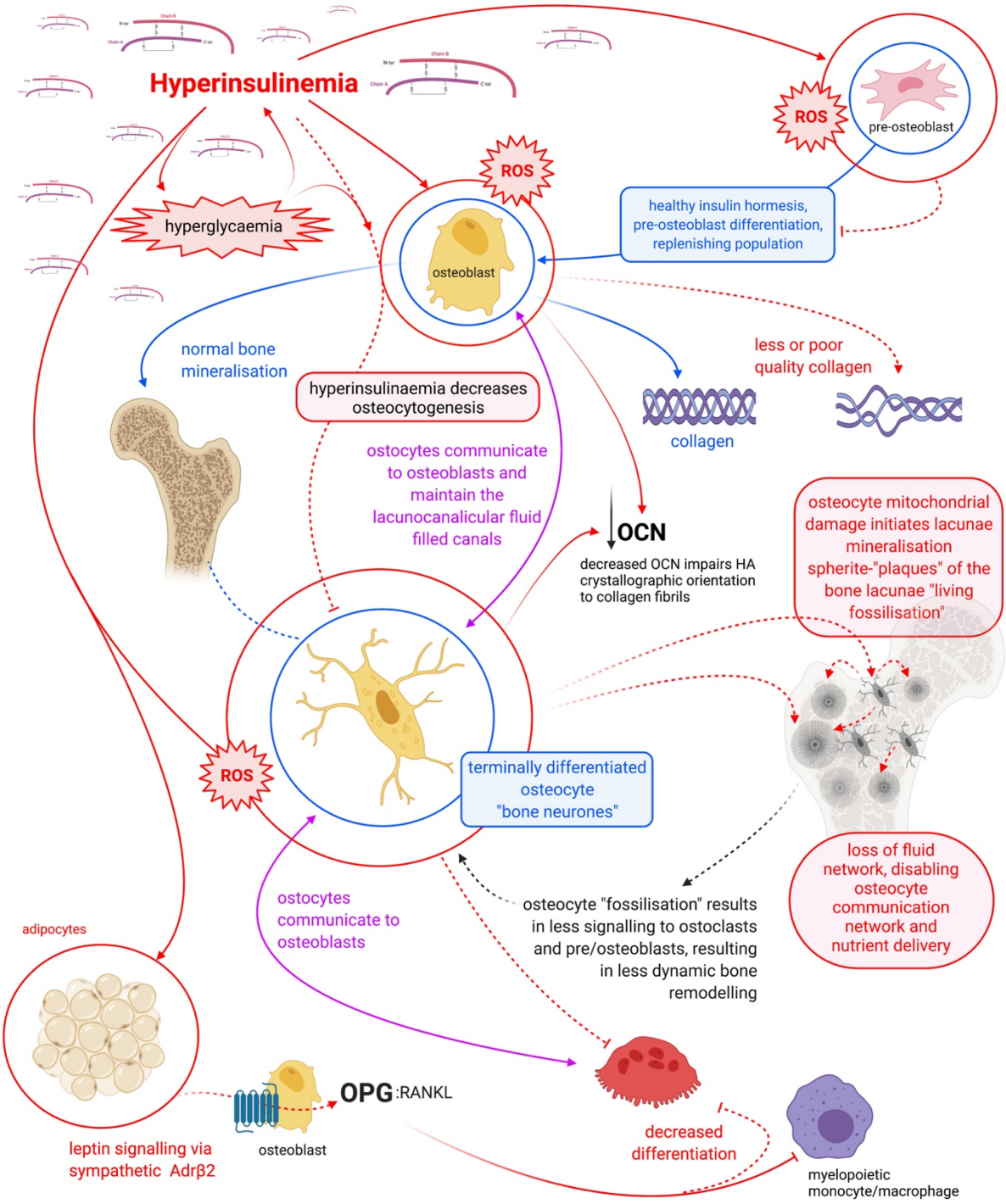

Figure 5. Schematic representation showing the dynamic role of osteocytes in the regulation of healthy and dysregulated bone. Beta-adrenergic receptor (Adrβ2), hydroxyapatite (HA), osteocalcin (OCN), osteoprotegerin (OPG), receptor activator of nuclear factor kappa-β ligand (RANKL), and reactive oxygen species (ROS). Red lines indicate hyperinsulinaemia-driven pathology pathways. Healthy physiology indicated with blue and purple lines. [Figure published in (Cooper, Brookler and Crofts, 2021)].

Interestingly, OCN significantly increases insulin-independent glucose uptake, and even more so in the presence of insulin. This leads to increased insulin sensitivity, through reducing the amount of insulin required to facilitate glucose uptake (Hill et al., 2014). Additionally, OCN production increases the expression of mitochondrial UCP1 in adipocytes, leading to increased thermogenesis and mitochondrial biogenesis, thus increasing glucose and fatty acid oxidation capacity. Furthermore, OCN increases adipocyte production of adiponectin. Osteocalcin exerts a large amount of its effects via adiponectin (Hill et al., 2014). However, under high insulin conditions, adiponectin receptors are downregulated via insulin activating the PI3K/FOXO1 signal transduction pathway. This diminishes the osteocalcin/adiponectin induction of AMPK, PGC-1a, mitochondrial biogenesis and increased thermogenesis, that all act to facilitate glucose uptake and oxidation, independent of insulin (Tsuchida et al., 2004).

Hyperinsulinaemia individuals have lower plasma levels of the bone hormone OCN, and a lower carboxylated to undercarboxylated ratio. Metabolically healthier individuals have higher levels of osteocalcin. Osteocalcin levels in phenotype 1 individuals may decrease with sustained suppression of ketosis (Saleem, Mosley and Kullo, 2010; Razny et al., 2017; Guney et al., 2019b; Riquelme-Gallego et al., 2020). Osteocalcin has been shown to function in glucose homeostasis, a decline in OCN would indicate a negative trend in bone metabolism (Ferron and Lacombe, 2014; Hill et al., 2014; Ferron, 2018).

Osteocalcin aids in regulating hepatic glucose output via glucagon like peptide-1 (GLP-1) and glucagon inhibition, and insulin independent skeletal and adipocyte glucose uptake, while further increasing uptake in the presence of insulin, thus acting as an insulin sensitiser which results in decreasing insulin requirement, which aids in decreasing basal insulin secretion and whole body overall excess insulin exposure (Cahill et al., 1966; Owen et al., 1967; Ferron et al., 2008; Kanazawa et al., 2009; Ferron and Lacombe, 2014; Rojas-Morales, Tapia and Pedraza-Chaverri, 2016). GLP-1 agonists are currently a major interest in drug management in T2DM and insulin resistance, the strongest risk factors for coronary heart disease (Lee and Lee, 2017; Mora et al., 2021). To date, OCN has not been investigated in longstanding metabolically flexible habitual ketosis individuals (phenotype 1), where its role in energy metabolism is not well defined (Ferron and Lacombe, 2014).

Suppression of ketosis may affect OCN levels, contributing to increased hepatic glucose output, a factor in the development of T2DM. Greater understanding of OCN levels between the metabolic phenotypes may provide further insight and resolution in detecting earlier transition towards hyperinsulinaemia potentially enabling earlier intervention.

2.10.9 Summary, Aims and Objectives

1. Hyperinsulinaemia is a common denominator of chronic diseases that constitute the major morbidities and mortality.
2. Hyperinsulinaemia drives intracellular changes that are consistently common in many of the vastly different types of cells that are in poor health, found in these common chronic diseases. Those commonalities are in fact a hyperinsulinaemia intracellular phenotype, that predicts disease manifestation depending on which cell type and location they are situated in, in the body. Furthermore, the hyperinsulinaemia state drives a dysregulated mitochondrial phenotype, in contrast, nutritional ketosis without prior hyperinsulinaemia pathology, prevents or reduces progression in dysregulated mitochondria and consequent cellular health.
3. To understand dysregulation of insulin, and its subsequent effects in the body, at the cellular and systemic level, we must understand and investigate insulin regulation and its broader effects in the healthy human physiological context. Defining metabolic phenotypes enables us to consider challenging the current accepted dogmas related to the role of insulin in pathophysiology. It is also to indicate that we may have been studying the "wrong" group of people, the common modern human (phenotype 2), as opposed to evolutionary human (phenotype 1) (Cooper et al., 2021). This control group (phenotype 2) often also (wrongly/mistakenly) includes phenotype-3 stage—1 people, resulting in more skewing/loss in resolution with regards to experimentation/interventional trials and results produced. This likely contributes to incorrect assumptions and conclusions in investigative studies.
4. Proposal of a phenotype descriptive organisation, of human metabolic and hyperinsulinaemia phenotypes, that present a sliding spectrum (see figure 1), in order to provide a more succinct, clear picture and ability to categorise and communicate what existing research has been done. For instance, to be able to better contextualise

existing research by answering the question: is a model truly reflective of healthy physiology, or does the model actually appear more reflective of type 1 diabetes? With consideration to the possibility that phenotype 1 may be the most "ideal" metabolic phenotype, reflective of a healthy human metabolic state maintained over time, this may impact future research, to be conducted with greater care, especially when creating genetic knock-out and animal model studies.

5. **Hypothesis 1**: 21 days of suppressing nutritional ketosis in metabolic phenotype 1 individuals, will result in changes to biomarkers associated with chronic diseases and ageing, primarily fasting insulin and IGF-1. Furthermore, phenotype 1 individuals will have a unique oral glucose tolerance test with insulin and ketone sensitivity curve pattern. It will be similar to phenotype 2, except they will have a distinctive concurrent beta-hydroxybutyrate curve, indicating hepatic selective insulin sensitivity. Their graph curve patterns should be the normative comparator. i.e., they are pattern Ia of pattern I using the Kraft model, where phenotype 2 have pattern Ib (Crofts et al., 2016).
6. **Hypothesis 2**: 21 days of suppressing nutritional ketosis will affect fasting OCN levels and potentially other markers that OCN regulates. OCN levels in phenotype 1, longstanding metabolically flexible habitual ketosis individuals has yet to be investigated, along with what would happen to OCN levels as ketosis is suppressed for three weeks. Osteocalcin (OCN) is a bone derived protein that functions in healthy bone formation and as an endocrine hormone. OCN has been shown to regulate glucagon like peptide-1 and thus likely to regulate glucagon effects on hepatic glucose output, and enhance insulin sensitivity through improving insulin independent glucose uptake, and increasing adipocyte adiponectin synthesis (Mizokami et al., 2013; Hill et al., 2014).

2.11 Conclusions

Ageing at the cellular level may be summarized as a cells' rate of damage verses rate of repair. Where over time, rate of repair cannot at least match the rate of damage (Harman, 2006; McHugh and Gil, 2018). The accumulation of damage within a cell ultimately begins to manifest either in cellular dysregulation, where the cell no longer functions correctly as part of a collective of cells that make up the tissues of the organ the cell is located in. This dissociation from regulated cellular behaviour and/or de-differentiation is the modus-operandi of the cancer cell phenotype (Sonnenschein and Soto, 2016). The ageing cell also begins to send out destructive inflammatory signals, such as from cytokines and reactive oxygen species (ROS) that signal neighbouring and distant cells in other organs, eliciting adaptive and often when chronic, maladaptive homeostatic responses, to these biological messengers (Harman, 2006).

2.11.1 Chronic oxidative stress decreases intracellular housekeeping capacity, a feature of chronic disease and ageing

In a healthy individual, old and young, wayward-cells implement active cellular response management systems of repair and/or removal and replacement, these include: autophagy, mitophagy and mitochondrial biogenesis, the "eating up, breaking down and recycling" of intracellular organelles, that have been sensed as damaged or impaired (Nourshahi et al., 2012; Schiavi et al., 2013; Ploumi, Daskalaki and Tavernarakis, 2016; Moloudizargari et al., 2017; Nakamura and Yoshimori, 2018; Bouchez and Devin, 2019). Thus, containing and removing intracellular debris accumulation, of which some "debris" may in turn drive further pathology such as accumulation of the protein amyloid beta Ab in AD (Qiu and Folstein, 2006; Ricciarelli and Fedele, 2017). Functional and arguably optimal intracellular housekeeping activities reduces: dysregulated cellular behaviour, and the production and release of inflammatory signalling molecules as a result of cellular decline in function. Apoptosis, controlled cell death enables the well-regulated removal of the whole cell, when the damage is beyond repair. This function is greatly diminished in individuals with chronic diseases and absent in cancer cells (Schiavi et al., 2013).

Intracellular housekeeping processes enable the culling of inefficient and even potentially toxic cells from the herd that makes up a tissue, and thus the upkeep of a healthy optimally

functioning organ (Herst et al., 2017). However, over time a cell's ability to trigger apoptosis becomes impaired for a variety of reasons. The stimulus may not be strong enough, or a counter pathological stimulus prevents apoptosis, and the gradual dysfunction within the cell persists, undetected thus able to keep causing pernicious destruction. Over time, accumulation of these cells begin to accumulate as a substantial population in an organ or tissues, at this point manifestations in overt pathogenesis of chronic disease symptoms may begin to present (Marchi et al., 2012).

This internal quality control is governed by a multitude of factors, such as the availability and utility of the type of cellular fuelling substrate that impacts intracellular cytosolic nicotine adenine dinucleotide (NAD+) availability, which may profoundly affect mitochondrial (mt) reactive oxygen species (ROS) production and defence management, and epigenetic gene regulation (Carrer and Wellen, 2015; Bouchez and Devin, 2019; Thakur and Chen, 2019; Luengo et al., 2021). In the generation of two acetyl-moieties, glucose fuelling consumes a greater amount of NAD+ than fatty acid, BHB and AcAc combined, two, one and zero NAD+ respectively. Therefore, cellular fuelling substrate type has consequences above energy provision, as many cellular repair enzymes are NAD+ dependent, such as SIRT1 and SIRT3, and the upregulation in transcription and activities of MnSOD2 and glutathione (Vassilopoulos et al., 2011; Newman and Verdin, 2014a; Scheibye-Knudsen et al., 2014; Verdin, 2015; Veech et al., 2017). The type of fuel cells use to power their energy dependent activities, instigate intracellular signals and phenotype adaptation. Furthermore, nutrient sensing hormones like insulin, not only aid in the uptake of glucose, insulin also inhibits beta-oxidation and ketolysis, thereby enforcing glucose fuelling as primary substrate (Muoio, 2014; Saxton and Sabatini, 2017), consequently playing an integral role in intracellular housekeeping functions and capacity.

2.11.2 Maintaining cellular differentiation, a marker of health

Humans are multi-cellular organisms. Healthy metazoan cells operate collectively, biological eusociality. When considering healthy ageing and extending lifespan, context must first be considered. At the cellular level, the cell must not only live longer, but must function correctly. Cancer cells are long-lived and capable of unlimited replicative cycles;

however, they do not function eusocialistically, they do not engage regulated apoptosis when they do not function in accordance to their tissue origin phenotype (Lineweaver, Davies and Vincent, 2014; Sonnenschein and Soto, 2016). Unicellular organisms such as bacteria and yeasts have a different concept of longevity. Indefinite numbers of replication, or stasis. In humans, the goal would be to maintain optimum organ function, enabling the whole system to thrive, with the aim of ensuring a long healthspan with negligible senescence, in the pursuit of immortality (Khokhlov, 2013) .

Chronic hyperinsulinaemia in effect is the metabolic opposite of ketosis, given insulin suppresses ketogenesis, and subsequently deprives the body of the anti-ageing properties that are being discovered in this small molecule BHB (Veech, 2004). Decreased insulin signalling has been shown to increase healthspan and lifespan (Newman and Verdin, 2014a). Although once again, insulin is life essential, it has a hormesis zone. Too much drives hyperinsulinemia, strongly associated with chronic diseases and ageing, such as chronic hyperinsulinaemia prevents cells from being able to commit apoptosis when needed (Wilcox, 2005; Crofts et al., 2015), and speeds up a cell's cycle, to replicate itself faster, preventing any pauses to check for DNA replication errors, and preventing a good dose of intracellular housekeeping (McHugh and Gil, 2018). Instead hyperinsulinaemia signals to the cell that energy is abundant, and therefore there is no need to be conservative with resources and keep a tight ship. Thus, although life essential, insulin may be viewed when in chronic excess, as an ageing hormone, one that inhibits the synthesis of BHB, a metabolite that appears to be a powerful anti-ageing factor in human metabolic phenotypes (Lizcano and Alessi, 2002; Narasimhan, Yen and Tissenbaum, 2009; Draznin, 2010; Cooper, Kyriakidou, Petagine, et al., 2023).

The combination of increased signalling from ketones, coupled with decreased signalling from the growth hormone insulin, propagates intracellular adaptive responses that result in increased efficiency in ATP production with increased intracellular housekeeping activity, resulting in cells that are able to remove and replace old organelles via autophagy and mitophagy, thus ensuring a decreased load from residual damage that gradually impedes a cell's function. This also translates into more time for DNA to be checked by DNA housekeeping proteins, of which there are also more as a result of being in ketosis,

that are able to prevent duplication errors from being propagated (ensuring copying fidelity) into the next cell division's daughter cells, thus avoiding a step closer to potential cancers or other age associated chronic diseases (Veech, 2004; Newman and Verdin, 2014a; Veech et al., 2017).

Understanding an underlying upstream common denominator in chronic diseases, hyperinsulinaemia, thus enables the development of effective prevention strategies based on targeting the logical mediators of hyperinsulinaemia. Furthermore, understanding that BHB with normo-glycaemia serves as a proxy indicator that insulin levels that are non-pathological. In this phenotype context, BHB not only independently confers cellular benefit, it produces the sum of actions that include systems level vascular health. This is via: prevention/reduction of excess glycation oxidative stress, and reduction in coagulopathy, through to intracellular modulations including improving mitochondrial function, mitophagy/biogenesis, autophagy, increased endogenous anti-oxidant transcription and activation, increased expression and activation of FOXO3a, AMPK, PGC- 1a, better regulation of mTOR, leading to protein preservation a critical factor in preventing cancer cachexia and sarcopenia (Cooper, Kyriakidou, Petagine, et al., 2023). Finally, preserving lean muscle mass facilitates maintaining a healthy glucose sink, while feed-back signals from myocytes to bone cells and adipocytes form a network in metabolic adaptive homeostatic regulation.(Ferron and Lacombe, 2014; Ferron, 2018; Kirk et al., 2020; Robling and Bonewald, 2020)

Deterioration in the body's systems with ageing, such as chronic insulin signalling is mechanistically associated with increased incidence of cognitive decline such as neurodegenerative diseases, malignancies, cardiovascular disease, deterioration of skin elasticity, decreased exercise performance, sarcopenia and loss of muscle strength (Draznin, 2010; Giovannucci et al., 2010; Kandimalla, Thirumala and Reddy, 2017). Many of these ageing associated conditions have been shown to be decreased by the production and use of ketone bodies, a normal and healthy metabolite that we are able to produce when we do not overstimulate the production of insulin, of which the greatest stimulant of insulin secretion is through our dietary choices (Kinzig, Honors and Hargrave, 2010; Veech et al., 2017). Beta-hydroxybutyrate is able to directly neutralise ROS, thus making it

a powerful endogenous anti-oxidant (Miller, Villamena and Volek, 2018; Pinto et al., 2018). The common recommendation often given to maintain or improve health, is to eat to keep up energy and health, however perhaps a little less, results in a little more with regards to healthspan and lifespan. Instead of having to achieve this by calorie restriction, other methods of inducing habitual ketosis may be applied in order to harness the bodies energy signalling system to improve/maintain health, such as by either eating only once a day *ad libitum* or eating foods that minimise stimulation and secretion of insulin. The result is the same as fasting and calorie restriction, less insulin secretion/requirement, and more ketones, this in turn is showing to translate into healthier cells, leading to healthy tissues and organs, thus providing a plausible chance to realise maximal lifespan potential.

2.12 List of common intracellular factors in hyperinsulinaemia chronic diseases and ageing

- Increased ROS
- Increased Drp1:Mfn2 ratio
- Increased mitochondrial fission
- Decreased NAD+: NADH ratio
- Decreased MnSOD, SIRT3
- Decreased FOXO3a transcription
- Increased FOXO3a phosphorylation (cytosolic sequestration)
- Decreased AMPK signalling
- Decreased PGC-1a activity
- Increased mTOR signalling
- Increased PI3K/Akt and MAPK signalling
- Increased NLRP3 inflammasome assembly
- Increased NFkB activation

Commonalities extracellular:

- Vascular inflammation, measured by glycation damage and coagulopathy indicators.
- Decreased anticoagulant factors such as heparan sulphate proteoglycans
- Hyperinsulinaemia with and without hyperglycaemia, marked by lack of BHB as insulin levels may appear to be “within” reference range.

Chapter 3

Ketosis Suppression and Ageing (KetoSAge): The effects of suppressing ketosis in long term keto-adapted non-athletic females

Chapter 3 comprises the introduction, and materials and methods of this thesis' published articles: Cooper et al., 2023 (Cooper, Kyriakidou, Edwards, et al., 2023) and Cooper et al., 2024 (Cooper et al., 2024).

A non-randomised cross-over trial testing the hypothesis proposed in chapter 1, that 21 days of suppressing nutritional ketosis in metabolic phenotype 1 individuals, will result in changes to biomarkers associated with chronic diseases and ageing, primarily fasting insulin and IGF-1. Furthermore, phenotype 1 individuals will have a unique oral glucose tolerance test with insulin and ketone sensitivity curve pattern. It will be similar to phenotype 2, except they will have a distinctive BHB curve that will not be present in phenotype 2.

KetoSAge Part 2: The Effect of Suppressing Ketosis on HOMA-IR, Leptin, Osteocalcin and GLP-1, Biomarkers Associated with Ageing in Healthy Females, is a follow up investigation of the open labelled non-randomised cross-over trial, testing a second hypothesis proposed in chapter 1. That 21 days of suppressing ketosis in metabolic phenotype 1 individuals, will result in changes to the biomarker osteocalcin, a bone protein and hormone involved in metabolism, insulin, HOMA-IR, leptin and GLP-1, markers associated with chronic diseases and ageing (Cooper et al., 2024).

https://doi.org/ 10.3390/ijms242115621

https://doi.org/10.3390/biomedicines12071553

Abstract

Metabolic disfunctions are among the best documented hallmarks of ageing. Cardiovascular disease, Alzheimer's disease, cancer, type 2 diabetes mellitus, metabolic dysfunction-associated steatosis liver disease, and fragility fractures are diseases of hyperinsulinaemia that reduce life and healthspan. Hyperinsulinaemia and obesity contribute to accelerated ageing and chronic disease risk. While ketosis mimics caloric restriction, promoting beta-hydroxybutyrate (BHB) production as an energy source, past studies have primarily focused on short-term effects, male athletes, or weight loss contexts. The impact of long-term ketosis in non-athlete, healthy females, and the effects of short-term ketosis suppression after long-standing ketosis remain unclear. Hereby, the effects of short-term ketosis suppression, was studied, in healthy women on long-standing ketosis. Ten lean (BMI 20.5 kg/m^2 ± 1.4), metabolically healthy, pre-menopausal women (age 32.3 ± 8.9 years) maintaining nutritional ketosis (NK) for an average 3.9 years (± 2.3), who underwent three 21-day phases: baseline nutritional ketosis (NK; P1), suppressed ketosis (SuK; P2), and returned to NK (P3). Adherence to each phase was confirmed with four times/day, evenly spread in awake hours, daily capillary D-beta-hydroxybutyrate (BHB) tests (P1 = 1.9 ± 0.7; P2 = 0.1 ± 0.1; and P3 = 1.9 ± 0.6 mmol/L). Ageing biomarkers and anthropometrics were evaluated at the end of each phase and an oral glucose tolerance test (OGTT) was given with real time BHB sensitivity testing. Ketosis suppression significantly increased: insulin, 1.78-fold from 33.60 (± 8.63) to 59.80 (± 14.69) pmol/L (p = 0.0002); IGF1, 1.83-fold from 149.30 (± 32.96) to 273.40 (± 85.66) ug/L (p = 0.0045); glucose, 1.17-fold from 78.6 (± 9.5) to 92.2 (± 10.6) mg/dL (p = 0.0088); respiratory quotient (RQ), 1.09-fold 0.66 (± 0.05) to 0.72 (± 0.06; p = 0.0427); GGT,1.29-fold from 9.63 (± 3.13) to 12.40 (±2.55) U/L (p = 0.0087), PAI-1, 13.34 (± 6.85) to 16.69 (± 6.26) ng/mL (p = 0.0428), HOMA-IR, 2.13-fold (p = 0.0008); leptin, 3.35-fold (p = 0.0010); total osteocalcin, 1.63-fold (p = 0.0138); and uncarboxylated osteocalcin, 1.98-fold (p = 0.0417), VEGF, EGF and monocyte chemotactic protein-1/CCL2 also significantly increased, indicating a pro-inflammatory shift. 21 days of ketosis suppression significantly decreased: beta-hydroxybutyrate, 13.50-fold (p = 0.0012) and glucagon-like peptide-1 (GLP-1), 2.40-fold (p = 0.0209). All markers returned or trended back to baseline in P3, indicating a dietary composition containing enough carbohydrates to SuK over 21 days, akin to 66 OGTTs was causal in the P2 changes. OGTT confirmed healthy glycaemic responses across phases, while U shaped curves for BHB in both ketosis phases defined participants' metabolic state. SuK P2 resulted in a very low BHB flat line. In all phases, markers remained within healthy reference ranges. Sustained ketosis showed no adverse health effects and may mitigate hyperinsulinemia without impairing metabolic flexibility and carbohydrate tolerance in metabolically healthy women. Reduced insulin requirements and IGF-1, with elevated BHB levels, lower chronic disease risk and may offer anti-ageing benefits.

3. Introduction

The 21st century bears the hallmark of an ageing global population, in an estimated 8 billion people by 2023 (O'Connor et al., 2023). Ageing is typically understood in its chronological context, the length of time passed since a person's birth, whereas biological age (BA) is the measure of functional age, often measured in terms of physical and mental performance as well as morbidities that decrease quality of life and youth-span (Cooper, Kyriakidou, Petagine, et al., 2023; Yang et al., 2023). By 2030, one in every six Europeans are expected to be aged over 60 years, and by 2040, a quarter of older adults will surpass 85 years of age (UN, 2019), reflecting chronological aged population. This demographic shift gains paramount significance when viewed through the prism of health implications associated with ageing. In 2019 and 2022, the leading cause of death for females in England and Wales, was Alzheimer's disease (AD) and other dementia's (ONS, 2023), followed by cardiovascular disease (CVD) and stroke, as well as cancers including: tracheae, bronchus and lung; colon and rectum; prostate; breast; and lymphomas and multiple myeloma (WHO, 2019). These diseases also top the leading causes of death in the United States, with CVD leading, closely followed by AD and cancers (WHO, 2019).

Concurrently, chronic non-communicable diseases promote an earlier decline in BA. This decline in BA is disturbingly evidenced in an analysis of 8,721 participants from the National Health and Nutrition Examination Survey (NHANES) 2009-2016 showed that the proportion of metabolically healthy Americans decreased from 19.9% to 12.2%, which means the metabolically unhealthy went from 80.1% to 87.8% and are on the hyperinsulinaemia spectrum (Araújo, Cai and Stevens, 2018; Cooper, Kyriakidou, Petagine, et al., 2023). Ageing is associated with increased risk and rates of non-communicable chronic diseases, including CVD, AD, hypertension, type 2 diabetes mellitus (T2DM), metabolic syndrome (MetS), metabolic dysfunction-associated steatotic liver disease (MASLD), chronic inflammation, and cancer (Cooper, Kyriakidou, Petagine, et al., 2023). These conditions detrimentally affect quality of life, healthspan, and lifespan. Specifically, MetS emerges as a direct consequence of chronic hyperinsulinaemia, which is closely linked to inflammation (Crofts et al., 2015; Cooper et al., 2021; Ambroselli et al., 2023; Cooper, Kyriakidou, Petagine, et al., 2023).

The chronic non-communicable diseases AD, CVD, cancer, type 2 diabetes mellitus (T2DM), metabolic syndrome (MetS), metabolic dysfunction-associated steatotic liver disease (MASLD) and chronic inflammation are the consequence of lifestyle factors that stimulate chronic excess insulin demand and secretion, termed hyperinsulinaemia (Crofts et al., 2015; Cooper et al., 2021; Younossi et al., 2021; Cooper, Kyriakidou, Edwards, et al., 2023; Cooper, Kyriakidou, Petagine, et al., 2023; Petagine, Zariwala and Patel, 2023). Hyperinsulinaemia is understood to be an ageing metabolo-endocrine state, and can be staged as metabolic phenotypes (MP) (Cooper et al., 2021; Cooper, Kyriakidou, Edwards, et al., 2023). Hyperinsulinaemia, when assessed as fasting insulin above the reference range only, likely does not capture the sub-clinical occult phase of hyperinsulinaemia (Yang et al., 2023). This thesis argues that hyperinsulinaemia can be detected before exceeding the broad reference ranges which are population derived, which are not filtered to exclude sub-clinically hyperinsulinaemia individuals, and do not reflect the individual's optimal level of insulin.

Lifespan, healthspan and cellular health are greatly influenced by nutrient availability. When the availability of nutrients is low, cells prioritise essential functions over cell division, which slows progression through their replicative cycles preserving their Hayflick limit (Hayflick and Moorhead, 1961). Carbohydrate restriction (CR) and fasting have been shown to extend lifespan (Veech et al., 2017). Cells are capable of sensing energy availability and nutrient types, activating intracellular signalling pathways to stimulate anabolic or catabolic processes which affect cellular health and longevity (Saxton and Sabatini, 2017; Pignatti et al., 2020; Ottens, Franz and Hoppe, 2021). Glucose, fatty acids, D-beta-hydroxybutyrate (BHB) and protein metabolic substrates serve as indicators of the body's overall metabolic condition and nutrient availability. CR and fasting induce a metabolic phenotype called ketosis (Cooper et al., 2021), characterised by decreased glucose and insulin levels and elevated BHB concentration; this state is termed nutritional ketosis (NK) when detectable plasma BHB concentration begins to exceed > 0.3 mmol/L and < 10 mmol/L, with endogenous insulin production below a level that inhibits ketogenesis (Cooper et al., 2021).

Chronic insulin secretion and signalling, driven by dietary sources of glucose, leads to hyperinsulinaemia and/or insulin resistance, and consequently chronic diseases which decrease healthspan by accelerating cellular growth and division whilst impeding apoptosis and promoting production of inflammatory cytokines. Reducing insulin and insulin-like growth factor receptor signalling (IIS) as well as increasing BHB has been found to increase lifespan and healthspan in model organisms and animal studies (Newman and Verdin, 2014a; Roberts et al., 2017; Veech et al., 2017; Napoleão et al., 2021). Conversely, ketosis has been shown to increase healthspan and lifespan through mechanisms, such as promoting transcription of longevity-related genes, increasing autophagy, mitophagy and mitochondrial biogenesis, and enhance antioxidant production (Shimazu et al., 2013; Newman and Verdin, 2014a; Grabacka et al., 2016; Wang, Chen and Xiao, 2021; Cooper, Kyriakidou, Petagine, et al., 2023). Fasting mimicking diets (FMDs), including ketogenic diets, upregulate beta-oxidation, ketogenesis and ketolysis, enhance mitophagy, increase mitochondrial biogenesis and alter gene expression promoting oxidative stress responses and cell survival (Puchalska and Crawford, 2017; Miller, Villamena and Volek, 2018; Qu et al., 2021; Benjamin et al., 2022; Cooper, Kyriakidou, Petagine, et al., 2023).

Historical and emerging research demonstrates the positive impact of ketogenic metabolic therapy (KMT) in treating and preventing neurological diseases, CVD, cancer, T2DM and chronic inflammation (Zhu et al., 2022). Insulin negatively regulates 3-hydroxy-3-methylglutaryl-COA (HMG-CoA) synthase the rate limiting enzyme for ketogenesis (Nadal, Marrero and Haro, 2002; Grabacka et al., 2016). Dietary farinaceous and sucrose rich foods are potent stimulators of bolus insulin secretion (DiNicolantonio et al., 2017; Cooper et al., 2021). Repeated bolus glucose excursions chronically stimulate bolus insulin synthesis and release, and over time downregulate ketogenesis enzyme expression, leading to chronic hypoketonaemia (Nadal, Marrero and Haro, 2002; Cooper et al., 2021; Cooper, Brookler and Crofts, 2021). There are a paucity of trials studying long-standing ketosis metabolically healthy individuals, that sustain ketosis as their normal metabolic phenotype 1 lifestyle (Cooper et al., 2021), and even fewer on active, yet non-athletic females. Therefore, this trial studied the effect of suppressing ketosis for 21 days in this demographic cohort. In order to suppress ketosis, participants followed the Standard U.K. (SUK) dietary guidelines, which recommend the daily consumption of at least 267 g of carbohydrate per day for

women (PHE, 2016). Following the intervention to suppress ketosis, participants returned to NK, and were reassessed 21 days later, to better understand if changes seen after suppression of ketosis for 21 days were due to the intervention, and to investigate metabolic flexibility.

A symphony of biomarkers taken together are more sensitive to aid in capturing hyperinsulinaemia as early as possible, especially in those with a body mass index (BMI) less than 25 kg/m^2 (Nabulsi et al., 1995; Kempf et al., 2006; Salvatore et al., 2022; Yang et al., 2023), thus enabling earlier intervention, preventive care and minimising the incorrect grouping of research participants which may lead to increased false negatives in study results. A prospective cohort study from the NHANES, n = 12,563 with median age 45 years (20-85 years), found hyperinsulinaemia to be a greater risk marker for increased mortality rather than BMI (Wiebe, Muntner and Tonelli, 2022). As this study was extensive with a vast number of biomarkers measured and data captured, it therefore required publication in parts. Part 1 published a report on the effect of suppressing ketosis on ageing and chronic disease associated biomarkers which included fasting insulin, insulin-like growth factor 1, glucose, beta-hydroxybutyrate (BHB), gamma-glutaryl transferase, plasminogen activator inhibitor-1, monocyte chemotactic protein and more (Cooper, Kyriakidou, Edwards, et al., 2023). Part 2 investigates additional biomarkers strongly associated with morbidity including; homeostasis model assessment for insulin resistance (HOMA-IR), osteocalcin (OCN), leptin and glucagon-like peptide-1 (GLP-1) which are biomarkers positively and negatively associated with chronic diseases and ageing. The aim was to investigate the effect of long term sustained nutritional ketosis (NK), also known as euketonaemia, on these biomarkers, and if suppressing ketosis for 21-days would result in any measurable changes, allowing us to understand one factor of lifestyle that may meaningfully impact these biomarkers, which could reduce the earlier onset of BA, meaning a potential to enhance youth-span as well as lifespan.

3.1 Materials and Methods

3.1.1 Ethical Approval

Ethical approval was obtained by the College of Liberal of Arts and Sciences Research Ethics Committee, University of Westminster, United Kingdom (ETH2122-0634). All procedures were conducted in accordance with the Declaration of Helsinki and UK legislation. Written informed consent was obtained from all participants prior to their participation.

3.1.2 Participants

Ten healthy pre-menopausal habitually keto-adapted women, "metabolic phenotype 1" as defined by capillary BHB (> 0.3 mmol/L) and low fasting insulin < 130 pmol/L, with normo-glycaemia (Cooper et al., 2021), [age, 32.30 years ± 8.97; body mass index (BMI), 20.52 kg/m^2 ± 1.39] were recruited to take part in this three phase study named KetoSAge (Cooper, Kyriakidou, Edwards, et al., 2023). Participants were not receiving hormonal birth control and self-reported adherence to a lifestyle that sustained NK for ≥ 6 months (mean 3.9 ± 2.3 years) ensuring sufficient time for metabolic adaptations. Ketosis-adaptation was proven during a 6-month lead-in period where participants were required to take a once daily capillary BHB reading between 4–6 p.m., before the evening meal, prior to commencement of the study. This standardised evening measurement was chosen due to it being a more rigorous threshold to pass in-order to be judged as sustaining NK over the majority of the 24-hour day, in comparison to morning fasted measurements, thus increasing confidence in the participants maintaining NK most of the time. Readings were taken with a Keto-Mojo™ GKI multi-function meter (Keto-Mojo, Napa, CA, United States; (Moore et al., 2021)). A summary of the participants' characteristics at baseline is given in **Table 1**.

Exclusion criteria for all participants included smoking, taking any medication, and no evidence nor history of metabolic syndrome, immunological, or CVD. Participants were required to complete a medical history questionnaire to confirm that they were free from any of the above diseases.

3.1.3 Study design

The 10 KetoSAge participants took part in an open-labelled, non-randomised cross-over trial with three 21 day phases: baseline NK defined as BHB ≥ 0.5 mmol/L (Phase 1; P1), suppression of ketosis (SuK) BHB < 0.3 mmol/L with dietary carbohydrate reintroduction following the UK Eatwell Guidelines, following Standard U.K. diet (SUK) (Phase 2; P2) which recommends consuming a predominance of calories from carbohydrates (e.g., 55% kcal from carbohydrates on a 2,000 kcal diet is 275 g/day net carbohydrate. The final phase is the removal of intervention, returning to NK (Phase 3; P3) (**Figure 6**). At the end of each of the 21-day study phases, on days 22, 44 and 66, the participants attended the laboratory for one day at 8 a.m. after a 12-hour fast to undertake anthropometric measurements and blood sampling (**Figure 6**).

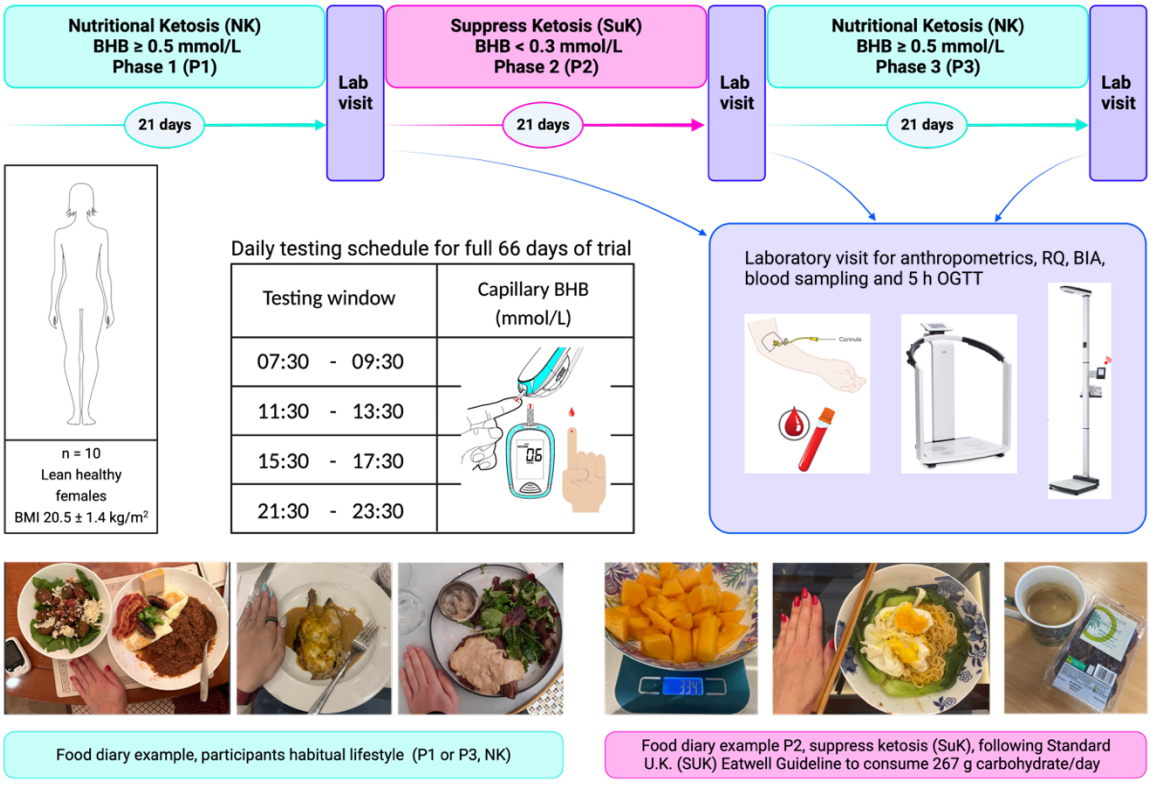

Figure 6. KetoSAge Study design. Phase 1 and 3 covered the participants' habitual ketosis lifestyle. Phase 2 was the interventional phase to suppress ketosis (SuK). Each phase was monitored by finger prick testing of capillary beta-hydroxybutyrate (BHB) concentration (mmol/L). Testing was conducted four times per day, prior to mealtimes at evenly spaced intervals. At the end of each phase, participants underwent a laboratory testing-day for body composition, blood sampling for biomarkers associated with chronic diseases and ageing and were given an oral glucose tolerance test (75g glucose in 250 mL water). Blood samples were taken at seven time points over 5 hours. Whole blood glucose, and BHB were measured sequentially in real time using

the Keto-Mojo™ Meter and plasma insulin sensitivity assay was conducted later using enzyme linked immunosorbent assay (ELISA).

For the duration of the study, participants were required to monitor their capillary glucose and ketone BHB concentrations (mmol/L) at four time points throughout the day to ascertain compliance (**Table 2 – 3**). Timepoints were between: 7:30-9:30 am, 11:30-13:30 pm, 15:30-17:30 pm and 21:30-23:30 pm. Participants determined capillary glucose and BHB using a Keto-Mojo™ GKI multi-function meter (Keto-Mojo, Napa, CA, United States). This equipment was selected for its reliability and good diagnostic performance (Moore et al., 2021).

During P1, participants maintained lifestyle NK, as determined by maintenance of capillary blood concentration of BHB ≥ 0.5 mmol/L, by *ad libitum* consumption of a very low carbohydrate high fat diet (VCHF), % carbohydrate:protein:fat = 8:17:75 (Miller, Villamena and Volek, 2018; Veyrat-Durebex et al., 2018) (this ratio is modulable according the metabolic health of the participant) or *ad libitum* feeding within a time-restricted feeding (TRF) window, or a mixture of both (**Table 2 – 3**).

On day 22 (visit 1), participants attended the Human Physiology Laboratory at the University of Westminster at the same time of day (8 am) in an overnight fasted state (> 12 hours) for baseline testing. The baseline visit included anthropometric measurements, metabolic measurements, including gas exchange analysis (VO_2, VCO_2), venous blood sample and an oral glucose tolerance test (OGTT) with BHB sensitivity. On day 23, participants suppressed ketosis (P2) and capillary BHB was targeted to be sustained at < 0.3 mmol/L for 21 days. Participants adapted out of ketosis during days 23 to 43 by following their healthiest interpretation (*ad libitum*) of the UK Eatwell Guidelines (% carbohydrate:protein:fat = 55:20:25) which recommend consuming at least 267 g of carbohydrate per day, divided over at least three meals.

On day 44 (visit 2), participants reported to the laboratory at 8 am having fasted overnight to complete the same measurements as during visit 1. On day 45, the trial intervention was removed, participants returned to their habitual lifestyle patterns resulting in a return to

NK (P3) and during days 45 to 65 they continued to monitor their capillary blood glucose and ketones, where BHB was maintained at ≥ 0.5 mmol/L, as in P1. On day 46 (visit 3), participants returned to the laboratory to repeat identical measurements as previous visits. An overview of the study design is presented in **Figure 6**.

3.1.4 Anthropometric Measurements

Upon arrival at the laboratory, height (to nearest 0.1 cm) was measured using a stadiometer (Marsden HM-250P Leicester Height Measure), and body weight (to nearest 0.1 kg), BMI, fat mass and total body water (TBW) were measured by bioelectrical impedance (BIA) using Seca® (mBCA 514 Medical Body Composition Analyzer, Gmbh&Co. KG, Hamburg, Germany). In addition, waist and hip circumference measures were obtained with a non-stretch anthropometric circumference measuring tape (Seca® 201) while participants stood upright on both feet. The average value (cm) of three measurements was used for analysis. All measurements were taken from following a 12-hour fast wearing standardised clothing with an empty bladder.

3.1.5 Metabolic Measurements

Respiratory quotient (RQ) was measured by indirect calorimetry using a Quark RMR (COSMED srl, Rome, Italy) and was defined as the ratio of carbon dioxide (CO_2) production to oxygen (O_2) consumption. RQ was determined with the participants lying down at rest and with 15 minutes of lead time to allow respiration to equilibrate before measurements were taken. After RQ was determined, blood pressure was taken using an automatic upper arm blood pressure monitor (OMRON HEALTHCARE Co., Ltd., Kyoto, Japan).

3.1.6 Blood collection and measurement

Following anthropometric measurements, a single-use sterile 22G Terumo (Japan, Tokyo) Versatus Winged and Ported IV Catheter (Cannula) was inserted into the participants antecubital vein for blood sampling. Saline solution flushes (0.9% NaCl, 5 mL, BD PosiFlush SP Syringe) were delivered in order to keep the intravenous line patent. 2 mL of blood was drawn and discarded prior to each blood draw to prevent blood sampling saline dilution.

Blood was drawn into tubes anti-coagulated with either ethylenediaminetetraacetic acid (EDTA) or lithium heparin (BD, Oxford, UK) ready for analysis by SYNLAB (Alexander Fleming, 3–6220 Heppignies–Company No: 0453.111.546). Blood was also drawn into serum SST™ II Advance tubes with thrombin rapid clot activator and separation gel (BD, Oxford, UK) and left for 30 minutes at room temperature. Serum tubes were then centrifuged (Hettich Zentrifugen, Universal 320 R, Tuttlingen, Germany) at 3,857 g for 10 minutes at room temperature. Serum samples were either sent to SYNLAB for analysis or aliquoted into cryovial tubes under sterile conditions and stored at -80°C for later analysis by Randox (Ardmore, 55 Diamond Road, Crumlin, Co. Antrim, BT29 4QY, company number: NI015738). Plasma was then aliquoted into airtight vials and frozen at -80°C for batch analysis later.

3.1.7 Blood marker analysis

Following blood draw, the blood samples were immediately sent to SYNLAB Belgium, to determine the concentrations of the following markers: insulin, insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein 3 (IGFBP-3), C-reactive protein (CRP), gamma-glutamyl transferase (GGT), cortisol, plasminogen activator inhibitor-1 (PAI-1), total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, triglycerides, thyroid stimulating hormone (TSH), free triiodothyronine (T3), reverse T3, and thyroxine (T4). In the publication KetoSAge part 2, serum insulin was measured via Simple Plex Assay (Ella™, Bio-Techne, USA), enabling further confirmation in confidence of results, as well as determining in house HOMA-IR calculation and for further studies already in progress with different metabolic phenotype cohorts, where it is deemed best practice to compare markers analysed using the same methods. Total osteocalcin (tOCN; DuoSet, R&D Systems, USA), uncarboxylated osteocalcin (unOCN; BioLegend, USA), melatonin, serotonin and serum GLP-1 (Abcam, Cambridge, UK), and leptin (DuoSet, R&D Systems, USA) were measured by enzyme linked immunosorbent assay (ELISA) from frozen EDTA plasma or serum samples, according to the manufacturer's instructions. Glucose concentrations were measured using Biosen C-Line Clinic Glucose and Lactate analyser (EKF-Diagnostic, GmbH, Germany).

At the end of the trial, frozen serum samples were sent to Randox Ireland to determine the concentrations of various cytokines and growth factors. These included: epithelial growth factor (EGF), vascular endothelial growth factor (VEGF), interferon-gamma (INF-**γ**), monocyte chemotactic protein (MCP-1), tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1 α, IL-1β, IL-2, IL-4, IL-6, IL-8, and IL-10.

3.1.8 Oral Glucose Tolerance Test

Following anthropometric and metabolic measurements and blood sampling, participants were subjected to an OGTT. 75 g of glucose in 250 mL water (prepared fresh on each day) was consumed by participants within 5 minutes. Blood samples were then drawn into EDTA tubes via cannula at 7 timepoints: 0 minutes (before glucose bolus), 30, 60, 120, 180, 240 and 300 minutes. All samples were immediately spun at 3,857 g for 10 minutes at 4°C to obtain the plasma fraction. Plasma was aliquoted under sterile conditions and stored at -80°C for later batch analysis. Plasma insulin concentrations were determined by Quantikine ELISA (R&D Systems), following the manufacturer's instructions. Samples were thawed once and analysed in triplicate. Throughout the OGTT at each timepoint, venous whole blood was used to measure glucose and BHB concentrations by a Keto-Mojo™ GKI multi-function meter.

3.1.9 Statistical analysis

Data was checked for normality using the Shapiro-Wilk test and parametric analyses were conducted for normally distributed data. Different markers measured in the plasma between study phases for KetoSAge participants were compared using the Friedman test with Dunn's correction for multiple comparisons or repeated measures one-way analysis of variance (ANOVA) was used to evaluate differences in various parameters between the three phases (baseline ketosis P1, suppression of ketosis P2 and return to ketosis P3), or across time points (0, 30, 60, 120, 180, 240, 300 minutes) for OGTT on glucose, insulin and BHB. Tukey's HSD correction test was used for *post hoc* analysis to perform pairwise comparisons, and p values < 0.05 were considered statistically significant. Data were presented as mean ± standard deviation, unless otherwise stated. Statistical analysis was

performed, and all figures were generated, in GraphPad Prism (version 9.1.2; San Diego, USA).

3.1.10 Sample size calculation

Sample size was calculated based on pilot feasibility data with 5 participants put through all 3 phases. Sample size was calculated using changes in fasted insulin and IGF-1 concentrations. The sample size was estimated using G*Power (v3.1) with an alpha level of 0.05, a power (1-β) of 0.80 and a medium effect size of f = 0.5 and a conservative intra-measurement correlation of 0.5. This analysis recommended a sample size of n = 9, that predicted to produce results with an effect size of 1.1 dz for paired comparisons.

Chapter 4

Chapters 4 and 5 comprise the results published in the two papers: Cooper et al., 2023 (Cooper, Kyriakidou, Edwards, et al., 2023) and Cooper et al., 2024 (Cooper et al., 2024).

https://doi.org/ 10.3390/ijms242115621

https://doi.org/10.3390/biomedicines12071553

4. Results part 1

4.1 Adherence

Based on the study protocol, participants were required to self-report 252 capillary BHB concentrations: 84 tests across each of the phases (Figure 1). The number of fulfilled tests and percentage of completed tests out of the possible 252 for all participants is shown in Table 2. The average percentage of successful tests was 99.37%, with 4 participants completing 100% of all 252 potential tests.

The mean capillary BHB concentration significantly decreased from 1.9 mmol/L (± 0.7) in the baseline ketosis phase (P1) to 0.1 mmol/L (± 0.1) following the suppression of ketosis phase (P2; $p < 0.0001$). During P3, mean capillary BHB concentration increased significantly ($p < 0.0001$) and returned to baseline (1.9 ± 0.6 mmol/L). The maintenance of high mean capillary BHB concentrations (> 0.5 mmol/L) during P1 and P3 indicated that all participants adhered to the requirements to maintain ketosis during these phases. Similarly, the low levels of BHB during P2 indicated adherence to the study protocol, whereby participants effectively suppressed nutritional ketosis (**Table 1**).

Table 1. Summary of fulfilled capillary BHB testing for all study participants across all phases (P1 – P3). Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention suppress ketosis (SuK) P2; and removal of SuK returning to NK P3. (n = 10)

			Mean Capillary BHB Concentration (mmol/L)		
Participant	**No of tests taken**	**% of tests fulfilled out of 252**	**P1**	**P2**	**P3**
1011	251	99.60	2.7	0.1	2.3
1021	252	100.00	2.8	0.1	2.2
1031	252	100.00	2.6	0.1	1.8
1041	252	100.00	1.5	0.2	1.6
1051	251	99.60	1.7	0.0	1.6
1061	245	97.22	0.7	0.1	0.8
1071	248	98.41	1.7	0.2	2.4
1081	250	99.21	2.0	0.1	1.2
1091	251	99.60	1.8	0.1	2.5
1101	252	100.00	1.5	0.1	2.4
Mean	250.40	99.37	1.9	0.1	1.9
±SD	2.15	0.85	0.7	0.1	0.6

There were variations in capillary BHB concentrations across the daily tests. The frequencies of tests which satisfied different cut-offs are summarised in **Table 2**. During P1 and P3, almost all reported capillary BHB concentrations were > 0.3 mmol/L or ≥ 0.5 mmol/L, which are generally considered the cut-off for ketosis or nutritional ketosis, respectively (Cooper et al., 2020, 2021). There were very few tests meeting these thresholds in P2, compared to P1 and P3 (**Table 2**).

During P1, 2/252 tests for two participants showed capillary BHB concentrations of 0.3 mmol/L, and 8/252 and 2/252 for the same two participants in P2 and P3 (**Table 1**) respectively. There were no reported capillary BHB concentrations of < 0.1 mmol/L in either P1 or P3 (**Table 2**).

During P2, the majority of capillary BHB concentrations were < 0.3 mmol/L, with a significant proportion of readings < 0.1 mmol/L. There were also some instances of capillary BHB readings > 0.3 mmol/L or ≥ 0.5 mmol/L during P2 (**Table 2**). These tests were often early in the morning after an overnight fast and during the first days of SuK (data not shown).

The high level of adherence to testing coupled with the expected high concentrations of capillary BHB in P1 and P3, and low concentrations of capillary BHB in P2, indicated high levels of adherence to the protocol throughout the entire study.

Table 2. Percentages of capillary BHB readings categorised as by different cut-offs across the study phases

	Capillary BHB (mmol/L)											
	≥ 0.5			> 0.3			≤ 0.3			< 0.1		
Participant	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
1011	100.00	2.38	95.18	100.00	4.76	98.80	0.00	95.24	1.20	0.00	28.57	0.00
1021	100.00	2.38	88.10	100.00	2.38	94.05	0.00	97.62	5.95	0.00	60.71	0.00
1031	100.00	2.38	92.86	100.00	2.38	95.24	0.00	97.62	4.76	0.00	59.52	0.00
1041	98.81	0.00	100.00	100.00	8.33	100.00	0.00	91.67	0.00	0.00	13.10	0.00
1051	100.00	0.00	97.62	100.00	1.19	98.81	0.00	98.81	0.00	0.00	94.05	0.00
1061	94.05	0.00	82.93	97.62	0.00	90.24	2.38	100.00	9.76	0.00	37.97	0.00
1071	96.30	4.82	97.62	97.53	4.82	97.62	2.47	95.18	2.38	0.00	1.20	0.00
1081	96.39	0.00	90.48	98.80	1.20	95.24	1.20	98.80	4.76	0.00	33.73	0.00
1091	98.81	0.00	98.80	100.00	1.19	100.00	0.00	98.81	0.00	0.00	21.43	0.00
1101	96.43	0.00	98.81	100.00	0.00	100.00	0.00	100.00	0.00	0.00	54.76	0.00

4.2 Frequency of rank ordered capillary BHB level

Assuming each test is independent of the other, then the mean of 21 days testing window has a 25% chance of being the highest. It can be seen in phase 1, 60% of the highest 21-day test window mean BHB concentration was found in the third test of the day (pre-dinner), with the remaining 40% as the pre-lunch test (**Table 3**).

Table 3. Frequency distribution of BHB (the mean of 21 days, BHB concentrations, per test window) expressed in rank, from 1 the lowest to 4 as the highest concentration. Each participant took 4 tests per day, totaling 840 capillary BHB tests in 10 participants sustaining nutritional ketosis over 21 consecutive days, in two NK phases (P1 and P3), totaling 1,680 capillary BHB tests. The rank is given to the mean BHB concentration for that test window of 21 days, per phase, per participant. If a participant's test 4 has a rank score of 1, this indicates the mean BHB concentration for 21 days of tests at bedtime, is the lowest of the mean values of all the other test windows.

Phase 1 NK											
Participant	1011	1021	1031	1041	1051	1061	1071	1081	1091	1101	Rank 4 frequency
Test 1 (Wake up)	2	1	2	1	2	1	1	1	1	1	0
Test 2 (Pre-lunch)	3	3	4	3	3	4	4	2	4	2	4
Test 3 (Pre-dinner)	4	4	3	4	4	2	3	4	3	4	6
Test 4 (Bedtime)	1	2	1	2	1	3	2	3	2	3	0
Phase 3 NK											
Participant	1011	1021	1031	1041	1051	1061	1071	1081	1091	1101	Rank 4 frequency
Test 1 (Wake up)	2	1	2	1	2	1	1	1	1	2	0
Test 2 (Pre-lunch)	4	3	4	3	2	3	4	3	3	4	4
Test 3 (Pre-dinner)	3	4	3	4	4	4	3	4	4	3	6
Test 4 (Bedtime)	1	2	1	2	3	2	2	2	2	1	0

4.3 Suppression of ketosis increases BMI and fat mass

Following P2, there were significant increases in both weight from 52.99 kg (± 4.24, P1) to 55.65 kg (± 4.10, P2; $p = 0.0002$) and BMI from 20.52 (± 1.39, P1) to 21.54 (± 1.30, P2; $p < 0.0001$) in all participants, compared to P1 (**Table 4**). Fat mass and TBW also increased from 14.21 kg (± 2.55, P1) to 15.88 kg (± 2.23, P2; $p = 0.0008$) and from 28.15 L (± 2.87, P1) to 29.15 L (± 2.96, P2; $p = 0.0016$), respectively (**Table 4**). Additionally, both waist-to-hip and waist-to-height ratios increased significantly in P2 compared to P1 (**Table 4**).

These trends then reversed after the removal of SuK, at the end of P3, compared to P2. Both weight (53.93 kg ± 4.04; $p < 0.0001$) and BMI (20.82 ± 1.46; $p = 0.0025$) trended back towards baseline in all but one participant, where only a small increase in weight was observed (+0.2 kg). Concordantly, the decreases in weight between the phases were accompanied by significant decreases in fat mass (14.78 kg ± 2.20; $p = 0.0057$) and TBW (28.42 L ± 3.15; $p = 0.0026$). These changes were also accompanied by decreases in both waist-to-hip and waist-to-height ratios (**Table 4**).

RQ increased in most participants (80%) following P2 (0.66 ± 0.05, P1 to 0.72 ± 0.06, P2; $p = 0.0427$; **Figure 7**). After removal of the intervention at the end of P3, there was a decrease in RQ in all participants, returning to their baseline (0.65 ± 0.06, P3; $p = 0.0005$; **Figure 7**). There were no changes in either systolic or diastolic blood pressure across all the study phases (**Table 4**).

Table 4. Participants' characteristics. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention suppress ketosis (SuK) P2; and removal of SuK returning to NK P3. Measurements were taken at 8 am after a 12-hour overnight fast; (n = 10)

	P1	P2	P3	ANOVA p value	P1 vs P2	P2 vs P3	P1 vs P3
Age (years)	32.30 (± 8.97)						
Height (cm)	160.95 (± 7.28)						
Weight (kg)	52.99 (± 4.24)	55.65 (± 4.10)	53.93 (± 4.04)	<0.0001	0.0002	<0.0001	0.7888
BMI	20.52 (± 1.39)	21.54 (± 1.30)	20.82 (± 1.46)	<0.0001	<0.0001	0.0025	0.0197
Waist/Hip	0.75 (± 0.03)	0.77 (± 0.03)	0.74 (± 0.03)	<0.0001	0.0015	<0.0001	0.5361
Waist/Height	0.43 (± 0.03)	0.45 (± 0.03)	0.43 (± 0.03)	<0.0001	0.0009	<0.0001	>0.9999
Fat mass (kg)	14.21 (± 2.55)	15.88 (± 2.23)	14.78 (± 2.20)	<0.0001	0.0008	0.0057	0.1016
TBW (L)	28.15 (± 2.87)	29.15 (± 2.96)	28.42 (± 3.15)	0.0005	0.0016	0.0262	0.3473
RQ	0.66 (± 0.05)	0.72 (± 0.06)	0.65 (± 0.06)	0.0096	0.0427	0.0005	0.8606
Systole (mmHg)	103.25 (± 6.24)	103.70 (± 10.17)	100.00 (± 9.54)	0.1455	0.9753	0.1746	0.2274
Diastole (mmHg)	70.75 (± 4.91)	69.45 (± 7.14)	68.15 (± 7.36)	0.3227	0.8044	0.7147	0.1715

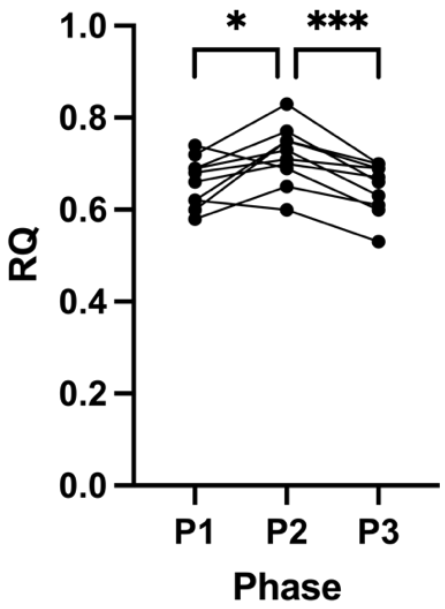

Figure 7. Respiratory quotient (RQ) measurements across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention suppress ketosis (SuK) P2; and removal of SuK returning to NK P3. RQ was determined by indirect calorimetry. Measurements were taken at 8 am after a 12-hour overnight fast; (n = 10); * $p < 0.05$; *** $p < 0.001$

4.4 Suppression of ketosis is associated with increases in insulin, IGF-1, glucose and T3

Following P2, fasting insulin significantly increased from 33.60 pmol/L (± 8.63, P1) to 59.80 pmol/L (± 14.69, P2; p = 0.0002; **Figure 8A**) and IGF-1 from 149.30 ug/L (± 32.96, P1) to 273.40 ug/L (± 85.66, P2; p = 0.0045; **Figure 8B**) compared to P1 (Table 4). This was accompanied by a significant increase in blood glucose from 4.36 (± 0.53) to 5.12 mmol/L (± 0.59, P2; p = 0.0088) (in mg/dL: 78.6 (± 9.5) to 92.3 (± 10.6)); **Figure 8C**) and decrease in BHB concentrations from 2.43 (± 1.28) to 0.18 mmol/L (± 0.13, P2; p = 0.0012); **Figure 8D; Table 5**). Free T3 also significantly increased from 3.81 pmol/L (± 0.28, P1) to 5.51 pmol/L (± 0.72, P2; p = <0.0001; **Figure 8B**) following P2.

These trends reversed following P3, with significant changes in the concentrations of insulin decreasing to 31.60 (± 9.38; p < 0.0001; **Figure 8A**), IGF-1 decreasing to 136.90 (± 39.60; p = 0.0055; **Figure 8B**), glucose decreasing to 4.41 (± 0.30; p = 0.0177; **Figure 8B**), BHB increased to 2.31 (± 0.71; p < 0.0001; Figure 8D) and free T3 decreasing to 4.05 (± 0.54; p = 0.0015; **Figure 9B; Table 5**), compared to P2.

	P1	P2	P3	ANOVA p value	P1 vs P2	P2 vs P3	P1 vs P3
Insulin (pmol/L)	33.60 (± 8.63)	59.80 (± 14.69)	31.60 (± 9.38)	<0.0001	0.0002	<0.0001	0.5361
IGF-1 (µg/L)	149.30 (± 32.96)	273.40 (± 85.66)	136.90 (± 39.60)	0.0015	0.0045	0.0055	0.4124
Glucose (mmol/L)	4.36 (± 0.53)	5.12 (± 0.59)	4.41 (± 0.30)	0.0015	0.0088	0.0177	0.9469
BHB (mmol/L)	2.43 (± 1.28)	0.18 (± 0.13)	2.31 (± 0.71)	0.0001	0.0012	<0.0001	0.9854
IGFBP-3 (mg/mL)	3.69 (± 0.56)	4.41 (± 1.27)	3.67 (± 0.70)	0.2357	0.3621	0.4272	0.9361
IGF-1/IGFBP-3[†]	0.14 (± 0.03)	0.25 (± 0.08)	0.15 (± 0.04)	0.0584	0.0870	0.1554	0.9049
TSH (mU/L)	1.40 (± 0.74)	1.56 (± 0.75)	1.25 (± 0.81)	0.3065	0.2334	0.4498	0.7742
Free T3 (pmol/L)	3.81 (± 0.28)	5.51 (± 0.72)	4.05 (± 0.54)	<0.0001	<0.0001	0.0015	0.3040
Reverse T3 (nmol/L)	0.29 (± 0.09)	0.26 (± 0.10)	0.25 (± 0.09)	0.6039	0.7030	0.9674	0.6323
T4 (pmol/L)	13.51 (± 1.61)	13.24 (± 1.49)	12.65 (± 0.66)	0.2125	0.8795	0.3059	0.2099

Table 5. Fasted insulin, IGF-1, glucose, and BHB across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention suppress ketosis (SuK) P2; and removal of SuK returning to NK P3. Measurements were taken at 8 am after a 12-hour overnight fast; (n = 10; [†]n = 5). Plasma insulin analysed with Synlab Belgium (Alexander Fleming, 3 – 6220 Heppignies – Company No: 0453.111.546).

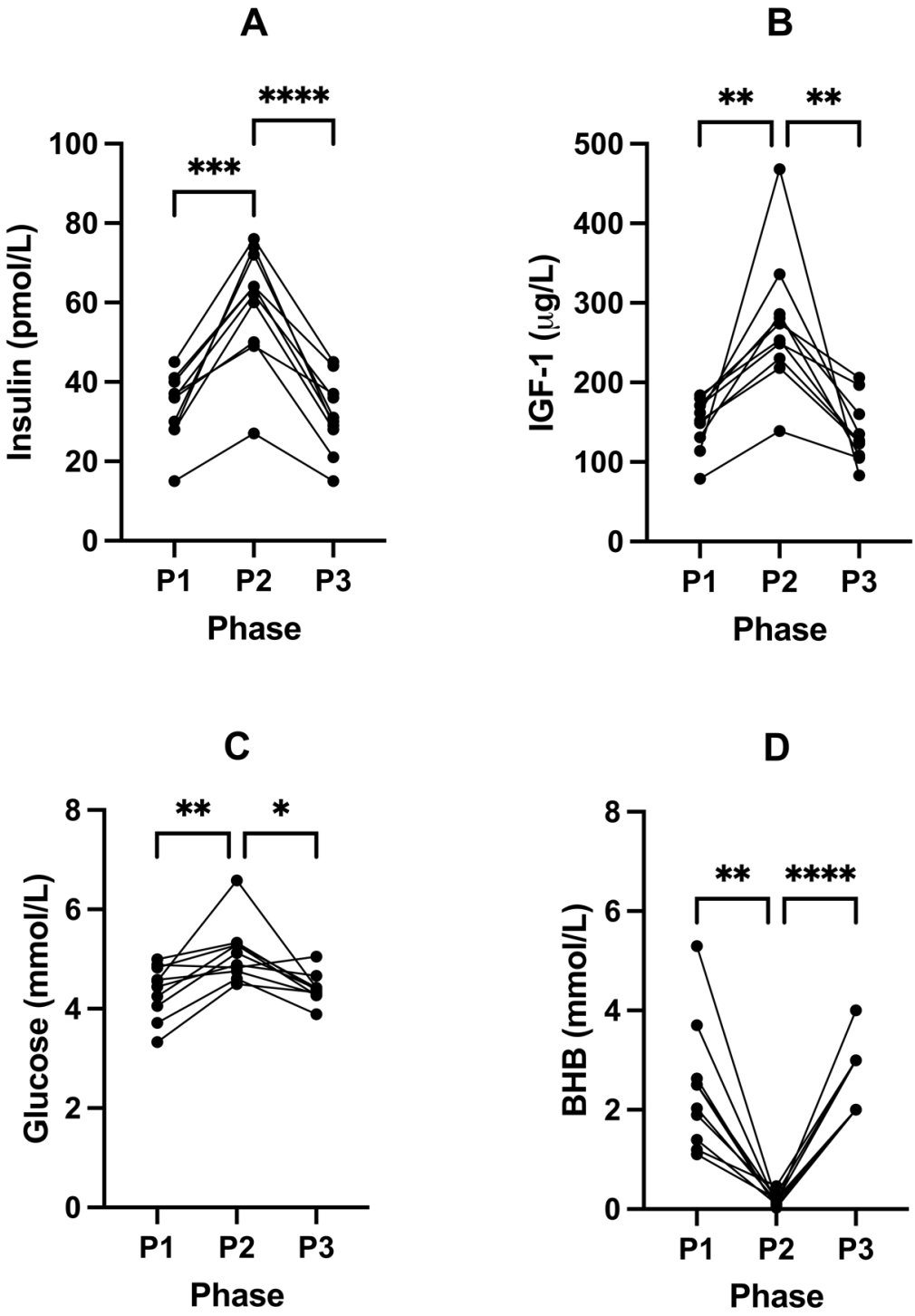

Figure 8. Changes in fasted blood insulin (A), IGF-1 (B), glucose (C) and BHB (D) concentrations across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3. Measurements were taken at 8 am after a 12-hour overnight fast; (n = 10). ** p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001*

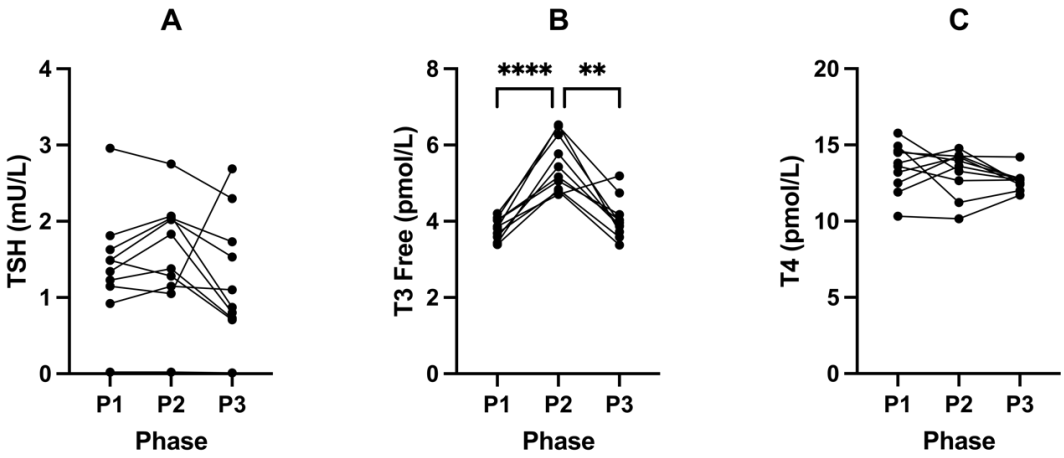

Figure 9. Changes in TSH (A), T3 Free (B) and T4 (C) concentrations across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention suppress ketosis (SuK) P2; and removal of SuK returning to NK P3. Measurements were taken at 8 am after a 12-hour overnight fast; (n = 10). *** p < 0.01; **** p < 0.0001*

4.5 Oral glucose tolerance tests

4.5.1 Between phases (P1 vs P2 vs P3) OGTT glucose response

Basal values of blood glucose measured by OGTT were lower in P1 and P3 (4.23 mmol/L ± 0.50 and 4.24 mmol/L ± 0.28, respectively) compared to P2 (5.01 mmol/L ± 0.70; **Figure 10A**). There were significant differences in mean glucose concentration amongst the three phases at baseline ($p = 0.0016$). *Post hoc* comparisons showed a significant difference in glucose concentration between P1 and P2 ($p = 0.0065$), and between P2 and P3 ($p = 0.0166$).

P1 and P3 showed a similar pattern in blood glucose response whereby glucose peaked at 60 minutes (8.31 mmol/L ± 2.78 and 7.48 ± 1.73, respectively; $p = 0.4385$). However, in P2, glucose concentration reached a peak earlier, at 30 minutes (6.63 mmol/L ± 1.20). P1 and P3 showed a trough in glucose response at 240 minutes (3.76 mmol/L ± 0.91 and 3.38 mmol/L ± 0.20, respectively; $p = 0.4137$), whereas glucose concentration in P2 dropped earlier at 180 minutes (3.64 mmol/L ± 0.61). P1 and 3, by minutes 240 and 300, glucose continued to trend down, whereas in P2, glucose trends up at these time points. By 300 minutes, values returned to their phase baselines.

4.5.2 Within-phase glucose response during a 5-hour OGTT

There were significant changes in glucose concentration across seven timepoints (0, 30, 60, 120, 180, 240 and 300 minutes) in P1 ($p < 0.0001$; **Figure 10A**). More specifically, there was a statistically significant increase in blood glucose change from 0 minutes (4.23 mmol/L ± 0.50) to 30 minutes (7.49 mmol/L ± 1.25; $p = 0.0009$), and to 60 minutes (8.31 mmol/L ± 2.78; $p = 0.0287$). There was also a significant difference between the peak of glucose at 60 minutes (8.31 mmol/L ± 2.78) and at 240 minutes (3.76 mmol/L ± 0.91; $p = 0.0070$), which continued to decrease at 300 minutes (3.52 mmol/L ± 0.55; $p = 0.0068$).

Following P2, there were significant changes in glucose concentration amongst all timepoints ($p = 0.0002$; **Figure 10A**). More precisely, glucose concentration was significantly increased from 0 minutes (5.01 mmol/L ± 0.70) to 30 minutes (6.63 mmol/L ± 1.20; $p = 0.0435$), but not between 0 and 60 minutes. There were also statistically

significant differences between the peak at 30 minutes (6.63 mmol/L ± 1.20) and the trough at 180 minutes (3.64 mmol/L ± 0.61; p = 0.0002).

After returning to P3, significant changes were observed in glucose concentration amongst all timepoints (p < 0.0001; **Figure 10A**). There was a statistically significant increase from 0 minutes (4.24 mmol/L ± 0.28) to 30 minutes (7.41 mmol/L ± 1.02; p = 0.0001), and to 60 minutes (7.48 mmol/L ± 1.73; p = 0.0059). Similarly, to P1, there was also a significant difference between the peak of glucose at 60 minutes (7.48 mmol/L ± 1.73) and the trough at 240 minutes (3.38 mmol/L ± 0.20; p = 0.0004).

4.5.3 Following plateau, blood glucose concentration increased during ketosis suppression

After 180 minutes in P2, glucose began to trend upwards, which was not observed in P1 or P3. At 240 minutes, whilst glucose concentration was higher in P2 (4.40 mmol/L ± 0.45) vs P1 (3.76 mmol/L ± 0.90), this difference was not statistically significant (p = 0.1342). However, glucose concentration was significantly higher in P2 (4.40 mmol/L ± 0.45) compared to P3 (3.38 ± 0.20; p = 0.0007). Similarly, at 300 minutes glucose concentration was significantly higher in P2 (4.70 mmol/L ± 0.38) compared to P1 (3.52 ± 0.55; p = 0.0001), and to P3 (3.53 ± 0.13; p < 0.0001). There was no difference in the concentration of glucose at 240 minutes (p = 0.4137) or at 300 minutes (p = 0.9988) when comparing P1 and P3; **Figure 10A**.

4.5.4 Between phases (P1 vs P2 vs P3) OGTT insulin response

Fasting insulin concentrations were also found to be lower in P1 and P3 (29.94 pmol/L ± 21.48 and 27.97 pmol/L ± 31.68, respectively) in comparison to P2 (98.19 pmol/L ± 107.69), although were not significantly different (P1 vs P2; p = 0.0971, and P2 vs P3, p = 0.0754; **Figure 10B**). Insulin concentration peaked at 30 minutes in P2 (411.07 pmol/L ± 226.59) and at 60 minutes in P1 and P3 (351.94 pmol/L ± 192.36 and 330.27 pmol/L ± 160.56, respectively). However, insulin concentration returned to baseline values in all phases with a similar pattern at the end of the experimental period (300 minutes). Changes in insulin concentration at 30 minutes amongst the three phases were also

analysed. Data showed a significant difference between P1 (256.27 pmol/L ± 112.59) and P2 (411.07 pmol/L ± 226.59; p = 0.0324), and between P2 (411.07 pmol/L ± 226.59) and P3 (278.23 pmol/L ± 137.20; p = 0.0161).

4.5.5 Within-phase insulin response during a 5-hour OGTT

Following P1, repeated measures one-way ANOVA illustrated statistically significant changes in insulin concentration across all timepoints ($p < 0.0001$; **Figure 10B**). More precisely, there was a significant increase from 0 minutes (29.94 pmol/L ± 21.48) to 30 minutes (256.27 pmol/L ± 112.59; p = 0.0013), and to 60 minutes (351.94 pmol/L ± 192.36; p = 0.0047). There was also a significant difference between the peak at 60 minutes (351.94 pmol/L ± 192.36) and 180 minutes (41.08 pmol/L ± 26.62; p = 0.0069).

P2 also showed significant changes in insulin concentrations amongst all timepoints ($p < 0.0001$; **Figure 10B**). More specifically, there was a statistically significant increase from 0 minutes (98.19 pmol/L ± 107.69) to 30 minutes (411.07 pmol/L ± 226.59; p = 0.0011), and to 60 minutes (366.68 pmol/L ± 204.21; p = 0.0291). Additionally, there was a statistically significant difference between the peak at 30 minutes (411.07 pmol/L ± 226.59) and 180 minutes (55.95 pmol/L ± 57.99; p = 0.0054), and between at 60 minutes (366.68 pmol/L ± 204.21) and 180 minutes (55.95 pmol/L ± 57.99; p = 0.0028).

Similarly, after returning to P3, insulin concentrations significantly changed across all timepoints ($p < 0.0001$; **Figure 10B**). Insulin levels were significantly increased from 0 minutes (27.97 pmol/L ± 31.68) to 30 minutes (278.23 pmol/L ± 137.20; p = 0.0024), and to 60 minutes (330.27 pmol/L ± 160.56; p = 0.0015). Like P1, there was a significant difference between the peak at 60 minutes (330.27 pmol/L ± 160.56) and 180 minutes (52.11 pmol/L ± 84.10; p = 0.0005). Notably, in all three phases, insulin concentrations began to converge and trend significantly downwards at 180 minutes. At 240 and 300 minutes, the concentration of insulin began to plateau.

4.5.6 Between phases (P1 vs P2 vs P3) OGTT BHB response

Basal values of BHB were higher in P1 and P3 (2.60 mmol/L ± 1.22 and 2.36 mmol/L ± 0.78, respectively) than in P2 (0.18 mmol/L ± 0.12), and they significantly differed between P1 vs P2 ($p = 0.0004$), and between P2 vs P3 ($p < 0.0001$; **Figure 10C**).

A statistically significant difference was also observed amongst three phases in mean BHB concentration at 30 minutes ($p = 0.0020$), at 60 minutes ($p = 0.0034$) and at 300 minutes ($p < 0.0001$). *Post hoc* testing indicated that BHB concentration significantly differed between P1 (2.22 mmol/L ± 1.51) and P2 (0.24 mmol/L ± 0.18; $p = 0.0078$), and between P2 (0.24 mmol/L ± 0.18) and P3 (1.89 mmol/L ± 0.77; $p = 0.0004$) at 30 minutes. In addition, BHB concentration at 60 minutes was significantly different between P1 (1.41 mmol/L ± 1.02) and P2 (0.19 mmol/L ± 0.17; $p = 0.0107$), and between P2 (0.19 mmol/L ± 0.17) and P3 (1.08 mmol/L ± 0.70; $p = 0.0039$). Further, there were significant differences between P1 (2.02 mmol/L ± 0.72) and P2 (0.36 mmol/L ± 0.28; $p < 0.0001$), and between P2 (0.36 mmol/L ± 0.28) and P3 (1.94 mmol/L ± 0.46; $p < 0.0001$) at 300 minutes.

4.5.7 Within-phase BHB response during a 5-hour OGTT

There were statistically significant changes in BHB concentration across time points overall in P1 ($p = 0.0006$; **Figure 10C**), with no significant changes between 0 minutes (2.60 mmol/L ± 1.22) and 30 minutes (2.22 mmol/L ± 1.51; $p = 0.2056$). However, there was a significant decrease from 0 minutes (2.60 mmol/L ± 1.22) to 60 minutes (1.41 mmol/L ± 1.02; $p < 0.0001$). Whilst there were minimal changes in BHB concentration across all timepoints following P2 ($p = 0.0961$; **Figure 10C**), after returning to P3 there were significant changes across time ($p < 0.0001$). More specifically, there was a significant decrease from 0 minutes (2.36 mmol/L ± 0.78) to 30 minutes (1.89 mmol/L ± 0.77; $p = 0.0444$), and to 60 minutes (1.08 mmol/L ± 0.70; $p = 0.0001$).

Following the carbohydrate-containing drink, both P1 and P3 demonstrated a similar pattern response in BHB change, with BHB concentration showing a steady time-dependent decrease until 120 minutes. Then, returning in a linear recovery from 180 minutes (0.42 mmol/L ± 0.37, P1; 0.52 mmol/L ± 0.42, P3), with a significant increase until

300 minutes (2.02 mmol/L ± 0.72; p = 0.0005, P1; 1.94 mmol/L ± 0.46; p < 0.0001, P3). Conversely, during SuK (P2), BHB concentration followed a similar response across time, whereby there were minimal changes throughout the experimental period (**Figure 10C**).

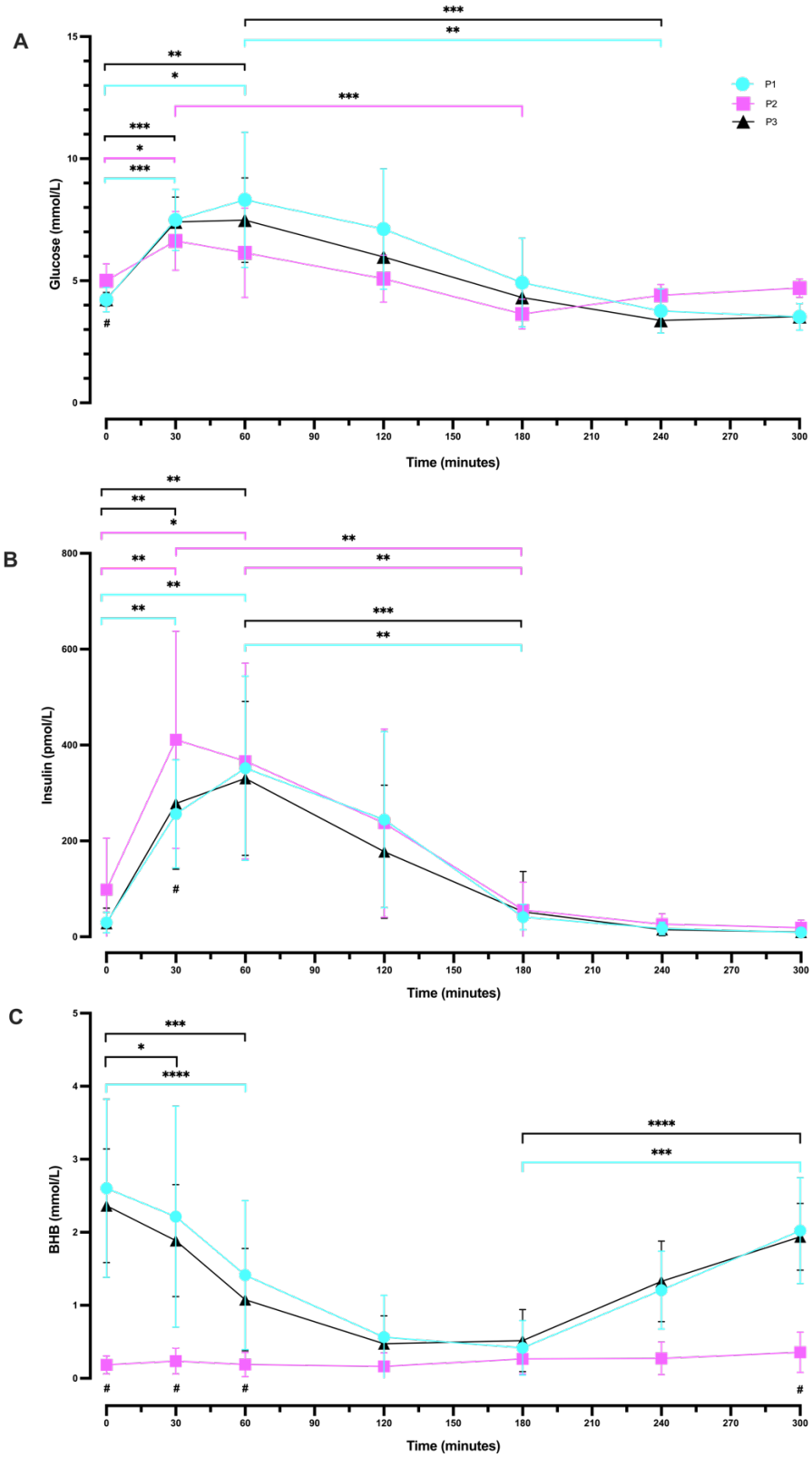

Figure 10. Differences in glucose (A), insulin (B) and BHB (C) as measured by oral glucose tolerance test across all study participants in all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1 (blue circles); intervention suppress ketosis (SuK) P2 (pink squares); and removal of SuK returning to NK, P3 (black triangles); (n = 10). *The connected line indicates group means (±SD); * indicates significant difference within each phase across different timepoints; # indicates significant difference between phases at the time point as indicated. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001*

Chapter 5

5. Results part 2

5.1 Suppression of ketosis is associated with increases in inflammatory liver markers

Following P2, GGT concentrations increased significantly in all participants from 9.60 U/L (± 3.13) in P1 to 12.40 U/L (± 2.55) in P2 ($p = 0.0087$; **Figure 11A**). From P2 to P3, GGT levels were significantly reduced to 9.70 U/L (± 2.50; $p = 0.0286$; **Figure 11A**; **Table 6**). It was also found that SuK (P2) significantly increased PAI-1 levels from 13.34 ng/mL (± 6.85, P1) to 16.69 ng/mL (± 6.26, P2; $p = 0.0428$). No changes in PAI-1 levels were observed following P3 (17.05 ng/mL ± 5.58) compared to P2 ($p = 0.9483$; **Figure 11B**; **Table 6**).

CRP was found to be low or less than the lowest detectable limit of the assay in all participants across all study phases (data not shown). CRP was therefore measured using a high sensitivity assay (ultra-sensitive CRP) in 5 participants (**Table 6**). Despite this, no significant changes were determined from P1 (1.00 mg/L ± 1.19) to P2 (1.16 mg/L ± 1.56; $p = 0.9938$); or from P2 to P3 (1.35 mg/L ± 2.23; $p = 0.7477$). There were no statistically significant changes in all other liver or lipid markers across all phases of the study (**Table 6**).

Table 6. Concentrations of fasted liver markers measured across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3. Measurements were taken at 8 am after a 12-hour overnight fast; (n = 10; [†]P3, n = 9; [§]P1, P2, P3, n = 5)

	P1	P2	P3	ANOVA p value	P1 vs P2	P2 vs P3	P1 vs P3
Triglycerides (mg/dL)	66.80 (± 28.00)	66.10 (± 21.09)	79.30 (± 45.88)	0.5018	0.9972	0.6629	0.6270
Total cholesterol (mg/dL)	231.50 (± 62.42)	188.50 (± 30.28)	210.20 (± 43.44)	0.0335	0.0802	0.2132	0.1061
HDL cholesterol (mg/dL)	70.10 (± 10.37)	72.70 (± 13.59)	69.80 (± 11.84)	0.6231	0.7460	0.6762	0.9943
LDL cholesterol (mg/dL)[†]	4.46 (± 2.03)	3.13 (± 0.91)	3.96 (± 1.34)	0.0888	0.1798	0.3280	0.1498
Triglycerides/HDL (mmol/L)	1.01 (± 0.55)	0.95(± 0.38)	1.25 (± 0.90)	0.3804	0.9478	0.5358	0.5515
CRP (Ultra-Sensitive) (mg/L)[§]	1.00 (± 1.19)	1.16 (± 1.56)	1.35 (± 2.23)	0.7103	0.9938	0.7477	0.7728
Gamma-GT (U/L)	9.60 (± 3.13)	12.40 (± 2.55)	9.70 (± 2.50)	0.0029	0.0087	0.0286	0.9885

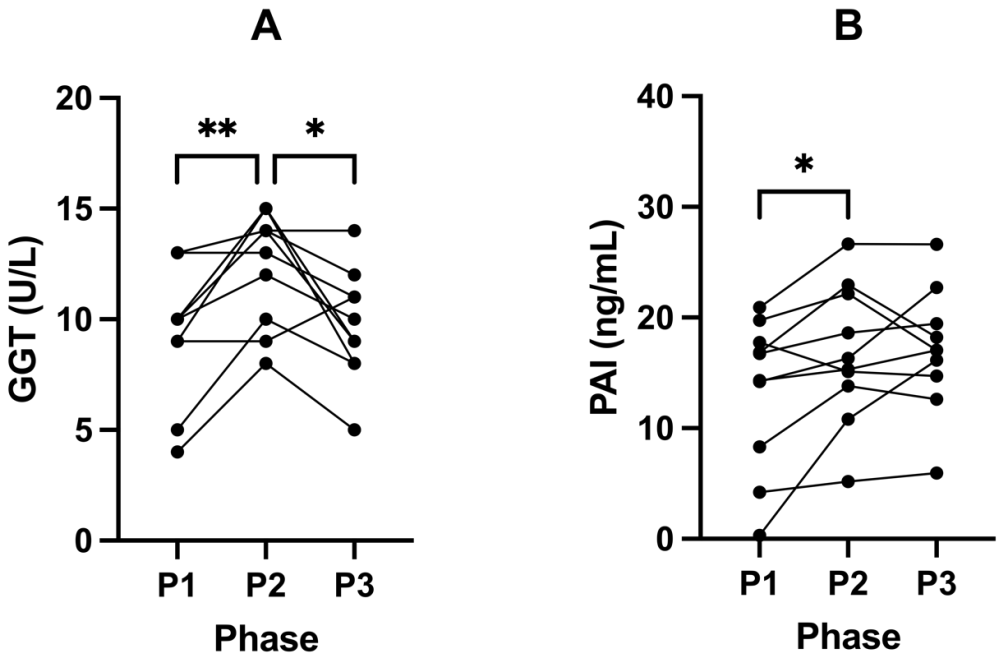

Figure 11. Changes in liver parameters GGT (A) and PAI-1 (B) in participants across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3. Measurements were taken at 8 am after a 12-hour overnight fast; (n = 10). ** $p < 0.05$; ** $p < 0.01$*

5.2 Ketosis maintains lower levels of EGF, VEGF and MCP-1

There were increases in EGF from 33.02 pg/mL (± 30.96) in P1 to 50.13 pg/mL (± 38.19) following P2 (p = 0.0450; **Figure 12A; Table 7**). VEGF also increased from 93.93 pg/mL (± 54.30) in P1 to 147.33 pg/mL (± 100.03) following P2 (p = 0.0314; Figure 7B; **Table 6**). MCP-1 significantly increased from 103.98 pg/mL (± 39.30) in P1 to 192.53 (± 84.73) following P2 (p = 0.0137; **Figure 12C; Table 7**).

Following P3, these growth factors and cytokines trended back to baseline and decreased significantly compared to P2. EGF (p = 0.3473; **Figure 12A**; **Table 7**) and VEGF (p = 0.2102; **Figure 12B**; **Table 7**) decreased to 37.82 pg/mL (± 26.81) and 134.80 pg/mL (± 98.79), respectively. Concentrations of MCP-1 also decreased significantly to 128.52 pg/mL (± 51.80) following P3 compared to P2 (p = 0.0175; **Figure 12C**; **Table 7**).

There were minimal changes in IL-1β following P2 (p = 0.7045). However, following P3 all participants had a significantly decreased expression of IL-1β (p = 0.0381; **Table 7**). Similarly, there were minimal changes following P2 in the expression of TNF-α (p = 0.3887); however, in P3, 86% of participants decreased their expression of TNF-α (p = 0.0785; **Table 7**). There were no changes in all other cytokines and growth factors across all phases of the study (**Table 7**).

Table 7. Concentrations of fasted growth factors and cytokines across the different phases of the study
Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3. Measurements were taken at 8 am after a 12-hour overnight fast; (P1, P2, n = 10; P3, n = 9)

	P1	P2	P3	ANOVA p value	P1 vs P2	P2 vs P3	P1 vs P3
EGF (pg/mL)	33.02 (± 30.96)	50.13 (± 38.19)	37.82 (± 26.81)	0.0139	0.0450	0.3473	0.0478
VEGF (pg/mL)	93.93 (± 54.30)	147.33 (± 100.03)	134.80 (± 98.79)	0.0147	0.0314	0.2102	0.0801
Interferon-γ (pg/mL)	1.14 (± 2.64)	0.72 (± 1.05)	0.57 (± 0.90)	0.3755	0.7019	0.2452	0.6019
(MCP-1) (pg/mL)	103.98 (± 39.30)	192.53 (± 84.73)	128.52 (± 51.80)	0.0026	0.0137	0.0175	0.2622
TNF-α (pg/mL)	2.23 (± 1.75)	2.66 (± 1.26)	2.09 (± 0.97)	0.1387	0.3887	0.0785	0.8430
IL-1α (pg/mL)	0.30 (± 0.40)	0.26 (± 0.25)	0.26 (± 0.25)	0.3230	0.6266	0.5406	0.5104
IL-1β (pg/mL)	2.23 (± 3.42)	1.85 (± 2.02)	1.71 (± 2.04)	0.3090	0.7045	0.0381	0.4989
IL-2 (pg/mL)	1.92 (± 1.48)	1.71 (± 1.16)	1.94 (± 1.37)	0.2932	0.4409	0.7569	0.3809
IL-4 (pg/mL)	2.14 (± 0.80)	2.06 (± 0.99)	2.25 (± 1.17)	0.4635	0.5358	0.5138	0.9090
IL-6 (pg/mL)	0.95 (± 0.80)	1.22 (± 1.11)	0.84 (± 0.56)	0.5034	0.9238	0.5677	0.5771
IL-8 (pg/mL)	8.91 (± 9.56)	8.60 (± 5.93)	8.08 (± 6.30)	0.6738	0.9966	0.5725	0.8009
IL-10 (pg/mL)	0.61 (± 0.37)	0.68 (± 0.46)	0.53 (± 0.25)	0.4323	0.9084	0.4420	0.5573

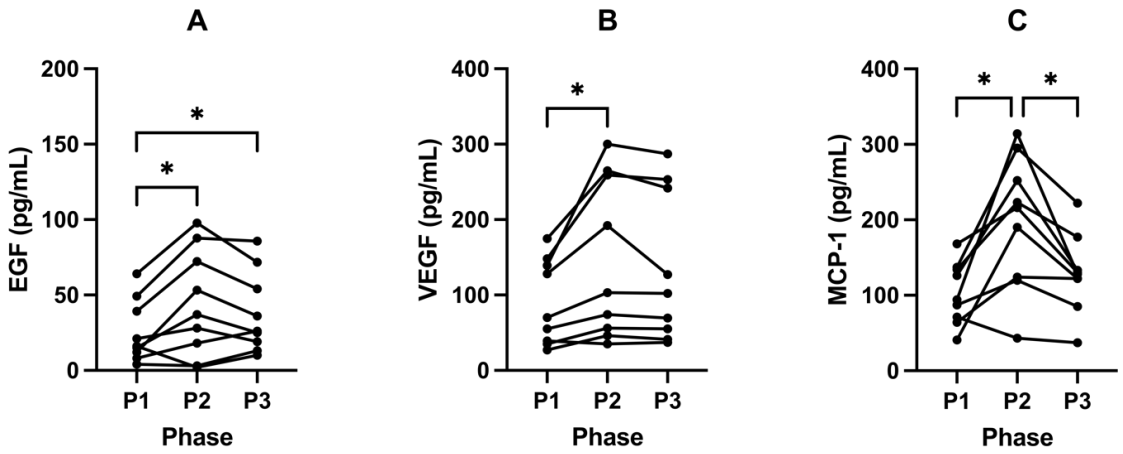

Figure 12. Fasted EGF (A), VEGF (B) and MCP-1 (C) in participants across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3. Measurements were taken at 8 am after a 12-hour overnight fast; *(A & B, n = 10; C, n = 9). * p < 0.05*

5.3 Suppression of ketosis is associated with increases in HOMA-IR

Following P2, HOMA-IR significantly increased from 0.97 (± 0.32, P1) to 2.07 (± 0.61, P2; p = 0.0008) This trend reversed following P3, where HOMA-IR significantly changed and returned to participants baseline levels of 1.12 (± 0.41, P3; p = 0.0013) compared to P2 (**Figure 13; Table 8**).

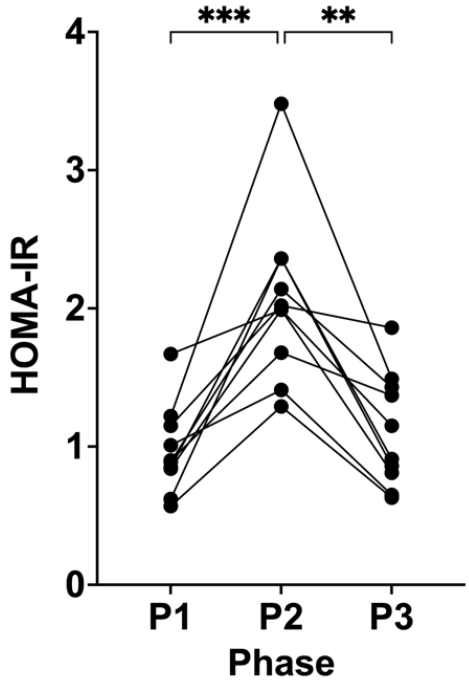

Figure 13. Homeostatic model assessment for insulin resistance (HOMA-IR) across all phases in KetoSAge participants. Fasting serum concentrations of insulin and plasma glucose were measured following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3; Insulin was determined by via Simple Plex Assay (Ella™, Bio-Techne, USA), glucose was measured by Biosen C-Line Clinic Glucose and Lactate analyser. HOMA-IR adopts the following formula to index insulin resistance: fasting plasma insulin (uIU/mL) × fasting plasma glucose (mmol/L)/22.5 [1,17,18]. Homeostasis model assessment for insulin resistance (HOMA-IR). Samples were taken at 8 a.m. after a 12-hour overnight fast; (n = 10). Data analysed by repeated measures one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 8. Fasted insulin, glucose, BHB, HOMA-IR, total, carboxylated and uncarboxylated osteocalcin, leptin, cortisol, serotonin and GLP-1 across all phases in KetoSAge participants. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention suppress ketosis (SuK) P2; and removal of SuK returning to NK P3; Measurements were taken at 8 am after a 12-hour overnight fast; (n = 10). Values are presented as mean ± SD. Serum insulin was measured via Simple Plex Assay (Ella™, Bio-Techne, USA).

	P1	P2	P3	ANOVA p value	P1 vs P2	P2 vs P3	P1 vs P3
Insulin (uIU/mL)	4.95 (± 1.24)	9.06 (± 2.14)	5.62 (± 1.83)	<0.0001	0.0006	0.0027	0.3995
Glucose (mmol/L)	4.36 (± 0.53)	5.12 (± 0.59)	4.41 (± 0.30)	0.0015	0.0088	0.0177	0.9469
HOMA-IR	0.97 (± 0.32)	2.07 (± 0.61)	1.11 (± 0.41)	<0.0001	0.0008	0.0013	0.4950
tOCN (ng/mL)	33.84 (± 13.66)	55.31 (± 29.71)	34.02 (± 12.05)	0.0049	0.0138	0.0253	0.9978
cOCN (ng/mL)	31.54 (± 12.59)	50.78 (± 26.22)	32.02 (± 11.13)	0.0040	0.0120	0.0246	0.9840
unOCN (ng/mL)	2.29 (± 1.25)	4.54 (± 3.79)	2.00 (± 1.16)	0.0004	0.0417	0.0010	0.7907
tOCN % (% relative to P1)	100.00	162.36 (± 46.53)	109.70 (± 40.36)	0.0005	0.0065	0.0094	>0.9999
cOCN % (% relative to P1)	100.00	161.56 (± 46.73)	111.52 (± 42.90)	0.0007	0.0062	0.0080	0.6836
unOCN % (% relative to P1)	100.00	176.67 (± 66.66)	87.11 (± 21.43)	0.0028	0.0135	0.0076	0.2476
Leptin (ng/mL)	4.50 (± 3.67)	15.08 (± 8.00)	4.57 (± 3.48)	<0.0001	0.0010	0.0052	>0.9999
Cortisol (ng/mL)	126.20 (± 52.67)	112.70 (± 58.46)	131.90 (± 52.18)	0.4362	>0.9999	0.5391	>0.9999
Serotonin (ng/mL)	21.05 (± 22.83)	18.38 (± 16.03)	21.77 (± 16.80)	0.6013	0.7907	>0.9999	>0.9999
GLP-1 (pg/mL)	1383.18 (± 911.36)	576.72 (± 452.43)	1471.85 (± 1,066.75)	0.0075	0.0209	0.0219	>0.9999

Beta-hydroxybutyrate (BHB); body mass index (BMI); carboxylated osteocalcin (cOCN); glucagon like peptide 1 (GLP-1); homeostasis model assessment for insulin resistance (HOMA-IR); total osteocalcin (tOCN); uncarboxylated osteocalcin (unOCN).

5.4 Suppressing ketosis increases all forms of osteocalcin

tOCN significantly increased from P1, 33.84 ng/mL (± 13.66) to 55.31 ng/mL (± 29.71, P2, p = 0.0138; **Figure 14**), and then significantly decreased following removal of SuK (P3), to 34.02 ng/mL (± 12.05, p = 0.0253) compared to P2, returning to similar baseline P1 values. cOCN significantly increased from P1, 31.54 ng/mL (± 12.59) to 50.8 ng/mL (± 26.2, P2, p = 0.0120), then significantly decreased following removal of SuK P3, to 32.0 ng/mL (± 11.1, P3, p = 0.0246), compared to P2, returning to similar baseline P1 values. unOCN increased from P1, 2.29 ng/mL (± 1.25) to 4.54 ng/mL (± 3.79, P2, p = 0.0417), then decreased following removal of SuK P3, to 2.00 ng/mL (± 1.16, p = 0.0010), compared to P2, returning to similar baseline P1 values.

Further analyses of tOCN, cOCN and unOCN as a percentage relative to each participants own baseline values in P1, tOCN significantly increased from P1, 100% as standardised baseline (± 0.00%) to 162.36% (± 46.53%, p = 0.0065; **Figure 15**) relative to P1, then decreased following removal of SuK P3, to 109.70% P1 (± 40.36%, P3, p = 0.0094) relative to baseline, compared to P2, returning to similar baseline P1 values. cOCN significantly increased from P1, 100% as standardised baseline (± 0.00%) to 161.56% (± 46.73%, P2, p = 0.0062) relative to P1, then decreased following removal of SuK P3, to 111.52% (± 42.90%, P3, p = 0.0080) relative to baseline, compared to P2, returning to similar baseline P1 values. unOCN significantly increased from P1, 100% as standardised baseline (± 0.00%) to 176.67% (± 66.66%, P2, p = 0.0135) relative to P1, then decreased following removal of SuK P3, to 87.11% (± 21.43%, P3, p = 0.0076) relative to baseline, compared to P2, returning to similar baseline P1 values

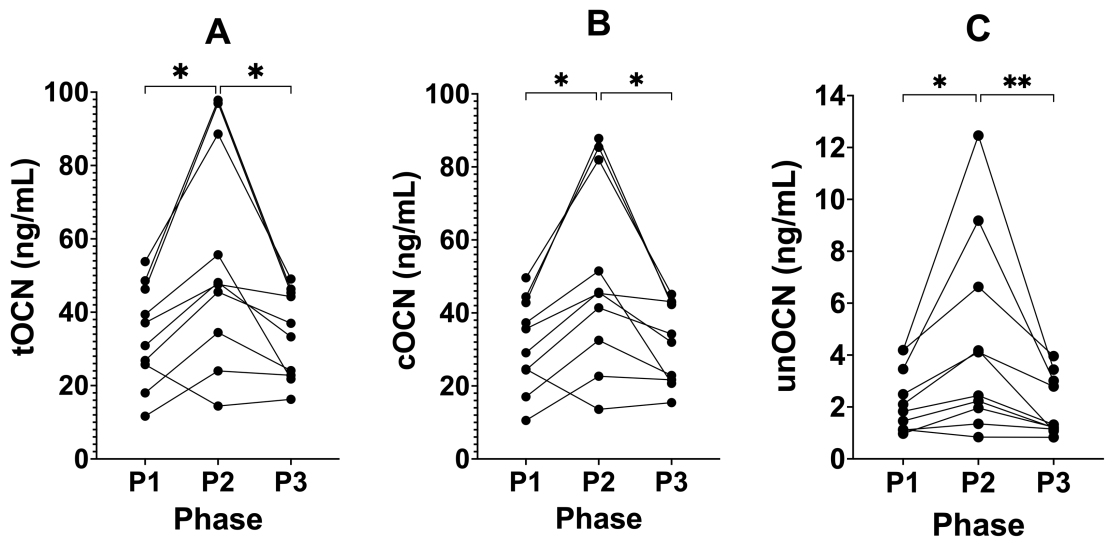

Figure 14. tOCN, cOCN and unOCN across all phases in KetoSAge participants. Fasting plasma concentrations of (A) tOCN, (B) cOCN, (C) unOCN were measured following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3; total OCN (tOCN) and uncarboxylated OCN (unOCN) were determined by ELISA, carboxylated OCN (cOCN) was calculated by subtracting unOCN from tOCN. Samples were taken at 8 a.m. after a 12-hour overnight fast; (n = 10); tOCN and cOCN data analysed by repeated measures one-way ANOVA with Tukey's correction for multiple comparisons, unOCN data analysed using the Friedman test with Dunn's correction for multiple comparisons. * $p < 0.05$; ** $p < 0.01$

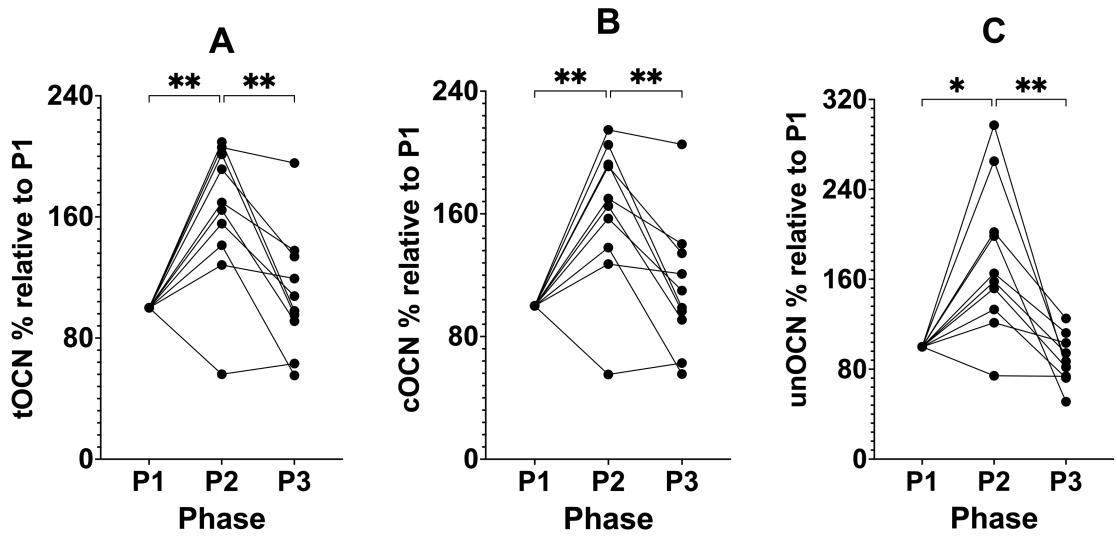

Figure 15. Percentage change from baseline P1 as 100% for tOCN, cOCN and unOCN across all phases in KetoSAge participants. Fasting plasma concentrations of (A) tOCN, (B) cOCN, (C) unOCN were measured following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3; total OCN (tOCN) and uncarboxylated OCN (unOCN) were determined by ELISA, carboxylated OCN (cOCN) was calculated by subtracting unOCN from tOCN. Samples were taken at 8 a.m. after a 12-hour overnight fast; (n = 10); data analysed by repeated measures one-way ANOVA with Tukey's correction for multiple comparisons. * $p < 0.05$; ** $p < 0.01$

5.5 Suppression of ketosis is associated with increased leptin

Following P2, leptin significantly increased from 4.50 ng/mL (± 3.67, P1) to 15.08 ng/mL (± 8.00, P2; p = 0.0010; **Figure 16**) This trend reversed following P3, where leptin significantly decreased and returned to participants baseline levels of 4.57 ng/mL (± 3.48, P3; p = 0.0005) compared to P2.

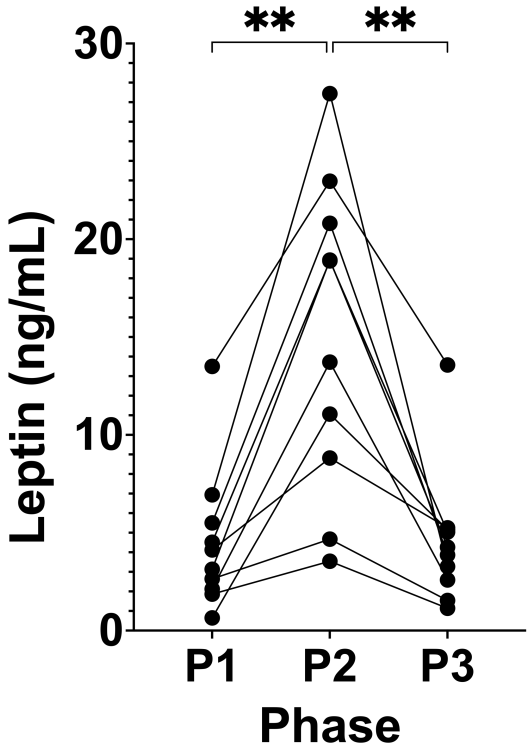

Figure 16. Serum leptin across all phases in KetoSAge participants. Fasting serum concentrations of leptin were measured following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3; Leptin was determined by via ELISA (DuoSet, R&D Systems, USA). Samples were taken at 8 a.m. after a 12-hour overnight fast; (n = 10). Data analysed by repeated measures one-way ANOVA. * $p < 0.05$; ** $p < 0.01$

5.6 Cortisol levels remain in healthy low levels in long term NK, and serotonin does not significantly change

Following P2, cortisol did not significantly change from P1, 126.20 ng/mL (± 52.67, P1) to 112.70 ng/mL (± 58.46, P2; p = > 0.9999; **Figure 17**). Similarly, following P3, there was no significant change compared to P2, 131.90 ng/mL (± 52.18, P3, p = 0.5391). Following P2, plasma serotonin did not significantly change from P1, 21.05 pmol/L (± 22.83, p = 0.7907; Figure 6). Removal of the intervention to SuK with a return to NK for 21 days, at the end of P3, there was no significant change in serotonin compared to P2, at 21.77 pmol/L (± 16.80, P3, p = > 0.9999).

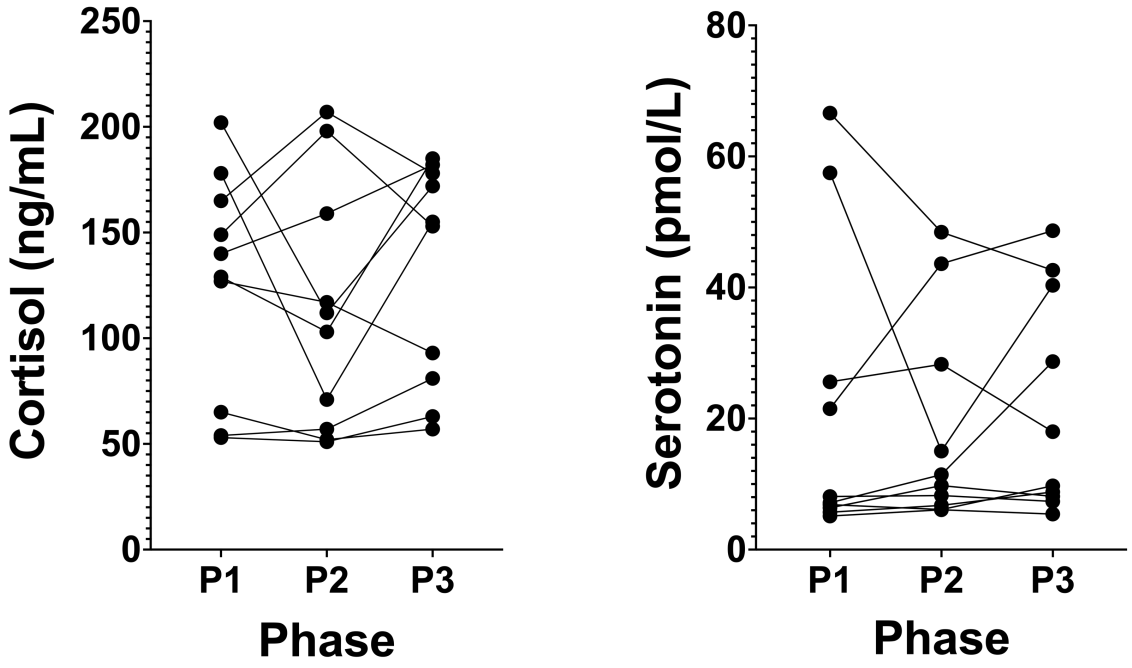

Figure 17. Serum cortisol and serotonin across all phases in KetoSAge participants. Fasting serum concentrations of cortisol and plasma serotonin were measured following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3; cortisol was measured externally by SYNLAB Belgium (Alexander Fleming, 3–6220 Heppignies–Company No: 0453.111.546), serotonin was measured by ELISA (Abcam, Cambridge, UK). Samples were taken at 8 a.m. after a 12-hour overnight fast; (n = 10). Data analysed by Friedman test with Dunn's correction for multiple comparisons.

5.7 Suppression of ketosis is associated with decreased GLP-1

Following P2, active GLP-1 significantly decreased from 1,064.98 pg/mL (± 500.15, P1) to 504.98 pg/mL (± 246.43, P2; p = 0.0209; **Figure 18**). This trend reversed following P3, where GLP-1 significantly increased and returned to participants' baseline levels of 1,032.26 pg/mL (± 453.88, P3; p = 0.0210) compared to P2.

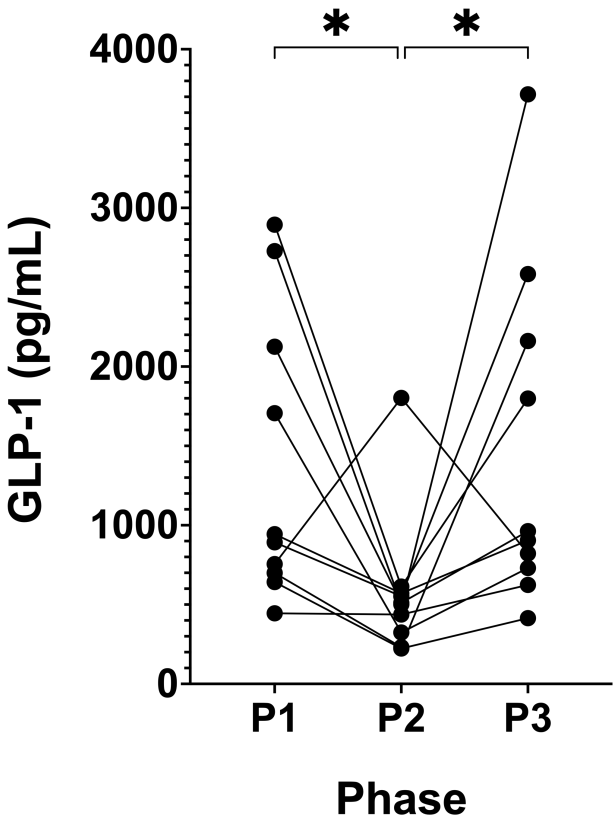

Figure 18. Serum concentrations of active GLP-1 across all phases in KetoSAge participants. Serum concentrations of active GLP-1 were measured following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3 (n = 9). GLP-1 was determined by ELISA (Abcam, Cambridge, UK). Glucagon-like peptide-1 (GLP-1). Samples were taken at 8 a.m. after a 12-hour overnight fast; (n = 10). Data analysed by repeated measures one-way ANOVA. * $p < 0.05$

Chapter 6

6. Discussion

There have been many studies investigating the effect of ketosis in humans, however, little is known about the physiological adaptations in individuals who have never had a metabolic illness and maintained long-term (> 1 year) habitual ketosis for more than 80% of their year. Furthermore, prior work has primarily examined males. This cohort self-reported to have sustained nutritional ketosis for an average of 3.9 years, with confirmed NK for at least 6 months in the lead-in period to the trial. Participants presented with healthy weights, BMI, waist-to-hip, waist-to-height, and blood pressure (**Table 4**). Molecular markers including lipid panels, liver enzymes (**Table 6**) and cytokines (**Table 6**) were also within healthy ranges. Thus, the data here indicates that long term NK, euketonaemia, does not have a negative effect on health in this cohort.

Euketonaemia is defined as a state of ketosis that is not associated with any harmful effects. Although in most cases the term has been used to refer to a state of normal ketonemia in patients with diabetes, the term has also been used in a broader sense to refer to a state of normal ketonemia in healthy individuals (Bronisz, Ozorowski and Hagner-Derengowska, 2018; Dilliraj et al., 2022). Euketonaemia in adults has been associated with improved insulin sensitivity and euglycaemia (Kinzig, Honors and Hargrave, 2010; Skow and Jha, 2022; Paoli et al., 2023). Moreover, euketonaemia is associated with reduced inflammation in the brain (Koh, Dupuis and Auvin, 2020; Jiang et al., 2022), is consistent with evolutionary biology and has a protective effect on mitochondria (Arima et al., 2021).

6.1 When to measure capillary BHB

The 6 months of 4-6 p.m. capillary ketone testing standardised evening measurement as a criterion for eligibility into the trial was chosen due to it being a more rigorous threshold to pass in-order to be judged as sustaining NK over the majority of the 24-hour day, in comparison to morning fasted measurements, thus increasing confidence in the participants maintaining NK most of the time. Most people embarking on a ketogenic lifestyle typically via carbohydrate restriction, time restricted feeding, or other forms of fasting mimicking diets, will measure their capillary ketones in the morning, much the

same way a morning fasting blood glucose is taken. However, a morning ketone reading for hyperinsulinaemic, and insulin resistant individuals will likely produce some measurable levels (potentially up to 0.5 mmol/L) due to an overnight fast. If this were the individuals only daily test, they could mistakenly then assume they are successfully maintaining a metabolic state of ketosis. However, if they were to measure their capillary ketones at least 3 to 4 hours after lunch, then this test would capture their breakfast and/or lunch if they had consumed anything, allowing them to truly know if their lifestyle was supportive of maintaining euketonaemia via minimising lifestyle practices that stimulate excess insulin demand which would inhibit (lower) ketone production.

In this cohort, these participants noted that their pre-lunch and dinner readings were often their highest level of ketones out of their four times a day tests, with their pre-dinner (4-6 p.m.) reading having a slightly higher frequency of being greater than their second test of their day (pre-lunch). This informed the participants of how their lifestyle was supporting them in maintaining therapeutic levels of ketones (> 0.5 mmol/L) throughout their day (Cooper, Kyriakidou, Edwards, et al., 2023).

6.2 Macroscopic changes/Anthropometrics

Throughout the intervention phase (P2), where participants were actively suppressing ketosis, participants often reported capillary BHB concentrations of > 0.3 mmol/L after the overnight fast, and even three hours after a carbohydrate-containing meal (**Table 2**). Together these data indicate that the participants were indeed highly fat-adapted and, even with the introduction of carbohydrates into their diet, their bodies reverted to beta-oxidation and ketolysis during periods of fasting. Following P3, participants tracked back to the baseline level of ketosis as indicated by their capillary BHB levels (**Figure 8D**). The participants enrolled in this study were able to tolerate 21 days of suppression of ketosis and the consequent upregulation of glucose metabolism and still return to their baseline level of ketosis. This suggests that metabolic flexibility is maintained in long-term habitual ketosis in metabolically healthy individuals.

Across all three phases, in two different metabolic states (ketosis vs glucose fuelling), participants' RQ values were indicative of individuals that were metabolically healthy, interestingly their values were superior to observations previously made in high performance athletes (Schutz and Ravussin, 1980; Bergman and Brooks, 1999; Ramos-Jiménez et al., 2008; Goldenshluger et al., 2021). Even after 21 days of SuK, a 12-hour overnight fast still induced higher fat oxidation, as evidenced by their P2 RQ values (**Figure 7; Table 1**). However, surprisingly, there was still a significant difference between P2 compared to P1 and P3, even within the overall highly fat-adapted state that all the participants were in, in all three phases after an overnight fast (**Figure 7**). Given that the baseline RQ values were indicative of a high state of beta-oxidation, it was not expected that a 21-day of SuK would result in RQ measurements that were statistically inferior to those observed at baseline (P1).

Three weeks of SuK resulted in changes in body composition, with increases in weight and BMI. This was largely accounted for by increases in TBW and total body fat (**Table 1**). However, the increased body composition measurements taken following P2 (and indeed, P1 and P3) were still within normal ranges (Nishida, Ko and Kumanyika, 2009; WHO, 2011), and tracked back to baseline levels following P3.

6.3 Insulin, IGF-1, and glucose

In the normative setting, the most influential pancreatic insulin secretagogue is dietary carbohydrate, whilst basal insulin release is regulated by a multitude of factors including hepatic glycogenolysis, which is further regulated by glucagon, osteocalcin (OCN) and other secretagogues (Cooper et al., 2021). It is interesting that with the increased repeated stimulation of bolus insulin during P2, fasting (basal) insulin and glucose subsequently increased (**Figure 8**). This is likely due to insulin's systemic effects, where enforced glucose fuelling results in increased glucose demand. In addition, insulin's suppressive effect on beta-oxidation, where ketogenesis nor lipid provision for beta-oxidation is sufficient due to insulin also inhibiting insulin sensitive lipase which is required to release lipids from adipocytes (Choi et al., 2010; Lan et al., 2019; Althaher, 2022). Therefore, the upregulation

of hepatic glycogenolysis occurs, in response to chronic insulin signalling. This begs the question; would this pattern continue if this intervention was sustained over 20 years?

It is arguable that insulin's primary function is not for glucose uptake in the fasted and ketosis state. Cells that must have glucose, uptake glucose via plasma membrane proteins, which are predominantly diffusion glucose uptake transporters (GLUT) 1-14, which fall into three subclasses I, II and III (Shao and Tian, 2015; Navale and Paranjape, 2016). Human pancreatic beta cells are mainly populated by GLUT1, the brain by highly glucose sensitive GLUT3 and intestinal and kidney cells GLUT2. GLUT4, 8 and 12 are insulin responsive (Shao and Tian, 2015). Whilst cardiac and skeletal muscle cells have GLUT4 transporters, these cells thrive on fatty acid metabolism; a well perfused heart will derive between 60 to 90% of its acetyl-CoA from the beta-oxidation of lipids (Navale and Paranjape, 2016; Chadt and Al-Hasani, 2020). An ischemic heart and CVD has been shown to be rescued by BHB (Yu et al., 2018; Wei et al., 2022; Dyńka et al., 2023). GLUT1 accounts for the predominance of basal glucose uptake (Shao and Tian, 2015), of which the heart and skeletal muscle cells are well populated with.

Participants' habitual ketosis lifestyle also demonstrated significantly lower IGF-1 levels in P1 and P3 (Figure 3). IGF-1 is regulated by insulin on multiple fronts; regulating synthesis and bioavailability via IGF1-binding proteins (Sandhu, Dunger and Giovannucci, 2002), as well as amplification of signal transduction capacity (Goalstone and Draznin, 1998; M L Goalstone et al., 1998; Draznin et al., 2000). Insulin and IGF-1 both transactivate each other's receptors, as well as form cross hybridised receptors (Cao and Yee, 2021). Chronically elevated IGF-1, and/or increased IGF-1 bioavailability and sensitivity, receptor expression and amount of Ras protein prenylation (Goalstone and Draznin, 1998) are strongly implicated in neoplasia and ageing (Sandhu, Dunger and Giovannucci, 2002; Barbieri et al., 2003; Pollak, 2008; Narasimhan, Yen and Tissenbaum, 2009; Zhang et al., 2020), whilst IGF-1 knockdown within *in vivo* models show improved longevity (Holzenberger et al., 2002; Barbieri et al., 2003).

In observational studies, low levels of insulin and IGF-1 have also been associated with reduced levels of pathologies. For example, elevated IGF-1 has been shown to correspond

to a 69% increase in colorectal cancer relative risk, a 49% increase in prostate cancer, 65% increase in breast cancer, and a 106% increase in lung cancer risk (relative risks) (Rahmani et al., 2022). Notably, a recent meta-analysis involving over 30,000 participants indicated that IGF-1 within the range of 120 - 160 ng/mL was the optimum range associated with the lowest risk of all-cause mortality (Rahmani et al., 2022). The participants in the present study fell well within this range during the P1 and P3 phase, however, during SuK (P2), IGF-1 significantly increased, which may confer an increased risk of all-cause mortality. Conversely, the lower levels of insulin and IGF-1 during the P1 and P3 phases may be of health benefit given that higher levels of IGF-1 and insulin are significant risk factors for ageing associated diseases including cancer.

Insulin/IGF-1 signalling inhibits FOXO activity via increasing phosphorylation causing cytosolic sequestration and suppressing BHB action on FOXO expression and nuclear translocation, through the Akt signalling pathway (Jag, Zavadil and Stanley, 2009). FOXO is a transcription factor which regulates the expression of a vast number of genes with functions associated with longevity, including cell cycle arrest, autophagy and DNA damage repair (Webb and Brunet, 2014), as well as regulating metabolism and antioxidant defence (Klotz et al., 2015). In addition to being a metabolic substrate, BHB also acts as a signalling molecule, modulating intracellular activity in cells across the body, such as regulating gene expression through inhibition of class I histone deacetylases (HDACs) via competitive inhibition (Newman and Verdin, 2017). Specifically, BHB prevents histone acetylation at the *FOXO* gene regions (Shimazu et al., 2013) and 12-hour fasted mice have significantly increased levels of FOXO protein expression in the liver (Miyauchi et al., 2019). Based on an understanding of these cellular and intracellular signalling and fuelling dynamics, it is proposed that the low levels of insulin and IGF-1 maintained by the participants in this study during P1 and P3, along with BHB ≥ 0.5 mmol/L, through their lifestyle habits, are a logical and potentially effective way to slow and/or reduce cellular ageing. It is unlikely that life-long sustained vs suppressed ketosis human trials will ever happen, and these indirect comparisons are the next best option, where it is seen in whole of life animal trials, maintaining minimal insulin demand and IGF-1 levels consistently results in optimum longevity (Barbieri et al., 2003; Willcox et al., 2008; Narasimhan, Yen and Tissenbaum, 2009; Zhang et al., 2020).

Considering that the study cohort is exclusively female, it is imperative to recognise, for global population health, the pressing importance of focusing on diabetes, hyperinsulinaemia (insulin may be inside reference ranges, however chronic hypoketonaemia may indicate an individual's hyperinsulinaemia threshold), obesity and breast cancer. Given their widespread prevalence (Visscher and Seidell, 2003; Kautzky-Willer, Harreiter and Pacini, 2016; Ahmed et al., 2023; WHO, 2023), understanding the intricate links between these conditions is critical in order to prevent occurrence and to improve outcomes. The data in this study shows long term NK reduces fasting insulin, IGF-1, and glucose. This data adds to existing evidence that sustaining a lifestyle which promotes euketonaemia is an effective modality for the prevention and management of both type 1 and type 2 diabetes (Bolla et al., 2019).

6.4 Thyroid - free T3

Along with increased glucose and insulin concentrations, SuK (P2) resulted in increased levels of free T3 (fT3). Elevated fT3 can be an indicator of several conditions, namely, thyroid hormones (TH) and T3 are effectors of healthy and dysregulated metabolism, and are strongly associated with T2DM, obesity, and hyperinsulinaemia (da Silva et al., 2017; Martinez and Ortiz, 2017; Tsatsoulis, 2018). Given fT3 is highly involved in the transcription and translation of OXPHOS proteins, it would be expected that being in ketosis would come with higher levels of fT3 than a suppressed ketosis state. Being in ketosis is highly dependent on OXPHOS capacity. It was found in this healthy long standing ketosis maintaining cohort, that their fT3 was significantly lower than after 21 days of suppressed ketosis. A plausible explanation is that ketosis is a fasting-mimicking metabolic state, which reduces thyroid hormone (TH) demand due to less ROS damage on OXPHOS proteins and IMM lipids, such as cardiolipin (CL) (Paradies et al., 2019), and may increase sensitivity, such as increasing mitochondrial fT3 receptors and/or increasing monocarboxylate transporter 8 (Martinez and Ortiz, 2017). In addition, BHB has an epigenetic regulatory role of its own, enabling increased transcription of OXPHOS proteins (Wang, Chen and Xiao, 2021).

Thyroid hormones, and more specifically T3, play a significant role in the regulation of OXPHOS, which affects glycolytic metabolism, in various organs including the liver, gastrointestinal tract, pancreas, adipose tissue, skeletal muscles, and the central nervous system (Short et al., 2001). In the liver, thyroid hormone increases hepatic glucose output by the upregulation of GLUT2, increasing gluconeogenesis and glycogenolysis, which can ultimately lead to hyperinsulinemia and glucose intolerance when there is chronic elevated TH signalling.

Chronic raised insulin levels (detected through chronic suppression of ketosis), both basal and bolus, where repeated bolus exposures are likely playing a role in the upregulation of basal levels, increase mtROS. Given hyperinsulinaemia has been shown to increased mitochondrial localised ceramide (Hansen et al., 2014; Hodson, Tippetts and Bikman, 2015), increasing mtROS and CL oxidation (Paradies et al., 2019), down regulating beta-oxidation (Muoio, 2014), and reduced redox antioxidant defence capacity, a suppressed ketosis state, may stimulate the thyroid to rescue OXPHOS, via increasing T3. Thyroid hormones directly affect mitochondrial biogenesis, fatty acid oxidation, and TCA cycle activity (Brenta, 2011). In-addition, hyperinsulinaemia inhibits lipolytic activity whilst promoting triacylglycerol accumulation and storage, via inhibition of hormone sensitive lipase (HSL), thereby inhibiting adipocyte fatty acid lipolysis and release (Lan et al., 2019). These ultimately increase glycolysis reliance, resulting in greater NAD+ catabolism, which subsequently decreases NAD+ dependant antioxidative redox management capacity. The subsequent increased basal ROS without commensurate antioxidant management, results in chronically upregulated oxidative stress and damage to mitochondrial CL and OXPHOS proteins. The increased insulin signalling, concurrently increases TH synthesis, secretion and signalling, to facilitate OXPHOS maintenance and repair (da Silva et al., 2017; Martinez and Ortiz, 2017; Tsatsoulis, 2018). For short durations, such as 21 days that evolutionarily would be akin to a short summer/autumn, is within the thyroid's capacity to deal with. However, in possible similarity to the pancreatic beta cells, chronic demand of the thyroid to produce increased amounts of TH, may result in mechanisms that down regulate either production or conversion of T4 to fT3, respectively. Those processes again may be due to thyroid cells being affected by intracellular fuelling dynamics and metabolism hormones (insulin) dictating antioxidant regulation, which either help or harm their mitochondria.

In P2, this cohort were not chronically long-term hyperinsulinaemic. Hence, they had the earlier phase of greater demand of thyroid hormone (TH). If those 21 days turned into 21 years, it is arguable that over that time frame, TH may become low, and more specifically T3, in concordance with the research literature in T2DM hyperinsulinaemic populations. The aforementioned healthy low T3 (euthyroid) levels in this cohort when in ketosis, P1 and P3, could lead clinicians to mistakenly diagnose a metabolic phenotype 1 individual (see methods **2.2**) with hypothyroidism. Therefore, it is worth highlighting that T3 levels were still within normative ranges, however, this information should assist clinicians and researchers, indicating a need for nuance and metabolic context when interpreting thyroid biomarkers.

When assessing biomarkers in a wider metabolic context, as can be seen in the data here, that the lower T3 levels were accompanied by significantly greater fat oxidation status understood via RQ values, along with a lower fasting glucose and insulin. In the metabolically unwell (phenotype 3, hyperinsulinaemia), a low T3 status would be accompanied by higher fasting glucose, insulin, and absence of NK, and with a higher RQ. It is likely that the increased T3 levels seen in P2 during the suppressed ketosis state, was due to insulin increasing oxidative stress on the mitochondrial OXPHOS proteins and CL, whilst increased glycolysis depletes the NAD+ cytosolic pool and consequently decreases NAD+ dependent antioxidant management. Finally, a lower T3 seen in T2DM/obesity is associated with poorer glycaemia and increased insulin, which was the contrary to this cohorts lower T3 levels whilst in ketosis with low insulin and glycaemia during P1 and P3. Therefore, there is a need for considering the metabolic context when interpreting thyroid biomarkers.

BHB directly neutralises $^{\cdot}OH$, protecting CL and signals to increase transcription of OXPHOS proteins (Robinson and Williamson, 1980; Achanta and Rae, 2017; Newman and Verdin, 2017). This likely protects the thyroid follicular cells directly, as well as reduces TH demand, thus reducing T3 levels. Interestingly, liraglutide, which increases the amount of insulin secreted in response to glucose, is not recommended for individuals with either a

family or personal history of medullary thyroid cancer or multiple endocrine neoplasia type 2 (MEN2). This may be due to the highly mitogenic effect of insulin (Draznin, 2011).

In the progression of hyperinsulinaemia stages, it is likely that TH demand and thus synthesis increases in the early stages to facilitate OXPHOS protein synthesis and repair. Over time as hyperinsulinaemia perpetuates and possibly worsens, TH, more specifically T3 levels may decline due to a number of factors which include; direct effect of hyperinsulinaemia on thyroid follicular cells (Biondi, Kahaly and Robertson, 2019), increasing mitochondrial ROS with subsequent previously described effects of chronic elevated ROS, such as reducing cytosolic NAD+ pool; and increasing pseudo-hypoxia glycolysis leading to increased mitogenic signalling (Draznin, 2011), reducing thyroid follicular cellular time in their G1 phase, the phase a cell spends performing it's tissue origin function, and instead increased rate of cell division, potentially leading to development of thyroid tumours. A chronically increased global demand for TH and T3 synthesis, would also increase selenium requirement for increased activity of glutathione peroxidase (GPx) and thioredoxin reductase (TRx), antioxidant selenoenzymes, in-order to protect cells from oxidative damage incurred whilst synthesising TH which produces H_2O_2. Furthermore, selenoprotein iodothyronine deiodinase enzymes (DIO), catalyse the conversion of thyroxine (T4) into triiodothyronine (T3) (Negro, 2008). Naturally, increased TH synthesis and use, therefore, increases iodine requirement. A low selenium and or iodine status would impair TH synthesis, and a low selenium status would potentially increase oxidative stress in thyrocytes due to the reduced selenium dependent antioxidant capacity (Carabotti, Annibale and Lahner, 2021).

Increased carbohydrate consumption that raises blood glucose, increasing hyperinsulinaemia and consequently suppressing ketosis, also raises stomach pH, thereby decreasing the strong stomach acidity required for the solubility of minerals. As stomach pH increases above pH 3, minerals precipitate out of solution and unbind from their amino acid ligands, rendering them less bioavailable (Drago, Binaghi and Valencia, 2005; Carabotti, Annibale and Lahner, 2021). Hyperinsulinaemia is strongly associated with conditions of hypochlorhydria, such as gastroesophageal reflux disease (GERD) and gastritis. A dietary intervention study using a high fat low carbohydrate diet to assess the

effects on GERD in 144 obese, hyperinsulinaemic/insulin resistant women found that carbohydrate ($p > 0.001$), sugar ($p = 0.005$), glycaemia load ($p = 0.001$) and HOMO-IR ($p = 0.004$) were all significantly associated with GERD, and after 10 weeks of implementing the LCHF dietary intervention, all symptoms of GERD and medications usage were resolved (Pointer et al., 2016). In another RCT, a higher carbohydrate intake was shown to raise stomach pH and increase GERD reflux symptoms (Wu et al., 2018). Hyperinsulinaemia and its common presentations such as obesity which is associated with T2DM and CVD, also has increased rates of gastrointestinal complications such as GERD (OR 1.94), erosive gastritis (OR 2.23), and many others including Barrett's oesophagus, gastric cancer, diverticulitis, gallstones, and pancreatic cancer (Camilleri, Malhi and Acosta, 2017). The prevalence of GERD appears to be occurring at younger ages and also in parallel to increases in sugar intake (Powell, Smith-Taillie and Popkin, 2016; Yamasaki et al., 2018).

Contrary to popular belief, microbiota diversity may not be desirable for optimal gastrointestinal health. In a study of 31 patients (80% female, 52.2 +/- 13.1 years of age), 75% with chronic atrophic gastritis, and all with an absence of any neuroendocrine tumours nor gastric adenocarcinomas, it was found that a higher gastric pH (lower acid), was contributory towards duodenal dysbiosis and the development of gastrointestinal tract pathology, and hypochlorhydria and increased chronic atrophic gastritis was associated with an increased duodenal microbiota diversity. This also has been shown to be correlated with increased rates of small intestinal bacterial overgrowth (SIBO) (Filardo et al., 2022). Proton pump inhibitors cause hypochlorhydria and achlorhydria, and are considered a contributory cause for the onset of SIBO, and reduce mineral absorption from food (Heidelbaugh, 2013). Foods that reduce stomach acidity (higher gastric pH), over time, increase hypochlorhydria. Diets higher in carbohydrates that suppress ketosis, increase hyperinsulinaemia, and reduce basal stomach acidity (raise stomach pH, hypochlorhydria) leading to gastrointestinal diseases such as GERD and gastritis, contribute to poorer uptake of essential minerals, affecting those involved in metabolism and regulation of antioxidant activities, such as iodine and selenium (Rannem et al., 1998; Drago, Binaghi and Valencia, 2005; Carabotti, Annibale and Lahner, 2021). This then compounds the need for TH in the context of a suppressed ketosis and hyperinsulinaemia state [phenotypes 2 and 3, discussed in (Cooper et al., 2021)].

The low levels of insulin with euglycaemia and euketonaemia observed in P1 and P3 may confer long term health benefits for the participants. Elevated insulin levels have been shown to be significantly associated with all-cause mortality and cancer-specific mortality in post-menopausal women (Pan et al., 2020). There are a number of studies which have demonstrated an association between elevated levels of insulin and risk of death in various cohorts across the globe (Ausk, Boyko and Ioannou, 2010; Loh et al., 2010; Perseghin et al., 2012; Tsujimoto, Kajio and Sugiyama, 2017; Lee et al., 2018). Thus, it is proposed that the sustained NK at baseline (P1) and after removal of the suppression of ketosis intervention (P3) were likely associated with lower risk of all cause insulin associated mortality.

The lower levels of basal insulin and IGF-1 were as a direct and indirect (respectively) result of reduced exposure to dietary induced elevated plasma glucose. Higher levels of basal glucose has been associated with increased risk of all-cause mortality (Crawley et al., 2014), however, this is likely due to the glucose induced stimulation of insulin and consequent increased IGF-1 pathways that become activated as a result of excess dietary carbohydrate. During P1 and P3, the blood concentration of glucose was considerably lower than during the suppression of ketosis P2. Notably, however, the concentrations of fasting glucose in all phases were under the threshold defined by the WHO (Alberti and Zimmet, 1998). In a prospective cohort of 28,024 US female health professionals, published in 2021, assessing clinical factors that increased the risk profile for premature coronary heart disease, the highest risk factor was T2DM across all ages, with aHR as high as 10.71, metabolic syndrome (aHR, 6.09), hypertension (aHR, 4.58), obesity (aHR, 4.33), and smoking (aHR, 3.92) which attenuated with age (Mora et al., 2021). It is important to recognise that T2DM, metabolic syndrome, hypertension and obesity are conditions of hyperinsulinaemia (Paoli et al., 2013; Crofts et al., 2015; Cooper et al., 2020), at various degrees of a spectrum and expression in different tissue/organ systems.

Hyperglycaemia causes oxidative damage, resulting in advanced glycation end-products (AGE), and receptor for AGE (RAGE), this increases heparanase expression (Maxhimer et al., 2005; An et al., 2011; Goldberg et al., 2019). Heparanase is an endoglycosidase that

enzymatically cleaves glycosaminoglycan heparan sulfate (HS) (Goldberg et al., 2019). The importance of this is understood by the following effects of loss of HS. Heparan sulphate proteoglycans (HSPG) are robust anticoagulants, buffering glycation damage (An et al., 2011). An increase in heparanase and a decrease in HS is implicated in endothelial cell dysfunction (An et al., 2011). HS is required for adipocyte to macrophage mitochondrial transfer, which is decreased in hyperinsulinaemia/obesity (Brestoff et al., 2021), potentially contributing to dysregulated immune system function in obesity, T2DM and other hyperinsulinaemia conditions (Liang et al., 2020). The effect of T2DM (hyperinsulinemia + glycaemia) on the immune system, results in defects in innate & adaptive immunity that play a large part in T2DM infection susceptibility. For example in T2DM Ig mass is higher versus controls, leading to reduced vaccine efficiency in stimulating humoral immunity (Daryabor et al., 2020).

Where you find increased AGE and RAGE, there is an increase in heparanase expression (hyperglycaemia/hyperinsulinaemia, insulin resistance, T2DM, CVD, obesity, cancer, dementia). Increased heparanase expression decreases extracellular plasma membrane localised HSPG, key mediators of anticoagulation. Hyperglycaemia upregulates heparan sulphate–degrading endoglycosidase heparanase-1 (HPR1) expression (Maxhimer et al., 2005; An et al., 2011). Hyperinsulinaemia drives lowered vitamin D hydroxylation/activation/transport, increasing sulphate wastage and oxidation damage to HSPG (Bolt et al., 2004; Cooper et al., 2020). Individuals with chronic hyperinsulinaemia already have increased risk of thrombi/clots. What propagates hyperinsulinaemia, drives a decrease in HS, leading to increased RBC and platelet agglutination, and thrombosis.

Logically, addressing hyperinsulinaemia, by restricting carbohydrate intake to below one's personal tolerance threshold, would be an appropriate measure to improve overall health and susceptibility to poorer outcomes from increased blood coagulability, and its downstream effects such as increased risk of hypertension, atherosclerosis, strokes, hypoxia. In a dose response meta-analysis, involving fifty trials with 4,291 patients, results showed carbohydrate restriction exerted a significant reduction on cardiometabolic risk factors in patients with T2DM and that those levels decreased linearly with the decrease in carbohydrate intake (Jayedi et al., 2022). Furthermore, analysis of 7 year primary care

data in 143 patients with T2DM implementing carbohydrate restriction, saw improvements in renal function (Unwin et al., 2021).

The participants data here shows that long term sustained nutritional ketosis serves to reduce fasting insulin, IGF-1, and blood glucose. These data further add to existing evidence that sustaining a lifestyle which promotes ketosis is an effective modality for the management of both type 1 and type 2 diabetes (Bolla et al., 2019) and reducing the risk and poorer prognosis for cancer and cachexia (Shukla et al., 2014). Cancer cachexia accounts for nearly 20% of cancer related deaths. Ketosis has been shown to protect against cachexia, diminish glutamine uptake, and induce apoptosis (Shukla et al., 2014). In a mouse model, mice given a ketosis-inducing high fat diet (using medium chain triglyceride - MCT oil as 80% of calories), this resulted in reduced tumour mass whilst maintaining body weight and reducing cachexia (Beck and Tisdale, 1989).

A unique aspect of this study is that the participants enrolled self-reported to be habitually adapted to ketosis for a significant period of time (mean 3.9 years ± 2.3), with confirmed NK in the lead-in period for at least 6 months. Participants presented with healthy weights, BMI, waist-to-hip, waist-to-height and blood pressure (**Table 3**). Molecular markers including lipid panels, liver enzymes (**Table 5**) and cytokines (**Table 6**) were also within healthy ranges. Thus, the data here indicate that long term ketosis in healthy women, does not have a negative effect on health.

6.5 OGTT

The data here indicate that the participants enrolled in this study were able to maintain normal glycaemic responses throughout the OGTT following each of the phases (**Figure 10**) highlighting that prolonged ketosis did not hinder metabolic flexibility. However, in the first 2 hours of insulin response, there appears to be a shift to the right for P1 and P3. This is likely due to a reduced frequency and load from a large glucose bolus exposure, therefore increasing the time to peak for bolus insulin synthesis and secretion. Concurrently, plasma glucose appears to be greater during the first 2 hours in these two phases, and also higher peaks of glucose in P1 and P3, compared to the P2 phase (although

not statistically significant). This pattern may be incorrectly labelled as a lack of sensitivity to insulin; on the contrary, this is the sum of the exogenous glucose from the OGTT, plus hepatic glucose output, which does not abate from the one-time glucose bolus.

The dosage of dietary glucose administered during the OGTT compounds with the hepatic glucose output, and therefore contributes to an elevated peak of glucose concentrations during P1 and P3 (**Figure 10a**). When the participants were fat fuelling (metabolic phenotype 1), their glucose needs were largely dependent on hepatic provision via gluconeogenesis and glycogenolysis. When glucose is restricted from the diet, the liver synthesises glucose mostly from the joining of two glycerol backbones from triacylglycerides during beta-oxidation, simultaneously supporting ketogenesis. These processes are enabled by the lower insulin levels, whereas greater levels of insulin inhibit beta-oxidation and subsequently ketogenesis. Sustained glycogenolysis during an OGTT is also seen in hyperinsulinaemia individuals (metabolic phenotype 3), where the liver is pathway-selective insulin signalling resistant, suppressing ketogenesis, and inhibiting beta-oxidation, whilst increasing, *de novo* lipogenesis and glycogenolysis is not inhibited (Petagine, Zariwala and Patel, 2023). The chronic hyperinsulinaemic state is also coupled with a higher glucagon state (DemantMia et al., 2018; Kumpatla et al., 2021), adding to hepatic signalling that maintains hepatic glucose provision to the wider system.

Hypothetically, under an evolutionary context, selection pressure would have favoured the ability to adapt to and maintain NK due to seasonal food availability and intermittent CR/fasting, meaning the body's glucose needs would have been met by hepatic gluconeogenesis and glycogenolysis. If an (infrequent) opportunity to consume a high carbohydrate load would have presented itself, the subsequent increased insulin secretion would likely not have inhibited gluconeogenesis and glycogenolysis during the one time exposure, given the body is adapted and reliant on hepatic glucose as its main glucose source (Cooper et al., 2021; Cooper, Brookler and Crofts, 2021). If insulin in this one instance were to inhibit gluconeogenesis and glycogenolysis, whilst facilitating oral glucose load myocyte uptake, a potential case of hypoglycaemia with hypoketonaemia and inhibition of beta-oxidation may simultaneously occur, which would be potentially fatal. In this metabolic phenotype 1 context (Cooper et al., 2021), an infrequent one time

bolus insulin secretion does not inhibit gluconeogenesis and glycogenolysis; this may incorrectly be interpreted as hepatic insulin resistance, as is the case for hyperinsulinaemic T2DM (stage-3 metabolic phenotype 3) individuals (Cooper et al., 2021). This has not been observed in this present cohort, as ketogenesis declined during the first 2 hours, indicating the liver is being affected by the bolus insulin release, and is selectively responding based on the participants metabolic phenotypic healthy physiological state and adaptation. Like switches and gates, the metabolic phenotype signature changes hepatic responses to a bolus insulin signal.

Overall, the data here indicate that long-term ketosis does not appear to negatively affect the insulin-dependent glucose uptake nor reduce carbohydrate tolerance. In fact, following SuK P2, participants demonstrated a significantly elevated peak level of insulin in response to a glucose challenge across the OGTT, compared to P1 and P3. Furthermore, in all phases, insulin levels were at their lowest and plateauing after 240 minutes, glucose was on a rise upwards to basal P2 fasting levels after 180 minutes in P2, whereas glucose was further declining back to basal fasting levels in P1 and P3. Together, these findings indicate a lower total insulin requirement to maintain lower glucose levels when in a state of NK, whereas SuK was associated with an increased insulin requirement. Maintaining lower-normal glucose levels with lower insulin and IGF-1 levels are associated with improved health outcomes, in decreasing risk of insulin resistance and T2DM, reducing chronic diseases and also improving longevity and healthy ageing (Holzenberger et al., 2002; Barbieri et al., 2003). This suggests that maintaining a long-term metabolic phenotype 1 profile may aid in maintaining a healthier healthspan and lifespan.

It was previously hypothesised that the addition of the BHB sensitivity assay in an OGTT challenge would help to differentiate between different metabolic phenotypes with improved resolution (Cooper et al., 2021; Cooper, Kyriakidou, Petagine, et al., 2023). Here it is shown that indeed, the combination of insulin and BHB measurements throughout the OGTT helps to differentiate early stage hyperinsulinaemic individuals (metabolic phenotype 3, stage 1 or 2), or prior metabolically unwell individuals who have restricted carbohydrates and gone into ketosis (metabolic phenotype 4), from long standing healthy ketosis living individuals (metabolic phenotype 1). The combination of glucose, BHB and

insulin measurement help to provide greater resolution in understanding metabolic health and, help clinicians and researchers to better classify individuals when designing trials or analysing data.

During P2, the participants were exposed to an increased frequency (*ad libitum*, spread over three times a day SUK diet recommended, which prevents TRF induced ketosis), dose (glycaemic load, SUK diet recommendation to consume at least 267 g of carbohydrate per day) and duration (21-day intervention) of dietary glucose, consequently repeatedly triggering bolus insulin release (equating to an equivalent carbohydrate exposure of approximately 63 OGTTs in 21 days). The increased bolus insulin secretion signals to the liver to temporarily reduce glycogenolysis; this is considered hepatic insulin sensitivity (response seen in metabolic phenotype 2 and not phenotype 3, stage 3), as hepatic glucose output is reduced in response to the insulin signal. Many have come to consider this the normal and healthy response, which is likely correct for those consuming a ketosis suppressive diet and do not have any chronic ageing and hyperinsulinaemia disease. However, if we were to consider humans under an evolutionary context, with less frequency, dose and duration of exogenous carbohydrate exposure, then it is arguable that what can be seen in this study's cohort's response curves in P1 and P3 would be the normal/healthy physiological responses. Hepatic glucose provision under the context of being in sustained NK would not be inhibited by bolus insulin secretion, and therefore this is not a case of pathological insulin resistance which logically only would be the case under a chronic hyperinsulinaemia and not an acute context (one-time OGTT for a metabolic phenotype 1 individual maintaining NK as a lifestyle). High levels of BHB would not be observed in hyperinsulinaemic/T2DM/CVD individuals, and therefore analysis of BHB response during an OGTT and/or for several consecutive days before the evening meal, is essential for resolving a T2D glycaemic response (metabolic phenotype 3 spectrum) and those on the hyperinsulinaemic spectrum, from those in ketosis (Cooper et al., 2021).

It can be seen that during both ketosis phases (P1 and P3), BHB concentrations began to recover following the glucose challenge at 180 minutes, with an overall U-shaped curve. However, participants in P2 did not mirror this response pattern and sustained low levels of ketones before and after 180 minutes following the glucose challenge, with a flat line

pattern. These data indicate that consumption of a carbohydrate diet that suppresses ketosis, for 21 days, results in limited BHB ketogenesis, even following a 12-hour fasting period, indicating adaptive changes and likely downregulation of cellular ketogenesis enzymes and activities.

6.6 Liver markers: GGT and PAI-1

There were significant findings regarding the effects of ketosis suppression and subsequent return to ketosis on liver markers. It is recognised that GGT is a diagnostic marker for many diseases in humans, including a fatty liver, T2DM, MetS and AD, which are typified by hyperinsulinaemia (Luchsinger et al., 2004; Grundy, 2007; Unwin et al., 2015). In this study, SuK (P2) resulted in a significant increase in GGT levels, an enzyme associated with oxidative stress, low-grade inflammation, and insulin resistance (Gohel and Chacko, 2013; Kwak, Seo and Lee, 2023). Furthermore, GGT participates in the direct generation of ROS, via a glutathione (GSH)/transferrin system, where in the presence of molecular oxygen and iron/copper ions from transferrin in the presence of cysteinylglycine (a product of GGT/GSH reaction), results in a paradoxical generation of ROS. This results in increased free radical and oxidative damage to nucleic acids, protein and lipid peroxidation (Drozdz et al., 1998). The findings here suggest that suppressing ketosis may impose some degree of oxidative stress and inflammation on the liver, leading to increased GGT levels. GGT levels returned near-baseline levels after these participants discontinued suppressing ketosis, indicating carbohydrate restriction is an effective tool in correcting the significant increases in GGT.

High levels of GGT have been found to be associated with increased risk of MetS and impaired fasting glucose (Wang et al., 2021; Xing et al., 2022; Kwak, Seo and Lee, 2023). The participants' GGT levels in ketosis (P1 and P3) and SuK (P2) were within standard reference ranges. However, a study in a large nonobese population of nondiabetics n = 1,309, showed that a moderate elevation in GGT within normal reference ranges is a strong risk marker predictor for T2DM, independent of visceral fat, obesity and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) (Bonnet et al., 2011). Unwin *et al.,* later corroborated this finding, in a primary healthcare setting. After

restricting dietary carbohydrates in 67 individuals, with a minimum 3 months adherence and an average follow-up of 13 months, they found the improvements in GGT (reduction) had no correlation to weight loss (Unwin et al., 2015). In a longitudinal study, n = 452, with average 80 years of age, GGT was shown to be associated with cognitive decline prior to vascular dementia (Björk and Johansson, 2018). With an increasingly growing aged population, monitoring GGT may also provide an ability to detect and intervene earlier in dementia prevention.

GGT is a glycosylated microsomal two substrate enzyme. It is ubiquitously expressed in cell types except myocytes (Brennan, Dillon and Tapper, 2022). In a two-step process, GGT catalyses the cleavage of the γ-glutamyl bonds from donor molecules such as glutathione (GSH) and glutamine, proceeding to transfer the γ-glutamyl moiety to acceptor substrates such as water molecules via hydrolysis, or via transpeptidation of short peptides or L-amino acids, particularly cysteine (Tate and Meister, 1981; Saini et al., 2021). GGT metabolises extracellular GSH, an antioxidant which plays an essential role in the removal of ROS (Lee, Blomhoff and Jacobs, 2004). GSH is a major mitochondrial antioxidant which protects against oxidative damage, a hallmark of various diseases including NAFLD, neurological disease, T2DM and cancer (Pizzino et al., 2017; Liguori et al., 2018). Higher levels of GGT can therefore lead to decreased GSH, causing an imbalance in the removal of ROS and increasing a state of oxidative stress. It is noteworthy that GGT levels returned near-baseline levels after the participants discontinued suppressing ketosis, carbohydrate restriction is shown here to be an effective tool in correcting the significant increases in GGT.

6.7 PAI-1

The antifibrinolytic plasminogen activator inhibitor-1 (PAI-1) is the primary inhibitor of plasminogen activators (PAs), via inhibition of tissue-type PA (tPA) and urokinase-type PA (uPA) that proteolytically cleave zymogen plasminogen to active plasmin (Sillen and Declerck, 2021). Elevated PAI-1 levels propagate a prothrombotic state (Sillen and Declerck, 2021). It can be seen that there was a significant increase in PAI-1 levels, during P2 compared to P1. Insulin has been shown to stimulate the secretion of PAI-1 by

adipocytes, and there is a strong positive correlation between hyperinsulinaemia and elevated PAI-1 (Samad et al., 2000; Yarmolinsky et al., 2016; Altalhi, Pechlivani and Ajjan, 2021). However, although fat mass increased after SuK (P2), it also returned to baseline return to ketosis (P3), whilst PAI-1 also significantly increased after P2, yet trended back but not significantly to baseline after P3. With the loss of gained fat mass after P3, with only a trend back for PAI-1, this indicates other mechanisms outside of adiposity were involved.

PAI-1 circulates in the plasma at low levels (5–50 ng/mL) and its main pool in platelets (approximately 300 ng/mL) (Booth et al., 1988). HSPG on the surface of endothelial cells and blood cells, are critical inhibitory regulators of platelets, maintaining them in their quiescent state (Vögtle et al., 2019). Hyperinsulinaemia decreases the amount of HSPG (Cooper, Brookler and Crofts, 2021). Hyperglycaemia upregulation of heparinase, (Maxhimer et al., 2005) proteolytically cleaves perlecan HSPG, releasing endorepellin the C-terminal fragment of HSPG. Endorepellin goes on to enhance collagen-mediated platelet activation via binding to integrin α2β1 (Bix et al., 2007). Renal sulphate wastage is increased via hyperinsulinaemia driven lowered vitamin D3 hydroxylation, activation, transport and adipocyte sequestration (Bolt et al., 2004; Cooper et al., 2020). Platelets remain quiescent when ``brushed" regularly by HSPG, maintaining healthy blood flow (Bye, Unsworth and Gibbins, 2016; Vögtle et al., 2019). Activation of platelets releases PAI-1 into the plasma, increasing plasma PAI-1 concentrations and subsequent downstream signalling effects (Vögtle et al., 2019; Sillen and Declerck, 2021). Platelet activation further increases *de novo* PAI-1 synthesis (Brogren et al., 2011). Platelet activation is increased via increased PI3K, Akt and PKC intracellular signalling, all of which are increased by hyperinsulinaemia and more so when glucose uptake insulin resistance develops (Leitner et al., 1997; Bye, Unsworth and Gibbins, 2016; Hopkins et al., 2018). In 2016, CVD mortality accounted for about 17.8 million deaths worldwide, where ischemic heart disease (IHD) and stroke contributed to 87% (WHO, 2019). Disseminated intravascular coagulopathy (DIC) and thrombosis cause blockages of the blood flow to either the heart or brain resulting in insufficient blood supply, as well as increased atherosclerosis (Juhan-Vague et al., 2003). These processes are strongly associated with increased levels of PAI-1. NK may provide an effective strategy to reduce risk of DIC, thrombosis (WHO, 2018).

Hyperinsulinaemia increases gene expression of and stabilises PAI-1. Semad *et al.,* demonstrated insulin mediated increase in PAI-1 gene expression is through a different signalling pathway to insulin mediated glucose transport. Indicating in the hyperinsulinaemia insulin resistant state, where glucose tolerance declines, signalling by hyperinsulinaemia to upregulate PAI-1 gene expression is unimpeded, and regulated by a pathway that does not become insulin resistant, directly contributing to elevated PAI-1 (Samad et al., 2000; Cooper et al., 2020).

Previous research has shown that PAI-1 is involved in inflammation, insulin resistance, obesity, cancer, Alzheimers, diabetes, metabolic syndrome and CVD (Mahmood, Mihalcioiu and Rabbani, 2018; Sillen and Declerck, 2021; Nawaz and Siddiqui, 2022). PAI-1 contributes to an inflammatory response via infiltration of immune cells, specifically macrophages, in adipose tissue (Sillen and Declerck, 2021). Adipocytes are a source of PAI-1 (Alessi et al., 1997; Sillen and Declerck, 2021). Findings from research conducted on obese mice revealed that adipocytes are a notable source of PAI-1 (Sawdey and Loskutoff, 1991; Sillen and Declerck, 2021). This evidence was later corroborated in human adipose tissue studies, emphasising the role of expanded adipose tissue in circulating PAI-1 levels (Alessi et al., 1997). The participants returned to their baseline mass with a concurrent loss of fat mass. Indicating the non-significant trend of PAI-1 returning to baseline from P2 to P3 was not associated with the significant loss of fat mass. As such, it may also be concluded that in these participants, the surge in PAI-1 during P2 was not due to enhanced adiposity. Potential explanations could be tethered to a prolonged interval in the downregulation of insulin-induced gene expression, or an extended duration required for optimising HSPG-mediated stimulation of platelet inactivity.

A state of chronic inflammation, typical of conditions like obesity, T2DM, and MetS, is distinguished by the augmented expression of inflammatory adipokines, such as IL-6 and TNF-α (Ellulu et al., 2017). These adipokines are known to upregulate PAI-1 expression within adipose tissue (Pandey, Loskutoff and Samad, 2005). In this study's data, however, there were no marked alterations in these inflammatory markers across the different phases. These observations may explain how the inflammatory cytokines, notably IL-6 and

TNF-α, did not precipitate PAI-1 increase from P1 to P2, suggesting that this increase was not caused by a surge in these cytokines.

Elevated, dose dependent levels of PAI-1 are pro-tumourigenic, pro-angiogenic and anti-apoptotic (Devy et al., 2002; Balsara and Ploplis, 2008). PAI-1 is one of the most highly induced proteins in metastatic invasive tumours and tumourigenesis process (Bajou et al., 1998; Sillen and Declerck, 2021). PAI-1 binds to the low density lipoprotein receptor-related protein 1 (LRP1) receptor, activating intracellular signalling cascades, modulating cell migration, such as mast cells in gliomas (Roy et al., 2015; Sillen and Declerck, 2021). PAI-1 is a highly reliable prognostic and biomarker in a host of cancers, including: breast (Harbeck et al., 2004; Leissner et al., 2006; Duffy et al., 2016; Jevrić et al., 2019; Sobocan et al., 2020), bladder (Becker et al., 2010; Chan et al., 2017), colon (Herszényi et al., 2008), gliomas (Iwadate et al., 2008; Roy et al., 2015), ovarian (Kuhn et al., 1999; Nakatsuka et al., 2017; Koensgen et al., 2018), non-small cell lung cancer (Ostheimer et al., 2018) and renal (Zubac et al., 2010) cancers.

During P2, levels of insulin and IGF-1 were increased indicating a synergistic relationship between markers of insulin resistance, whereby higher levels of insulin, IGF-1 and PAI-1 are seen in combination with low levels of BHB. However, PAI-1 levels did not show any significant changes upon returning to ketosis. Suppression of ketosis for 21 days produced significant changes in GGT, and PAI-1. Therefore, P2 in these participants may be associated with lipid peroxidation, oxidative stress, and inflammation.

With ageing comes increased rates of chronic diseases. At the cellular microscopic level, cells express a senescence associated secretory profile (SASP), resulting in loss of cellular physiological function, arrested growth/division, and increased oxidative stress and ROS production. This SASP profile triggers paracrine and endocrine signalling domino effect, inducing more cells to adopt a SASP profile, culminating in accelerated tissue, organ and systemic ageing (López-Otín et al., 2013). PAI-1 is seen to increase with age, furthermore, PAI-1 is a part of the SASP paracrine signalling pathway, inducing the SASP profile in neighbouring cells, therefore acting as both a marker and maker of cellular ageing and ageing related pathologies (Vaughan et al., 2017; Sillen and Declerck, 2021; Jiang et al.,

2023). The data from this trial adds to the suggestion that reducing PAI-1 through adopting a ketogenic diet may have the potential to carry wide health benefits.

6.8 Cytokines

It is often posited that inflammation precedes hyperinsulinaemia, however, this study's data does not support this. Where there was an observed increase in insulin and glucose, at the end of P2, CRP, interleukins and TNF-α remained unchanged. With no increase in CRP nor interleukin cytokines or TNF-α, indicating that inflammation by these molecules was not mediating the increased insulin levels that suppresses ketogenesis. There were, however, increases in growth factors VEGF, EGF and MCP which are discussed below.

6.9 VEGF and EGF

Following SuK (P2), there were significant increases in VEGF and EGF compared to baseline ketosis (P1) (**Table 6; Figures 12A & B**). This indicates that being in a state of ketosis does not over stimulate the production of these growth factors and chemokines, whereas being in a state of carbohydrate metabolism promotes their production. The concentration of these growth factors then trended back towards the baseline values after 21 days of returning to ketosis (P3). However, the concentrations were not significantly different compared to P2. This indicates that the relatively short period of carbohydrate fuelling is a sufficient time to elevate the concentrations of these growth factors in a way that cannot be fully recovered in 21 days after returning to ketosis.

Pericytes are supportive cells which wrap around blood vessels, serving as multilineage progenitor cells and are essential for the development of new blood vessels (Ahmed and El-Badri, 2018). Insulin stimulates pericytes to increase their production of VEGF which in turn stimulates endothelial cells to grow and proliferate, facilitating angiogenesis (Escudero et al., 2017). The lower levels of insulin in the NK phases likely account for the lower levels of VEGF. Insulin and IGF-1 have been shown to promote the upregulation of VEGF or EGF (Hill and Milner, 1985; Steller, Delgado and Zou, 1995; Miele et al., 2000; Hale et al., 2013). EGF signalling is one of the key pathways involved in tumour development

(Ayati et al., 2020; Mabeta and Steenkamp, 2022). Ketogenic metabolic therapy (KMT) may aid in reducing the expression of EGF and VEGF.

Considering this trial cohort is female, it is imperative to recognise the links between hyperinsulinaemia, metabolic health and breast cancer. Women with diabetes have been shown to exhibit poorer outcomes for breast cancer compared to their non-diabetic counterparts (Ahmed et al., 2023). Consistent with these findings, *in vitro* research has shown that treating cancer cells, particularly breast and pancreatic, with high glucose initiates molecular alterations such as phosphorylation of EGFR, which promotes their proliferation (Han et al., 2011; Flores-López et al., 2016; Hou et al., 2017). The implications of hyperglycaemia also extend to treatment outcomes, with heightened glucose levels during chemotherapy leading to increased chemoresistance in tumour cells (Zhao et al., 2015). Beyond direct cellular growth effects, the hyperglycaemic state appears to compromise the body's innate anti-tumour defences, notably by inhibiting neutrophil mobilisation, thereby granting tumour cells an immunological escape route and enhancing their metastatic capabilities (Fainsod-Levi et al., 2017).

In cancer, the aberrant activation of the EGF pathway is characterised by upregulated downstream signalling cascades, including the PI3K/Akt/mTOR and the Ras/Raf/MEK/ERK pathways, both of which modulate processes such as cell proliferation, differentiation, and apoptosis evasion. In Glioblastoma for example, amplification or mutation of EGFR can result in constitutive activation of these signalling cascades, conferring a survival advantage to tumour cells. Hyperinsulinaemia is a potent stimulator of the PI3K/Akt/mTOR pathway (Lopez et al., 2013; Noch et al., 2023), which is intricately linked to the mechanisms of both EGF and VEGF. High insulin levels, as seen in hyperinsulinaemia, increase VEGF production, leading to enhanced blood supply to growing tumours. This augmented vascular network can facilitate delivery of nutrients to the tumour, promoting its growth.

SuK (P2) involved routine 3x a day feeding containing around 267 g of carbohydrate resulting in the occurrence of hyperglycaemic and hyperinsulinaemic excursions equivalent to 3 OGTTs per day for 21 days, totalling 63 OGTTs. These periodic increases in

glucose and consequent bolus insulin would not be captured in a fasting glucose/insulin test. This likely contributed to the upregulation of VEGF, EGF and PAI-1.

6.10 MCP-1

Following SuK (P2), MCP-1 expression significantly increased, returning to baseline following P3 (**Table 6; Figure 12C**). MCP-1 is a chemokine (also termed CCL2) involved in the recruitment of monocytes and produced by a range of cell types including monocytes/macrophages, epithelial, adipocytes, endothelial and smooth muscle cells (Deshmane et al., 2009), cells which express high levels of insulin receptors. Insulin has been shown to increase levels of MCP-1 in adipose tissue of both lean and obese individuals (Westerbacka et al., 2008). Thus, carbohydrate rich diets that suppress ketosis resulting in elevated insulin during P2 may help to explain the significantly increased levels of MCP-1.

There are multiple lines of evidence from both human and murine studies which suggest that MCP-1 appears to be a key player in insulin resistance. MCP-1-deficiency ameliorates insulin resistance in mice via downregulation of ERK and p38 mitogen activated protein kinase (MAPK) phosphorylation in the liver (Nio et al., 2012). Moreover, MCP-1 has been shown to mediate skeletal muscle inflammation and localised insulin resistance in mouse muscle in T2DM models (Patsouris et al., 2014). Thus, NK may not only assist with regulating glycaemic control in T2DM, reducing insulin demand and exposure (Yuan et al., 2020), but may help to ameliorate further MCP-1-mediated insulin resistance, further reducing insulin demand.

The reduction in MCP-1 during the ketosis phases indicate one manner by which a ketogenic state may possess protective effects. Murine studies have shown that insulin can increase the expression of MCP-1 by adipocytes (Sartipy and Loskutoff, 2003), and stimulation of adipose tissue with MCP-1 can also induce dedifferentiation, which may contribute to the pathologies observed in obesity, such as cancer cell dedifferentiation which occurs in their malignant transformation (Sartipy and Loskutoff, 2003). Elevated levels of MCP-1 have also been indicated in the pathophysiology of many other diseases

inducing age-related macular degeneration (Du et al., 2016), allergic asthma (Gonzalo et al., 1998; Quoc et al., 2023), COVID-19 and CVD (Singh, Anshita and Ravichandiran, 2021). Thus, ketogenic diets may reduce pathological burden in these conditions through reducing MCP-1 levels.

Hyperinsulinaemia often coexists with obesity, where adipose tissue is metabolically active, secreting adipokines and cytokines, including MCP-1. Adipocyte secreted MCP-1, transforms monocytes into activated macrophages, and attracts them to fat tissue (Kanda et al., 2006), further fuelling inflammation and release of additional MCP-1, inducing insulin resistance and contributing to tumourigenesis potential. In the early stages of hyperinsulinaemia induced weight gain, adipocytes undergo hypertrophy. If hyperinsulinaemia persists over time, it drives adipocytogenesis and hyperplasia, which bears similarities to neoplasia. Chronic adipocyte hyperplasia is strongly associated with increased rates of malignant neoplasms, likely due to the hormonal and metabolite signalling profile that upregulates growth and division pathways (Janssen and Street, 2021; Zhang et al., 2021). It is often claimed that obesity causes cancer, however, it can be seen that adipocyte hyperplasia and malignant neoplasia's share an upstream root cause. Although, once obesity occurs, it may go from sharing the same effectors, to becoming an instigator itself.

This cohort showed no significant changes in the interleukins, except for a decrease in IL-1β from P2 to P3 (**Table 6**). IL-1β is a potent pro-inflammatory cytokine which becomes upregulated in response to pathogens and also in chronic disease (Kaneko et al., 2019). IL-1β is a cytokine mainly produced by activated monocytes/macrophages (Lopez-Castejon and Brough, 2011). Elevation of IL-1β in P2 compared to P3 correlates with the increased expression of MCP-1 was observed in this phase. However, there was no observable increase in IL-1β after SuK P2 from P1, this may be because these participants were in an anti-inflammatory state (P1) that persisted during the early days of SuK, which may have buffered/slowed down any change during that time.

Given VEGF, EGF and MCP-1 are often elevated in many cancers (Abdulla et al., 2021; Wang et al., 2022; Yang and Cao, 2022; Mulholland et al., 2023), KMT may be an effective

way to support the action of certain cancer therapies, along with using the glucose-ketone index (GKI) calculator to measure therapeutic efficacy in metabolic management of brain cancers and likely other cancers (Meidenbauer, Mukherjee and Seyfried, 2015). Furthermore, KMT may be an effective stand-alone therapy for cancer. There have, indeed, been various human studies indicating that a ketogenic diet is tolerable for individuals with cancer (Tan-Shalaby, 2017), but also effective in reducing tumour burden and symptomatic disease (Weber et al., 2020). Tumour cells are not well adapted to metabolising ketones, especially in the presence of abundant glucose, but instead predominantly depend on glucose for fuelling (Liberti and Locasale, 2016). Limiting glucose availability for tumours by adapting into ketosis may therefore create a metabolically unfavourable environment for tumour growth, whilst also reducing insulin and IGF-1's growth and division stimulating signals (Draznin, 2010). The data presented here indicate that long-term ketosis is safe in healthy populations; well-designed clinical trials would elucidate the value of such an approach in cancer therapy.

6.11 Hypoketonaemia, HOMA-IR and fasting insulin

Throughout the KetoSAge study, participants maintained healthy fasting glucose levels in all phases, 4.36 mmol/L (± 0.53, P1), 5.12 mmol/L (± 0.59, P2) and 4.41 mmol/L (± 0.30, P3), which are considered extremely healthy (The Emerging Risk Factors Collaboration, 2011). HbA1c was not measured in this study, due to the three-phase blood sampling timing that would have resulted in prior blood sampling affecting subsequent next blood sampling results (P2 affected by P1, and P3 affected by P2). HbA1c is used to assess cumulative glucose exposure which glycates haemoglobin (HbA1c) in blood, this provides evidence of average blood glucose levels over the previous two to three months, which is the average half-life of red blood cells (Sherwani et al., 2016). The percentage glycation damage as a result of hyperglycaemia which is associated with increased risk for atherosclerosis, CVD, cerebrovascular disease, all-cause dementia (ACD) and AD. For example, in the AgeCoDe cohort study of 1,342 elderly individuals, a higher HbA1c level was found to be associated with an increased ACD and AD. Where an HbA1c ≥ 6.5% was associated with an increased risk of ACD by 2.8-fold ($p = 0.027$) and for AD ($p = 0.047$). There was an associated 5-fold increased risk of incident ACD ($p = 0.001$) and a 4.7-fold increased risk of AD ($p = 0.004$)

with HbA1c levels ≥ 7% (Ramirez et al., 2015). However, hyperinsulinaemia has been shown to precede hyperglycaemia by up to 24 years (Crofts et al., 2016). There is, therefore, a weakness when relying on HbA1c to detect earlier disease risk, in that there is a sub-clinical phase (occult stage) during which a healthy HbA1c value is seen. This is due to an increased insulin concentration that is reducing plasma glucose, whilst the elevated basal insulin is still having its non-glucose related signalling effects, such as increasing PI3K and MAPK signalling for growth and division (Goalstone and Draznin, 1998; M L Goalstone et al., 1998; Draznin, 2010, 2011), and increasing ceramide production that increases mitochondrial reaction oxygen species generation, and therefore increasing oxidative stress in the system (Smith et al., 2013; Hansen et al., 2014; Hodson, Tippetts and Bikman, 2015).

An example of how a reduced HbA1c, due to more insulin exposure can turn out to be less positive for health, is found in the ACCORD group randomised controlled trial (RCT) study of 10,251 patients, mean age 63 years who had an 8.1% median HbA1c. All participants were already on exogenous insulin. Half of the group was randomised to a more tailored intensive insulin therapy regimen to achieve a lowering of their HbA1c to 6%, whilst the other group was to follow their standard insulin therapy to target a value of 7.0 - 7.9%. The three primary outcomes measured in this RCT were non-fatal stroke, non-fatal myocardial infarction, and CVD death. This trial was stopped after 3.5 years due to ethical reasons, as the intervention group receiving the intensive insulin therapy (more insulin) in order to lower their HbA1c a greater amount, had a significantly higher mortality rate (Veech et al., 2017). Whilst successfully lowing their HbA1c with more insulin, there was an increase in deaths, indicating that relying on a lower HbA1c may lead physicians and patients into a false sense of security whilst patients are actually being exposed to greater insulin levels and thus, its subsequent potential harms (Draznin, 2011; Hansen et al., 2014; Cooper et al., 2020; Ahmed et al., 2023; Cooper, Kyriakidou, Petagine, et al., 2023; Zhou et al., 2023).

HOMA-IR is recognised as a diagnostic marker for insulin resistance, where a higher concentration of fasting insulin to maintain a low fasting glucose results in a higher HOMA-IR value (Yang et al., 2023). Insulin resistance is generally accepted as having a

HOMA- IR > 2 (Paoli et al., 2013; Gershuni, Yan and Medici, 2018; Cooper, Sanchez-Pizarro, Norwitz, et al., 2023), whilst hyperinsulinaemia is considered where fasting plasma insulin is > 28.8 uIU/mL [8,9,22,36]. The participants' group mean of fasting insulin levels was 9.06 uIU/mL (± 2.13) after 21 days of suppressing ketosis, with the highest individual fasting insulin measuring at 11.92 uIU/mL. However, HOMA-IR was able to detect a significant change, where in both NK phases, the participants' HOMA-IR were at the very healthy levels of 0.97 (± 0.32, P1) and 1.11 (± 0.41, P3). Conversely, suppressing ketosis managed to significantly double their mean HOMA-IR to 2.07 (± 0.61, P2), bringing it into the beginning range of insulin resistance, with one participant having a HOMA-IR > 3 after suppressing ketosis for 21 days. All participants' HOMA-IR values returned to their healthy baseline values with only 21 days of returning to NK by removing the intervention to SuK via ingestion of carbohydrates. Given HOMA-IRs strong association and even predictive value in chronic disease risk, it would be of high value to understand how lifestyle may modulate this marker index measured alongside other biomarkers strongly associated with disease risk, such as gamma-glutamyl transferase (GGT), insulin like growth factor-1 (IGF-1) and monocyte chemotactic protein 1 (MCP-1) and ageing to provide further confidence and resolution in diagnostics and more accurately phenotyping/categorising patients in studies (Cooper, Kyriakidou, Edwards, et al., 2023).

In a cross-sectional analysis study of 12,266 participants from the NHANES data, a positive correlation was found between participants' HOMA-IR levels and their BA in US adults (Yang et al., 2023). A prospective cohort study of 3,741 asymptomatic employees of Santander Bank in Madrid, aged between 40-55 years at recruitment, with no known CVD, investigated the relationship between early insulin resistance as measured using the HOMA-IR index in normoglycaemic individuals assumed to be at low risk of atherosclerosis, in addition to the effect of cardiovascular risk factors in individuals with normal HbA1c. The presence and progression of sub-clinical atherosclerosis (SA) was assessed using non-invasive vascular imaging modalities, multiterritorial vascular ultrasound and coronary arterial calcification (CAC) scans (Iglesies-Grau et al., 2023). The study found HOMA-IR had a direct association with the multiterritorial extent of SA and CACs ($p < 0.001$). 85.1% of the reference group (HOMA-IR < 2) were free of coronary artery calcium (14% had a CACS of > 0). This proportion of participants with CAC increased as

insulin resistance increased, with a HOMA-IR = 2 to 3 the proportion increased to 25.6%, and with a HOMA-IR > 3 the proportion increased further to 39.3%. A bibliometric study of 1,500 publications found HOMA-IR/insulin resistance strongly associated with cerebral small vessel disease, ischemic stroke incidence, post-stroke depression and early neurological deterioration in patients who suffered stroke (Zhou et al., 2023).

The 21 days of suppressing ketosis resulted in hypoketonaemia, due to insulin's suppressive control of ketogenesis (Newman and Verdin, 2017). Whereas P1 and P3 were typified by euketonaemia along with healthy low insulin and HOMA-IR values. As this cohort was exclusively female, it is imperative to recognise the links between hyperinsulinaemia, metabolic health, and breast cancer (WHO, 2019; Ahmed et al., 2023). In a retrospective case-control study, 80 non-diabetic patients with pre-menopausal and post-menopausal breast tumours were compared to 60 women with normal mammography's as a control. Hyperinsulinaemia and insulin resistance/HOMA-IR have been found to be associated with an increased risk of breast cancer in non-diabetic (normoglycaemic) women. Whilst hyperinsulinaemia determined by a fasting insulin levels > 28 uIU/mL, may proceed hyperglycaemia for up to 24 years (Crofts et al., 2015; Cooper et al., 2020, 2021; Cooper, Kyriakidou, Edwards, et al., 2023; Cooper, Kyriakidou, Petagine, et al., 2023), early sub-clinical (occult) hyperinsulinaemia may be considered when there is chronic hypoketonaemia measured between 4-6 p.m. for at least three consecutive days (a longer duration would increase confidence), as this time of day would reduce the false positive test result coming from waking up with nutritional ketosis levels (> 3 mmol/L) due to an overnight fast (Cooper, Kyriakidou, Edwards, et al., 2023; Cooper, Sanchez-Pizarro, Norwitz, et al., 2023).

In this KetoSAge trial, the chronic suppression of ketosis (P2) intervention resulted in significantly increased fasting insulin levels that would still be considered healthy values, and significantly increased HOMA-IR values into the insulin resistant range. The participant data show that long term NK maintains and reduces HOMA-IR to very healthy levels, adding to existing evidence that sustaining a lifestyle that promotes ketosis (minimising insulin demand and secretion), is an effective modality in preventing insulin resistance and its associated diseases including cancer, CVD, dementia, T2DM and earlier biological

ageing (Paoli et al., 2013; Crofts et al., 2015; Gershuni, Yan and Medici, 2018; Janssen and Street, 2021; Cooper, Kyriakidou, Edwards, et al., 2023; Yang et al., 2023). It was very reassuring to see that the participants' HOMA-IR levels returned to their baseline mean value 1.11 (± 0.41, P3) after the removal of carbohydrates returning to sustained NK, indicating that restricting carbohydrates is a viable and practical method to reduce risk of insulin resistance related morbidities known to affect healthspan and lifespan. Additionally, the data indicates that the effect of suppressing ketosis, for 21 days, on HOMA-IR days can be reversed by a subsequent period of sustained NK, in individuals who habitually maintain a ketogenic supportive lifestyle.

6.12 Osteocalcin

Human OCN has a molecular weight of 5 kDa, 49 amino acids long and is the tenth most abundant protein in the body (Hauschka et al., 1989), and has been shown to have an endocrine function in glucose homeostasis, insulin sensitivity, neurogenesis, cognitive health and mitochondrial biogenesis (Ferron and Lacombe, 2014; Mizokami et al., 2016; Puig et al., 2016; Nakamura, Imaoka and Takeda, 2020; Cooper, Brookler and Crofts, 2021; Wang, Mazur and Wein, 2021). The majority of OCN is synthesised by osteoblasts and osteocytes in the bone. The carboxylated form (cOCN) is both deposited into the bone and released into the system by the osteoblasts and osteocytes. unOCN is synthesised and released into the circulation by osteoblasts and osteocytes, as well as resorbed from the bone by osteoclasts (Cooper, Brookler and Crofts, 2021). cOCN is required for the correct alignment of hydroxyapatite (HA) which confers bone its torsion and tensile ductility, giving it resistance to fragility fractures which is not captured via bone mineral density (BMD) assessment (Dede et al., 2014; Cooper, Brookler and Crofts, 2021).

In the KetoSAge trial, all forms of osteocalcin (tOCN, cOCN and unOCN) significantly increased after 21 days of suppressing ketosis and then decreased back to baseline with the return to ketosis. Interestingly, OCN is synthesised and secreted from adipocytes only during adipogenesis (Foresta et al., 2010). Weight gain as fat mass occurred during the 21 days intervention phase (P2) to suppress ketosis via re-introduction of dietary carbohydrates following the SUK Eatwell Guideline recommendations (data previously

published in (Cooper, Kyriakidou, Edwards, et al., 2023)). This may explain the increase in all forms of OCN.

tOCN, cOCN and unOCN increased from P1 to P2 in all but one participant (ID: 1091). From P2 to P3, tOCN and cOCN decreased in all but one participant, which was the same individual (ID: 1091). Upon further investigation, participant ID: 1091 regularly reported consuming beef bone broth during P1 and P3. Since OCN is the second most abundant protein in bone, it is plausible that this broth may have served as an exogenous source of OCN, thus explaining the increase (Mizokami et al., 2016). Removal of their data in unOCN analysis results in a significant decrease from P2, 4,691.87 pg/mL (± 3,751.66 pg/mL) to P3, 1,873.10 pg/mL (± 1,173.00 pg/mL; $p = 0.0489$). Further investigation into exposure of exogenous OCN from foodstuffs is therefore warranted.

An increased HOMA-IR increases coronary atherosclerosis risk, insulin resistance has a sub-clinical occult phase potentially detectable via repeated consecutive days blood ketosis and glucose testing (between 4-6 p.m.), where a capillary BHB value < 0.3 mmol/L would indicate hypoketonaemia due to occult hyperinsulinaemia. Endothelial progenitor cells (EPC) which aid in the repair of the vasculature, may also come with vascular calcification as they have been shown to express OCN genes in insulin resistant chronic disease conditions such as atherosclerosis (Iglesies-Grau et al., 2023). EPCs from 72 patients with coronary atherosclerosis undergoing invasive coronary assessment have been shown to express OCN genes indicating epigenetic osteogenic transformation (Gössl et al., 2008). Other studies of patients with hyperinsulinaemia conditions, such as in CVD and chronic kidney disease, have detected vascular smooth muscle cell osteogenic differentiation and aortic valve tissue having increased expression of sclerostin (Zhu et al., 2011; Brandenburg et al., 2013; De Maré, D'haese and Verhulst, 2020; Tyson et al., 2020; Cooper, Brookler and Crofts, 2021), corroborating the relationship between chronic increased insulin exposure altering gene expression to the osteogenic profile.

Interestingly, in a study of 2,493 individuals with MetS, plasma OCN levels were found to be inversely correlated with HOMA-IR, fasting insulin and glucose, leptin, and BMI ($p < 0.0010$ for each biomarker) (Saleem, Mosley and Kullo, 2010). Interestingly, OCN is

found to be lower in obese (Bao et al., 2013; Yaylali et al., 2019) and T2DM people, such as seen in a correlation analysis of 204 patients with T2DM, an inverse relationship between OCN and HOMA-IR was found (Yaylali et al., 2019; Lei et al., 2022). With this information, it would be easy to then make the assumption that a higher level of OCN would therefore be better. However, the KetoSAge trial shows an opposite pattern, where P1 and P3 had the healthiest low HOMA-IR values, and significantly lower OCN (all forms) relative to P2. Indicating OCN is positively associated with HOMA-IR in this cohort. Furthermore, as BMI and fat mass increased in P2 (Cooper, Kyriakidou, Edwards, et al., 2023), so too did all forms of OCN in the present study. In corroboration with Ferron's work (Ferron, 2018), OCN increases in P2 where insulin also increases, indicating a potential relationship of OCN increasing insulin secretion. However, this trial does not corroborate an increase in sensitising glucose uptake, given P2 came with a significant increase in fasting blood glucose, although the mean value was still in a healthy range. It is possible that OCN was rescuing the situation, meaning if OCN did not increase, perhaps there would have been a greater increase in glycaemia and trend towards IR. OCN patterns in MP1 individuals may behave much like free triiodothyronine (T3), where T3 demand increases as mitochondrial reactive oxygen species increases, causing oxidative damage to mitochondrial (mt) oxidative phosphorylation (OXPHOS) proteins, and resulting in increased demand of T3 to upregulate synthesis of mtOXPHOS electron transport chain proteins. Furthermore, like the pattern seen in the prior work (Cooper, Kyriakidou, Edwards, et al., 2023; Cooper, Sanchez-Pizarro, Norwitz, et al., 2023), a lower T3 can be observed when in long-term ketosis and an increase in T3 after 21 days of suppressing ketosis, likely indicating an increased T3 sensitivity and decreased demand, which may be a similar case with OCN. If we assume in a healthy setting OCN would be synthesised and deposited into the bone, and only a low dose of OCN is steadily released into the circulation, therefore the bone OCN deposition would confer a healthier bone tensile strength, and there may be a potential greater sensitivity to the OCN that is in the circulation (Cooper, Kyriakidou, Edwards, et al., 2023; Cooper, Sanchez-Pizarro, Norwitz, et al., 2023).

OCN has been shown to stimulate GLP-1 synthesis (Mizokami et al., 2013). A pattern that was not corroborated in this trial. However, there may be a difference between

endogenous versus oral exposure to OCN. It is interesting that as OCN significantly increased in P2, GLP-1 conversely decreased, which is an opposite trend to other experiments showing OCN activating GLP-1 synthesis (Mizokami et al., 2013, 2020). Research into human physiology often investigates pathology and tries to reverse engineer the relationships, or very often uses a control group labelled as the "healthy" group, where in actual fact they are the "common" group and not necessarily a group reflecting human evolutionary phenotypic living which is likely to be in a state of ketosis for a majority of each 24-hour day (Ben-Dor et al., 2011; Cooper et al., 2021; Cooper, Kyriakidou, Petagine, et al., 2023). This would result in a metabolic signature that is different to non-pathology presenting long-term suppressed ketosis metabolic phenotype 2 (MP2) people (Cooper et al., 2021). A low OCN concentration in healthy long-term ketosis should not be confused with a low OCN level as seen in obesity, T2DM, CVD and AD, where these cohorts are also seen to have higher rates of fragility fractures with normal to high bone mineral density. This indicates poor bone health with hyper mineralisation as a form of osteocyte fossilisation, rendering them unable to thrive nor survive, nor produce the necessary OCN not only for the alignment of HA to confer bone tensile strength, but to also release into the circulation to play its part in healthy metabolic homeodynamic regulation (Cooper, Brookler and Crofts, 2021). In this context, a low plasma OCN concentration is indicative of pathology. It is therefore clear that clinicians and researchers need to be aware of understanding biomarkers within the metabolic phenotype profile of each individual, so as not to misdiagnose/categorise according to a single marker out of the patient's metabolic context.

6.13 Leptin increases with chronic suppression of ketosis, a risk of hyperleptinaemia

Leptin is an adipokine, synthesised and secreted by white adipocytes, which has a 24-hour circadian rhythm (Boden et al., 1997) and may be a more sensitive marker for hyperinsulinaemia than BMI. In a study of 119 normal-weight 18 to 24 year old participants, with a BMI < 25, found fasting leptin levels were significantly associated with fasting insulin ($\beta \pm SE = 0.30 \pm 0.06$, $p < 0.001$) and HOMA-IR ($\beta \pm SE = 0.41 \pm 0.20$, $p < 0.001$) (Kempf et al., 2006). These relationships were independent of age, gender and total body fat, indicating that although leptin increases with obesity, it may be, that which

causes obesity or excess body fat, which can exist in normal BMI individuals often termed TOFI (thin on the outside, fat on the inside) (Ruderman, Schneider and Berchtold, 1981; Pluta, Dudzińska and Lubkowska, 2022), also causes the hyperleptinaemia and hyperinsulinaemia. These share a common root cause, and therefore, explaining why it can be seen, leptin and insulin rising where obesity may not, or may rise later on, meaning obesity was not the first mover in causing the rises in these biomarkers associated with chronic metabolic diseases. Chronic excess insulin is the likely root cause, since insulin is so intrinsically linked to the regulation of leptin, and that excess may not be captured by a fasting insulin nor an oral glucose tolerance test (OGTT). Insulin tightly regulates ketogenesis, therefore many consecutive days of BHB testing between 4-6 p.m., acts as a proxy to understanding individual hyperinsulinaemia, if we accept hyperinsulinaemia to mean "more than is ideal for optimal health" for an individual (Veech, 2004; Newman and Verdin, 2017). The KetoSAge participants' leptin levels were healthily low in their natural habitual ketosis states, at levels classified/categorised as the healthiest, in terms of CVD risk (Reilly et al., 2004). With the 21 days suppression of ketosis, which resulted in a significant increase in fasting insulin, there was a significant increase in fasting leptin too, to levels that re-classify into less healthy ranges associated with poorer health outcome (Reilly et al., 2004). Returning to ketosis showed a complete return to baseline levels, indicating how responsive leptin is to dietary carbohydrate-stimulated insulin secretion, in long-term habitually keto-adapted females.

The KetoSAge trial participants' weight and fat mass did increase significantly after 21 days of SuK, however, their BMI stayed well within the healthy range. This corroborates the understanding that although BMI is a good tool to investigate disease risk, there are subsets of people with sub-clinical (occult) conditions that are being pooled into research which decreases accurate reflection of physiology and pathophysiology in trial findings and in data analysis. A BMI < 25 kg/m^2 is considered healthy, however, there are a subset of people with normal BMI who have metabolic disease and even those with BMI > 25 kg/m^2 may have occult hyperinsulinaemia for many years when their BMI was < 25 kg/m^2 (Ruderman, Schneider and Berchtold, 1981; Ruderman et al., 1998; Zdrojewicz et al., 2017; Ghosh et al., 2019; Pluta, Dudzińska and Lubkowska, 2022). Boden *et al.* showed using a 72-hour euglycaemia-hyperinsulinaemia and hyperglycaemic clamp, that

prolonged hyperinsulinaemia and not hyperglycaemia increased serum leptin in 28 healthy normal weight males. Furthermore, those high levels of free fatty acids also did not affect leptin release (Boden et al., 1997). Whilst this trial intervention to SuK for 21 days resulted in these significant changes, it is thought provoking to consider if it had lasted months if not years.

Higher levels of leptin have been shown to be associated with increased frailty in older adults. Fragility fracture rate increases with hyperinsulinaemia (Cooper, Brookler and Crofts, 2021), which is also associated with ageing given the "chronic" in chronic-elevated insulin indicates a long duration of time exposure, which chronological age reflects. In an investigation between leptin levels and incident frailty in 1,573 individuals from the Seniors-ENRICA cohort, aged ≥ 60 years and without T2DM (Lana et al., 2017), those with leptin levels in the highest tertile, had a significantly increased risk of frailty (odds ratio [OR]: 2.12; 95% confidence interval [CI]: 1.47-3.06; p-trend < 0.001). In a multivariate linear regression analysis, leptin levels were shown to be positively associated with insulin resistance in 398 middle-aged and elderly Taiwanese individuals ($\beta = 0.226$, $p < 0.01$) (Shih et al., 2022). Leptin has also been shown to be positively associated with atherosclerosis assessed by CAC in a cross-sectional study in 200 participants, age between 35-75 years with T2DM (which is hyperinsulinaemia with hyperglycaemia, with over insulin resistance) (Reilly et al., 2004). Given the HOMA-IR and progression of early SA-CNIC-Santander study (NCT01410318) showed a significant increased risk of SA with increase in HOMA-IR, the KetoSAge trial provides us with valuable information on increasing our knowledge about how to maintain or modulate these biomarkers associated with one of the global leading causes of chronic disease mortality, CVD (Iglesies-Grau et al., 2023). The KetoSAge trial showed lower leptin levels during ketosis coupled with very low HOMA-IR (no insulin resistance), and suppression of ketosis increased leptin in lockstep with increasing insulin resistance. The removal of dietary farinaceous carbohydrates to no longer stimulate and increased insulin demand repeatedly, showed how responsive these chronic disease biomarkers are to ketosis, the metabolic state that throughout history humans likely spent most of their time in.

6.14 Cortisol and serotonin

The steroid glucocorticoid hormone cortisol is mainly synthesised in the adrenal cortex by the zona fasciculata, and to a much lesser degree by the thymus, brain, intestine and skin (Stalder and Kirschbaum, 2020). Further, cortisol is involved in the regulation of gluconeogenesis, and is often considered the stress response or related hormone (Yan et al., 2016). In 919 participants aged 60-75 years, from the Edinburgh Type 2 Diabetes prospective study, elevated fasting plasma cortisol was positively associated with increased ischaemic heart disease (Reynolds et al., 2010). The univariate analysis found an associated increased odds of ischaemic heart disease with cortisol concentrations > 800 nmol/L (290 ng/mL). The standard reference range for cortisol in plasma is 33- 246 ng/mL (Kushnir et al., 2004). The KetoSAge cohorts mean fasting serum cortisol levels were within healthy ranges in all phases of the trial, with both ketosis phases mean concentrations of 126.20 ng/mL (± 52.67, P1) (348.13 nmol/L) and 131.90 ng/mL (± 52.18, P3) (363.90 nmol/L), and after 21 days of SuK (P2) 112.70 ng/mL (± 58.46, P2) (310.90 nmol/L). It has been suggested that chronic gluconeogenesis can induce a "fight or flight" emergency survival process, resulting in chronic elevated cortisol secretion (Seal and Turner, 2021). Interestingly, although there were no significant changes in cortisol in the KetoSAge trial, the overall healthy low levels of cortisol should be noted, which indicate ketosis via carbohydrate restriction with subsequent reliance on gluconeogenesis for a long period of time, does not appear to result in hypercortisolaemia. Further, as the KetoSAge participants were on average, in long-term ketosis for 3.9 years, their plasma glucose source was predominantly from gluconeogenesis, despite perfectly healthy cortisol levels. Together, these data strongly negate claims that long-term ketosis gluconeogenesis is a fight or flight emergency survival process.

OCN has been shown to modulate insulin sensitivity and secretion via stimulating β-cell serotonin synthesis and secretion, to modulate pancreatic islet α cells (Cooper et al., 2021; Cooper, Brookler and Crofts, 2021), however, this modulation of serotonin may be intracellular, and its secretory actions act in a paracrine manor within the pancreas and thus be undetectable in the blood stream. No significant changes in peripheral vascular plasma serotonin were seen in the KetoSAge trial.

5.15 GLP-1

The current "blockbuster" drugs on the market for diabetes and weight loss are classed as GLP-1 agonists. These participants in long-term NK, showed significantly higher levels of GLP-1, whilst suppressing ketosis significantly decreased GLP-1. Lifestyle practices that stimulate chronic excess insulin demand and secretion results in lower GLP-1 levels and therefore less GLP-1 receptor (GLP-1R) activation, which is associated with increased insulin resistance, obesity and T2DM (Popoviciu et al., 2023). Interestingly, in a competing risk regression analysis of 462 incident first cancer cases with 2,417 controls, it was found that a higher fasting GLP-1 level was significantly associated with a lower risk of incident first cancer (sub-hazard ratio 0.90; 95% CI 0.82–0.99; p = 0.022) (Jujić et al., 2023).

GLP-1 is excreted primarily by ileo-colonic (L) enteroendocrine cells in the distal small bowel and colon (Drucker, 2006). Interestingly, GLP-1 stimulates proinsulin gene expression resulting in activating the replenishment of insulin stores (Drucker et al., 1987). GLP-1 activates GLP-1R on pancreatic islet α cells, inhibiting glucagon secretion resulting in a lowering of plasma glucose independent of insulin (Kreymann et al., 1987; Ramracheya et al., 2018). It was not possible to measure glucagon in these participants' samples due to low sensitivity of the assays trialled and potentially as a result of glucagon's rapid degradation *ex vivo* (Albrechtsen et al., 2015). However, fasting glucose and insulin were both significantly lower in both NK phases along with significantly higher concentration of GLP-1, whilst fasting glucose was significantly higher during P2 suppression of ketosis with significantly lower GLP-1 levels and higher fasting insulin. This suggests the lower fasting glucose levels during both NK phases, were likely due to GLP-1 lowering of glucagon thus resulting in a lower fasting glucose, independent of insulin, and subsequently the NK phases having lower fasting insulin values. GLP-1 is an incretin, however, it enhances insulin secretion in a glucose-dependent manor (Ebou et al., 2016). Participants' low HOMA-IR values in both NK phases, were half that of when suppressing ketosis (P2), meaning they had significantly greater insulin sensitivity when in ketosis, which would mean less basal insulin would be required to "do the job" of regulating plasma glucose, if one were to ascribe this as the primary role of insulin. However, in the long-term NK state, basal insulin's primary role is not likely to be the regulation of plasma glucose, and may instead be the regulation of fat oxidation and ketogenesis, as well as

modulating hormones and growth factors (Cooper, Kyriakidou, Edwards, et al., 2023; Cooper, Kyriakidou, Petagine, et al., 2023), whilst GLP-1 may be regulating basal glucose levels via glucagon.

OCN, more specifically the uncarboxylated form unOCN, stimulates GLP-1 synthesis via the promiscuous GPRC6A receptor (Pi, Nishimoto and Quarles, 2017). However, the KetoSAge participants had lower OCN (all forms) in both NK phases with higher GLP-1, indicating the elevated GLP-1 was not likely due to endogenous OCN action. As the incretin effect of GLP-1 is glucose dependent, therefore elevated basal GLP-1 in long standing euketonaemia is likely acting on its none incretin actions, such as the slowing of gastric emptying (Müller et al., 2019). This may aid in increasing nutrient uptake during food scarcity periods, or when only consuming one meal a day, then maximal nutrient absorption would be beneficial. The slowing of gastric emptying may assist in extending the time provided for intestinal processing and maximising absorption of nutrients from the acidic chyme. This may explain an evolutionary purpose of elevated basal GLP-1 levels in humans who likely spent a great amount of time in ketosis and thus not exposed to dietary farinaceous nor sucrose rich foods that would trigger bolus GLP-1 secretion.

Euketonaemia is achievable without fasting, through carbohydrate restriction which results in a fasting mimicking state. Palmitic-acid-9-hydroxy-stearic-acid (9-PAHSA), a branched fatty acid ester of hydroxy fatty acids (FAHFAs), stimulates GLP-1 secretion. 9- PAHSA is endogenously synthesised and regulated by a fasting state (euketonaemia/NK) and dietary saturated fatty acid consumption (Yore et al., 2014). Insulin resistant people have lower levels of 9-PAHSA in their serum and adipose tissue, and serum 9-PAHSA levels are significantly correlated to insulin sensitivity shown in humans assessed via euglycaemic clamp (Yore et al., 2014). FAHFAs are found in the fat of ruminant meat, milk, eggs, fish (Manful et al., 2022; An et al., 2023), and breast milk; although lower in the breast milk of obese lactating mothers (Brezinova et al., 2018). Interestingly, omnivores have significantly higher Older serum levels of FAHFA than vegetarians/vegans, and in a 1-week over-feeding study in 15 lean males with BMI < 27, the consumption of saturated fatty acids from whipping cream was shown to increase serum FAFHA (Ott et al., 2018; Kellerer et al., 2021). All of these foods are common in many ketosis-supportive lifestyles.

The stimulation of basal GLP-1 secretion has been mainly studied in people in suppressed ketosis (hypoketonaemia) states, who are in lean and overweight categories, or in pathology. It is arguable that stimulators of basal GLP-1 secretion should be studied in healthy long term sustained NK (euketonaemia) individuals, who may better reflect the metabolic state that humans likely evolved in, where they would have had less frequent meals, with only seasonally available low farinaceous carbohydrate availability, and thus would have been naturally in ketosis for a greater number of hours in their 24-hour day and overall year (Rollo et al., 2002; Ben-Dor, Sirtoli and Barkai, 2021; Cooper, Brookler and Crofts, 2021; Cooper, Kyriakidou, Edwards, et al., 2023; Cooper, Kyriakidou, Petagine, et al., 2023).

Trials administering oral glucose to hypoketonaemic participants have shown dietary glucose causing bolus associated GLP-1 secretion (Kreymann et al., 1987). During the NK phases of the KetoSAge trial, participants were not consuming enough carbohydrates to cause the suppression of ketosis, whilst they were consuming 267 g of carbohydrate spread over three meals a day during phase 2, to suppress ketosis. As it can be seen, basal GLP-1 was significantly higher in the two phases with the least dietary glucose exposure, whilst after the participants had followed the SUK dietary guidelines to habitually consume the equivalent of three OGTTs worth of glucose for 21 days straight, which would be equivalent to 63 OGTTs, the KetoSAge participants' mean basal fasting GLP-1 levels were significantly lower, with higher fasting glucose and insulin. This could be explained by increased GLP-1 clearance in a hypoketonaemia state, via increased DDP-4 enzymatic breakdown or increased renal clearance. Alternatively, euketonaemia may decrease DDP- 4 activity, thus reducing GLP-1 clearance. This warrants further investigation.

Given the "blockbuster" drugs for the current treatment for T2DM and are now used for the treatment of obesity to induce weight loss are GLP-1R agonists (Fareed and Hussain, 2023), it would be beneficial to understand the physiological role of basal GLP-1 in healthy humans in a metabolic state reflective of how we evolved, namely euketonaemia MP1. The fasted mimicking state euketonaemia effectuates weight normalisation, shown to bring back weight homeostasis, meaning the overweight will lose weight and the underweight will return to a healthier weight, whilst resolving many metabolic and

neurological health conditions in the process (Hallberg et al., 2018; Athinarayanan et al., 2019; Norwitz, Hu and Clarke, 2019; Kelly, Unwin and Finucane, 2020; Danan et al., 2022; Norwitz et al., 2023).

6.16 Strengths and Limitations

This study is the first investigating a non-athletic, healthy pre-menopausal female population living in a long-term (> 1 year, group average of 3.9 years) habitual ketosis lifestyle for more than 80% of their year. These participants were not on hormonal birth control, making this cohorts data more reflective of natural responses in a female population, without being timed to menstrual cycles. This study was also culturally and ethnically diverse, whereby participants followed their own lifestyle and food preferences. However, the participants were controlled (via photos and daily dietary diary) for each meal throughout the day during the 9-week experimental period. Extensive nutritional instructions/guidance (e.g., reminders to follow the SUK dietary recommendations during the SuK phase) were also given to each participant.

Another strength of this study was participant adherence and compliance, as participants recorded and photographed daily capillary measurements (glucose and BHB) for a 6-months pre-trial, and throughout the experimental period (9 weeks) 4 times per day. The use of standardised procedures, including laboratory visit, blood sampling time and testing measurements is another strength of this study. The combination of anthropometric and metabolic indices as well as various biomarkers and OGTT assessments provides further knowledge of the underlying metabolic responses of hyperinsulinaemia, and subsequently on potential healthspan/lifespan. Participants maintained a similar level of activity throughout the study period. Finally, all the main results withstood a p-value correction, indicating this study was fully powered.

On the other hand, variations of findings between studies may occur due to the study population (e.g., females vs males); female participants may respond differently to male cohorts, due to hormonal changes. In addition to this, training status of the participants (trained vs untrained) (Volek et al., 2015; Kyriakidou et al., 2021) or the duration of the

study protocol may play a role (Jansen et al., 2022). Further work on metabolic health between different age groups (e.g., young vs elderly population) and in healthy individuals in NK (as controls) vs people with pathologies (i.e., cancer, T2D or elderly population with ageing-associated diseases, such as sarcopenia) in larger cohorts is also needed. Future investigations should also conduct in depth profiling analysis using RNA sequencing, proteomics, and metabolomics in response to hyperinsulinaemia.

Being a study on a wide range of physiological biomarkers (with different intrinsic magnitudes of variance) it is expected to have a large variability of p-values. It should be highlighted that while only insulin and IGF were used to calculate the sample size, most biomarkers showed statistical significance clearly below a multiple comparisons corrected p-value. Therefore, the borderline statistically significant findings in the measured cytokines and growth factors should be interpreted within the context of likely statistically underpowered tests on highly variable biomarkers and warrant further investigation as their trends are in resonance with the protective metabolic and inflammatory patterns observed here and have been documented elsewhere.

This study is a novel investigation of biomarkers in chronic diseases of ageing in a healthy pre-menopausal female population living in a long-term ketosis. OCN and GLP-1 have not been measured in this cohort before. Further work assessing these biomarkers in larger cohorts across different ages and in pathologies should be considered. Due to the low sensitivity of several trialled glucagon assays, it was not possible to measure glucagon in this cohort of participant samples.

6.17 Translational Importance

Recently, it has been shown in a large cohort study that a diet with a high proportion of carbohydrates significantly increases the risk of CVD (Jo and Park, 2023). This study, coupled with the studies discussed above indicate that higher levels of insulin and IGF-1 are associated with increased morbidity and mortality risk. The current SUK Eatwell guideline recommendation to consume at least 267 g of carbohydrates a day, effectively suppressed ketosis, to a degree that by the end of this 21-day intervention, the

participants were waking up with undetectable ketones on a capillary meter, indicating insulin demand, secretion and exposure had been enough to down regulate ketogenesis to even prevent return to ketosis after an overnight fast. It is therefore proposed that the reduced concentration of BHB accompanied by higher concentrations of insulin and IGF-1 may confer increased risk of morbidity and mortality over time, potentially increasing biological ageing rate.

The data here suggests that the Standard UK Eatwell guidelines should be revised, given that it results in repeated daily exposure to excess bolus glucose and insulin, which is implicated to increased morbidity and mortality risks. Recommendations would benefit the public by including directions to restrict carbohydrate intake to below personalised tolerance levels, in order to not stimulate excess insulin both in concentration and frequency, and thus not suppress nutritional ketosis. It is proposed that maintaining a metabolic phenotype of nutritional ketosis in the long term is an effective and safe way to mitigate these risks, without compromising metabolic flexibility. This study's data suggests that those in habitual ketosis are able to withstand a significant period (21 days) of glucose fuelling before returning to their original status of fat metabolism, which would be in agreement with our understanding of evolutionary human practices. It has been hypothesised that humans evolutionarily spent more time in a metabolically fasted state of ketosis than current modern-day humans (phenotype 2 and 3, where BHB is consistently < 0.3 mmol/L for several consecutive days if not months and years) (Thomas, no date; Rollo et al., 2002; Cooper et al., 2021). Where historically people would eat one to two meals a day, which were limited in carbohydrate, low in glycaemic load, minimally processed and were seasonally available (Mattson, 2005; Cooper et al., 2021; Cooper, Brookler and Crofts, 2021).

Chapter 7

7. Conclusion

Evolutionary evidence suggests that ancestral populations were predominantly adapted to patterns of intermittent and time restricted feeding as opposed to continuous nutritional intake, rich in farinaceous and sucrose carbohydrates that stimulate bolus insulin secretion. The escalating prevalence of T2DM, obesity, CVD, AD and cancer observed in populations adhering to multiple substantial carbohydrate dominated meals in developed nations is a testament to this. Individuals maintaining long-standing habitual NK, when subjected to 21-days of consuming carbohydrate to suppress ketosis, followed with restricting carbohydrate, reverting to an evolutionary ketotic state within one day, indicate metabolic flexibility and health.

Optimal metabolic health is having all five of the following anthropometric and blood biochemistry markers fulfil these thresholds, whilst metabolic syndrome is defined as failing to achieve three out of the five (Malhotra, Kamepalli and Bamrah, 2020):

1. Waist circumference: Women: < 88 cm (< 85 South Asian women)
2. Fasting glucose or HbA1c: < 5.6 mmol/L [100 mg/dL], < 5.7%
3. Blood Triglycerides < 1.7 mmol/l (< 150 mg/dL)
4. HDL > 1mmol/l (> 40/50mg/dL for men/women)
5. Blood Pressure: systolic <120, and diastolic <80mmHg (> 135/850)

Having a waist to height ratio of < 0.5 in women is also a strong predictor of metabolic health, and may be used in addition or alternative to just waist circumference (Shen et al., 2017). It can be seen that the KetoSAge cohort fulfilled the optimal metabolic health profile in all three phases of the trial (**Table 9**), indicating that their lifestyle of long term sustained euketonaemia is both safe and sustainable, and they also demonstrated metabolic flexibility.

Table 9. Optimal metabolic health marker panel, across all phases in KetoSAge participants. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention suppress ketosis (SuK) P2; and removal of SuK returning to NK P3; Measurements were taken at 8 am after a 12-hour overnight fast; (n = 10). Values are presented as mean ± SD.

	P1	P2	P3	ANOVA p Value	P1 vs. P2	P2 vs. P3	P1 vs. P3
BMI	20.52 (± 1.39)	21.54 (± 1.30)	20.82 (± 1.46)	< 0.0001	< 0.0001	0.0025	0.0197
Waist (cm)	68.92 (± 2.83)	72.43 (± 1.81)	69.23 (± 2.38)	< 0.0001	0.0002	< 0.0001	0.7888
Waist/Hip [3]	0.75 (± 0.03)	0.77 (± 0.03)	0.74 (± 0.03)	< 0.0001	0.0015	< 0.0001	0.5361
Waist/Height [4, 5]	0.43 (± 0.03)	0.45 (± 0.03)	0.43 (± 0.03)	< 0.0001	0.0009	< 0.0001	> 0.9999
Glucose (mmol/L)	4.36 (± 0.53)	5.12 (± 0.59)	4.41 (± 0.30)	0.0015	0.0088	0.0177	0.9469
Systole (mmHg)	103.25 (± 6.24)	103.70 (± 10.17)	100.00 (± 9.54)	0.1455	0.9753	0.1746	0.2274
Diastole (mmHg)	70.75 (± 4.91)	69.45 (± 7.14)	68.15 (± 7.36)	0.3227	0.8044	0.7147	0.1715
Triglycerides (mg/dL)	66.80 (± 28.00)	66.10 (± 21.09)	79.30 (± 45.88)	0.5018	0.9972	0.6629	0.6270
HDL cholesterol (mg/dL)	70.10 (± 10.37)	72.70 (± 13.59)	69.80 (± 11.84)	0.6231	0.7460	0.6762	0.9943

Long-term ketosis does not result in chronic elevated cortisol levels, indicating ketosis is not a chronic stress inducing, nor fight or flight state. Long-term NK, euketonaemia, results in elevated GLP-1 levels that would presumably stimulate GLP-1R, that is associated with maintaining a healthier weight or weight loss and satiety, a method employed by the T2DM and weight loss GLP-1R agonists. Long-term sustained euketonaemia results in lower levels of leptin, which is associated with increased satiety sensitivity and healthier metabolic profiles. Whilst OCN is considered necessary for insulin secretion, given that it is understood that a low level of insulin that does not suppress ketogenesis, with euglycaemia, is a healthy level of insulin, this MP1, euketonaemia, presents with lower plasma OCN levels. This likely indicates OCN is remaining in the bone, which would confer greater bone health. This is understood to be corroborated in those with low HOMA-IR

values also having lower risk of fragility fractures, indicating insulin sensitivity and thus not being hyperinsulinaemic. Furthermore, the increased plasma OCN seen after suppressing ketosis, may be from osteoclast bone resorption which may over time result in hyper-mineralisation of the bones (increased BMD seen in T2DM and obesity) but be coupled with increased brittleness resulting in increased fragility fracture rates, as is observed in obesity and T2DM individuals. Lower OCN levels are found in the healthy euketonaemic state, and is accompanied with lower fasting glucose, insulin, insulin sensitivity HOMA-IR, leptin and higher GLP- 1.

The data here show long-standing NK appears to provide major health benefits in the maintenance of euglycaemia, with low insulin and IGF-1, the triad of markers most strongly associated with chronic diseases and biological ageing. Sustained NK serves as a reliable surrogate marker for these parameters to understand an individual's metabolic phenotype, and therefore risk. This study was conducted to establish a detailed metabolic phenotype biomarker profile in a long-standing healthy ketosis cohort, providing a NK control group for other studies to establish metabolic phenotypes in people with cancer, CVD, AD, T2DM and ageing, and to assess treatment efficacy using KMT in gaining better health. Overall, sustained NK may mitigate hyperinsulinemia without impairing metabolic flexibility and carbohydrate tolerance in metabolically healthy individuals. Maintaining low insulin requirement and IGF-1 levels, through endogenous ketogenesis sustained above NK levels, may offer lower chronic disease risk resulting in benefits to both lifespan and healthspan.

Author Contributions to the IJSM MDPI published version of KetoSAge part 1 (Cooper, Kyriakidou, Edwards, et al., 2023): Isabella D. Cooper (I.D.C.) conceived the hypothesis and study design, wrote the original draft, designed the figures, and reviewed and edited the final published manuscript. I.D.C. and Yvoni Kyriakidou (Y.K.) conducted the trial. I.D.C., Y.K., Sandra L. Jacome (S.L.J.), Valentina Borgognoni (V.B.), Vanusa Novaes (V.N.) and Rima Al-Faour (R.A.-F). collected and compiled data. I.D.C., Y.K., Kurtis Edwards (K.E.) and Lucy Petagine (L.P.) performed *ex vivo* bench top assays. I.D.C., Y.K., K.E. and L.P. analysed data, designed tables and graphs. I.D.C., Y.K., K.E., L.P., Thomas N. Seyfried (T.N.S.), Tomas Duraj (T.D.), Adrian Soto-Mota (A.S.-M.), Andrew Scarborough (A.S.), Kenneth Brookler (K.B.) and Bradley T. Elliott (B.T.E). contributed to writing and reviewed and edited the final manuscript. All authors have read and agreed to the published version of the manuscript.

Author Contributions to the Biomedicines MDPI published version of KetoSAge part 2 (Cooper, Kyriakidou, Petagine et al., 2024)**:** I.D.C. conceived the hypothesis and study design, wrote the original draft, designed the figures, and reviewed and edited the final manuscript. I.D.C. and Y.K. conducted the trial. I.D.C., Y.K. and L.P. collected and compiled data. I.D.C., Y.K., L.P. and K.E. performed *ex vivo* bench top assays. I.D.C., Y.K., L.P. and K.E. analysed data. I.D.C. designed tables and graphs. I.D.C., Y.K., L.P., K.E., A.S.-M., K.B. and B.T.E. contributed to writing and reviewed and edited the final manuscript. All authors have read and agreed to the published version of the manuscript.

References

Abdulla, M.H. et al. (2021). Expression of VEGF, EGF and HGF in early- and late-stage colorectal cancer. *Molecular and Clinical Oncology*, 15 (6). Available from https://doi.org/10.3892/MCO.2021.2413.

ACCORD et al. (2008). Effects of intensive glucose lowering in type 2 diabetes. *The New England Journal of Medicine*, 358 (24), 2545–2559. Available from https://doi.org/10.1056/NEJMoa0802743.

Achanta, L.B. and Rae, C.D. (2017). β-hydroxybutyrate in the brain: one molecule, multiple mechanisms. *Neurochemical Research*, 42 (1), 35–49. Available from https://doi.org/10.1007/s11064-016-2099-2.

Adijanto, J. et al. (2014). The retinal pigment epithelium utilizes fatty acids for ketogenesis implications for metabolic coupling with the outer retina. *Journal of Biological Chemistry*, 289 (30), 20570–20582. Available from https://doi.org/10.1074/jbc.M114.565457.

Agius, L., Ford, B.E. and Chachra, S.S. (2020). The metformin mechanism on gluconeogenesis and AMPK activation: The metabolite perspective. *International Journal of Molecular Sciences*, 21 (9). Available from https://doi.org/10.3390/ijms21093240.

Aguayo-Ruiz, J.I. et al. (2020). Effect of supplementation with vitamins D3 and K2 on undercarboxylated osteocalcin and insulin serum levels in patients with type 2 diabetes mellitus: A randomized, double-blind, clinical trial. *Diabetology & Metabolic Syndrome*, 12 (1), 73. Available from https://doi.org/10.1186/s13098-020-00580-w.

Aguiló, F. (2015). The expression pattern of the acetoacetyl-CoA synthetase and its kinetic parameters facilitate the use of ketone bodies in liver during feeding. *Journal of Proteomics & Enzymology*, 04 (02). Available from https://doi.org/10.4172/2470-1289.1000124.

Ahmed, S.B.M. et al. (2023). Assessing the link between diabetic metabolic dysregulation and breast cancer progression. *International journal of molecular sciences*, 24 (14), 11816. Available from https://doi.org/10.3390/IJMS241411816/S1.

Ahmed, T.A. and El-Badri, N. (2018). Pericytes: The role of multipotent stem cells in vascular maintenance and regenerative medicine. *Advances in experimental medicine and biology*, 1079, 69–86. Available from https://doi.org/10.1007/5584_2017_138.

Ahrén, B. and Thorsson, O. (2003). Increased insulin sensitivity is associated with reduced insulin and glucagon secretion and increased insulin clearance in man. *The Journal of Clinical Endocrinology & Metabolism*, 88 (3), 1264–1270. Available from https://doi.org/10.1210/jc.2002-021547.

Alberti, K.G.M.M. and Zimmet, P.Z. (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Provisional report of a WHO Consultation. *Diabetic Medicine*, 15 (7), 539–553. Available from https://doi.org/https://doi.org/10.1002/(SICI)1096-9136(199807)15:7<539::AID-DIA668>3.0.CO;2-S.

Albrechtsen, N.J.W. et al. (2015). Stability of glucagon-like peptide 1 and glucagon in human plasma. *Endocrine Connections*, 4 (1), 50. Available from https://doi.org/10.1530/EC-14-0126.

Alessi, M.C. et al. (1997). Production of plasminogen activator inhibitor 1 by human adipose tissue: possible link between visceral fat accumulation and vascular disease. *Diabetes*, 46 (5), 860–867. Available from https://doi.org/10.2337/DIAB.46.5.860.

Altalhi, R., Pechlivani, N. and Ajjan, R.A. (2021). PAI-1 in diabetes: Pathophysiology and role as a therapeutic target. *International Journal of Molecular Sciences*, 22 (6), 1–15. Available from https://doi.org/10.3390/IJMS22063170.

Althaher, A.R. (2022). An overview of hormone-sensitive lipase (HSL). *The Scientific World Journal*, 2022. Available from https://doi.org/10.1155/2022/1964684.

Ambrogini, E. et al. (2010). FoxO-mediated defense against oxidative stress in osteoblasts Is indispensable for skeletal homeostasis in mice. *Cell Metabolism*, 11 (2), 136–146. Available from https://doi.org/10.1016/j.cmet.2009.12.009.

Ambroselli, D. et al. (2023). New advances in metabolic syndrome, from prevention to treatment: The role of diet and food. *Nutrients*, 15 (3), 640. Available from https://doi.org/10.3390/NU15030640/S1.

An, N. et al. (2023). A dataset of branched fatty acid esters of hydroxy fatty acids diversity in foods. *Scientific Data*, 10 (1). Available from https://doi.org/10.1038/S41597-023-02712-Z.

An, X.-F.F. et al. (2011). Advanced glycation end-products induce heparanase expression in endothelial cells by the receptor for advanced glycation end products and through activation of the FOXO4 transcription factor. *Molecular and Cellular Biochemistry*, 354 (1–2), 47–55. Available from https://doi.org/10.1007/S11010-011-0804-7/FIGURES/8.

Anderson, R.M., Shanmuganayagam, D. and Weindruch, R. (2009). Caloric restriction and aging: Studies in mice and monkeys. *Toxicologic Pathology*, 37 (1), 47–51. Available from https://doi.org/10.1177/0192623308329476.

Anello, M. et al. (2005). Functional and morphological alterations of mitochondria in pancreatic beta cells from type 2 diabetic patients. *Diabetologia*, 48 (2), 282–289. Available from https://doi.org/10.1007/s00125-004-1627-9.

Araújo, J., Cai, J. and Stevens, J. (2018). Prevalence of optimal metabolic ealth in american adults: National health and nutrition examination survey 2009-2016. *Metabolic Syndrome and Related Disorders*, 17 (1), 46–52. Available from https://doi.org/10.1089/met.2018.0105.

Arima, Y. et al. (2021). Murine neonatal ketogenesis preserves mitochondrial energetics by preventing protein hyperacetylation. *Nature metabolism*, 3 (2), 196–210. Available from https://doi.org/10.1038/S42255-021-00342-6.

Arismendi-Morillo, G.J. and Castellano-Ramirez, A. V. (2008). Ultrastructural mitochondrial pathology in human astrocytic tumors: Potentials implications pro-therapeutics strategies. *Journal of Electron Microscopy*, 57 (1), 33–39. Available from https://doi.org/10.1093/jmicro/dfm038.

Athinarayanan, S.J. et al. (2019). Long-term effects of a novel continuous remote care intervention including nutritional ketosis for the management of type 2 diabetes: A 2-year nonrandomized clinical trial. *Frontiers in Endocrinology*, 10 (JUN), 348. Available from https://doi.org/10.3389/fendo.2019.00348.

Auestad, N. et al. (1991). Fatty acid oxidation and ketogenesis by astrocytes in primary culture. *Journal of Neurochemistry*, 56 (4), 1376–1386. Available from https://doi.org/10.1111/j.1471-4159.1991.tb11435.x.

Augustin, K. et al. (2018). Mechanisms of action for the medium-chain triglyceride ketogenic diet in neurological and metabolic disorders. *The Lancet Neurology*, 17 (1). Available from https://doi.org/10.1016/S1474-4422(17)30408-8.

Ausk, K.J., Boyko, E.J. and Ioannou, G.N. (2010). Insulin resistance predicts mortality in nondiabetic individuals in the U.S. *Diabetes Care*, 33 (6), 1179–1185. Available from

https://doi.org/10.2337/DC09-2110.
Ayati, A. et al. (2020). A review on progression of epidermal growth factor receptor (EGFR) inhibitors as an efficient approach in cancer targeted therapy. *Bioorganic Chemistry*, 99, 103811. Available from https://doi.org/10.1016/J.BIOORG.2020.103811.
Babu, K. and Tay, Y. (2019). The yin-yang regulation of reactive oxygen species and microRNAs in cancer. *International Journal of Molecular Sciences*, 20 (21), 5335. Available from https://doi.org/10.3390/ijms20215335.
Bae, H.R. et al. (2016). β-Hydroxybutyrate suppresses inflammasome formation by ameliorating endoplasmic reticulum stress via AMPK activation. *Oncotarget*, 7 (41), 66444–66454. Available from https://doi.org/10.18632/oncotarget.12119.
Bajou, K. et al. (1998). Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nature Medicine 1998 4:8*, 4 (8), 923–928. Available from https://doi.org/10.1038/nm0898-923.
Balsara, R.D. and Ploplis, V.A. (2008). Plasminogen activator inhibitor-1: The double-edged sword in apoptosis. *Thrombosis and Haemostasis*, 100 (6), 1029–1036. Available from https://doi.org/10.1160/TH08-07-0427/ID/JR10626-3/BIB.
Bao, Y. et al. (2013). Inverse relationship between serum osteocalcin levels and visceral fat area in chinese men. *The Journal of Clinical Endocrinology & Metabolism*, 98 (1), 345–351. Available from https://doi.org/10.1210/JC.2012-2906.
Barbato, D. et al. (2012). Caloric restriction and the nutrient-sensing PGC-1α in mitochondrial homeostasis: New perspectives in neurodegeneration. *International Journal of Cell Biology*. Available from https://doi.org/10.1155/2012/759583.
Barbieri, M. et al. (2003). Insulin/IGF-I-signaling pathway: An evolutionarily conserved mechanism of longevity from yeast to humans. *American Journal of Physiology - Endocrinology and Metabolism*, 285 (5 48-5). Available from https://doi.org/10.1152/AJPENDO.00296.2003/ASSET/IMAGES/LARGE/H11131489001.JPEG.
Bause, A.S. and Haigis, M.C. (2013). SIRT3 regulation of mitochondrial oxidative stress. *Experimental Gerontology*, 48 (7), 634–639. Available from https://doi.org/10.1016/j.exger.2012.08.007.
Beck, A.S. and Tisdale, J.M. (1989). Nitrogen excretion in cancer cachexia and its modification by a high fat diet in mice. *American Association for Cancer Research*, 49 (14), 3800–3804.
Becker, M. et al. (2010). Prognostic impact of plasminogen activator inhibitor type 1 expression in bladder cancer. *Cancer*, 116 (19), 4502–4512. Available from https://doi.org/10.1002/CNCR.25326.
Bellucci, A. et al. (2020). Nuclear factor-κB dysregulation and α-synuclein pathology: critical interplay in the pathogenesis of Parkinson's disease. *Frontiers in Aging Neuroscience*, 12, 68. Available from https://doi.org/10.3389/fnagi.2020.00068.
Ben-Dor, M. et al. (2011). Man the fat hunter: The demise of Homo erectus and the emergence of a new Hominin lineage in the middle pleistocene (ca. 400 kyr) Levant. *PLOS ONE*, 6 (12), e28689. Available from https://doi.org/10.1371/JOURNAL.PONE.0028689.
Ben-Dor, M., Sirtoli, R. and Barkai, R. (2021). The evolution of the human trophic level during the Pleistocene. *American Journal of Physical Anthropology*, 175 (S72), 27–56. Available from https://doi.org/10.1002/AJPA.24247.
Benjamin, D.I. et al. (2022). Fasting induces a highly resilient deep quiescent state in muscle

stem cells via ketone body signaling. *Cell Metabolism*, 34 (6), 902-918.e6. Available from https://doi.org/10.1016/j.cmet.2022.04.012.
Bergman, B.C. and Brooks, G.A. (1999). Respiratory gas-exchange ratios during graded exercise in fed and fasted trained and untrained men. *Journal of Applied Physiology*, 86 (2), 479–487. Available from https://doi.org/10.1152/JAPPL.1999.86.2.479/ASSET/IMAGES/LARGE/JAPP05216005X.JPEG.
Bikle, D. (2017). Vitamin D: Production, metabolism, and mechanisms of action. *Endotext*. Available from https://doi.org/http://www.ncbi.nlm.nih.gov/pubmed/25905172.
Biondi, B., Kahaly, G.J. and Robertson, R.P. (2019). Thyroid dysfunction and diabetes mellitus: Two closely associated disorders. *Endocrine Reviews*, 40 (3), 789. Available from https://doi.org/10.1210/ER.2018-00163.
Birnbaum, A. et al. (2019). Age-dependent changes in transcription factor FOXO targeting in female drosophila. *Frontiers in Genetics*, 10 (MAY), 312. Available from https://doi.org/10.3389/fgene.2019.00312.
Bix, G. et al. (2007). Endorepellin, the C-terminal angiostatic module of perlecan, enhances collagen-platelet responses via the α2β1-integrin receptor. *Blood*, 109 (9), 3745–3748. Available from https://doi.org/10.1182/BLOOD-2006-08-039925.
Björk, M.P. and Johansson, B. (2018). Gamma-Glutamyltransferase (GGT) as a biomarker of cognitive decline at the end of life: contrasting age and time to death trajectories. *International Psychogeriatrics*, 30 (7), 981–990. Available from https://doi.org/10.1017/S1041610217002393.
Blasiak, J. et al. (2020). The aging stress response and its implication for AMD pathogenesis. *International Journal of Molecular Sciences*, 21 (22), 1–22. Available from https://doi.org/10.3390/ijms21228840.
Blázquez, C. et al. (2002). The AMP-activated protein kinase Is involved in the regulation of ketone body production by astrocytes. *Journal of Neurochemistry*, 73 (4), 1674–1682. Available from https://doi.org/10.1046/j.1471-4159.1999.731674.x.
Boden, G. et al. (1997). Effects of prolonged hyperinsulinemia on serum leptin in normal human subjects. *Journal of Clinical Investigation*, 100 (5), 1107. Available from https://doi.org/10.1172/JCI119621.
Bolla, A.M. et al. (2019). Low-carb and ketogenic diets in type 1 and type 2 diabetes. *Nutrients*, 11 (5). Available from https://doi.org/10.3390/NU11050962.
Bolt, M.J.G.G. et al. (2004). Critical role of vitamin D in sulfate homeostasis: Regulation of the sodium-sulfate cotransporter by 1,25-dihydroxyvitamin D3. *American Journal of Physiology-Endocrinology and Metabolism*, 287 (4), E744–E749. Available from https://doi.org/10.1152/ajpendo.00151.2004.
Bonnet, F. et al. (2011). Liver enzymes are associated with hepatic insulin resistance, insulin secretion, and glucagon concentration in healthy men and women. *Diabetes*, 60 (6), 1660. Available from https://doi.org/10.2337/DB10-1806.
Booth, N.A. et al. (1988). Plasminogen activator inhibitor (PAI-1) in plasma and platelets. *British Journal of Haematology*, 70 (3), 327–333. Available from https://doi.org/10.1111/J.1365-2141.1988.TB02490.X.
Bos, J.L. (1995). p21ras: An oncoprotein functioning in growth factor-induced signal transduction. *European Journal of Cancer*, 31 (7–8), 1051–1054. Available from https://doi.org/10.1016/0959-8049(95)00168-I.
Bots, M.L. et al. (1998). Coagulation and fibrinolysis markers and risk of dementia.

Pathophysiology of Haemostasis and Thrombosis, 28 (3–4), 216–222. Available from https://doi.org/10.1159/000022433.
Bouchez, C. and Devin, A. (2019). Mitochondrial biogenesis and mitochondrial reactive oxygen species (ROS): a complex relationship regulated by the cAMP/PKA signaling pathway. *Cells*, 8 (4), 287. Available from https://doi.org/10.3390/cells8040287.
Brandenburg, V.M. et al. (2013). Relationship between sclerostin and cardiovascular calcification in hemodialysis patients: A cross-sectional study. *BMC Nephrology*, 14 (1). Available from https://doi.org/10.1186/1471-2369-14-219.
Brandes, N., Schmitt, S. and Jakob, U. (2009). Thiol-based redox switches in eukaryotic proteins. *Antioxidants and Redox Signaling*, 11 (5), 997–1014. Available from https://doi.org/10.1089/ars.2008.2285.
Brenmoehl, J. and Hoeflich, A. (2013). Dual control of mitochondrial biogenesis by sirtuin 1 and sirtuin 3. *Mitochondrion*, 13 (6), 755–761. Available from https://doi.org/10.1016/j.mito.2013.04.002.
Brennan, P.N., Dillon, J.F. and Tapper, E.B. (2022). Gamma-Glutamyl Transferase (γ-GT) – an old dog with new tricks? *Liver International*, 42 (1), 9–15. Available from https://doi.org/10.1111/LIV.15099.
Brenta, G. (2011). Why can insulin resistance be a natural consequence of thyroid dysfunction? *Journal of Thyroid Research*, 2011, 1–9. Available from https://doi.org/10.4061/2011/152850.
Brestoff, J.R. et al. (2021). Intercellular mitochondria transfer to macrophages regulates white adipose tissue homeostasis and is impaired in obesity. *Cell Metabolism*, 33 (2), 270-282.e8. Available from https://doi.org/10.1016/j.cmet.2020.11.008.
Brezinova, M. et al. (2018). Levels of palmitic acid ester of hydroxystearic acid (PAHSA) are reduced in the breast milk of obese mothers. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1863 (2), 126–131. Available from https://doi.org/10.1016/J.BBALIP.2017.11.004.
Brogren, H. et al. (2011). Platelets retain high levels of active plasminogen activator inhibitor 1. *PLOS ONE*, 6 (11), e26762. Available from https://doi.org/10.1371/JOURNAL.PONE.0026762.
Bronisz, A., Ozorowski, M. and Hagner-Derengowska, M. (2018). Pregnancy ketonemia and development of the fetal central nervous system. *International Journal of Endocrinology*, 2018. Available from https://doi.org/10.1155/2018/1242901.
Bye, A.P., Unsworth, A.J. and Gibbins, J.M. (2016). Platelet signaling: a complex interplay between inhibitory and activatory networks. *Journal of Thrombosis and Haemostasis*, 14 (5), 918. Available from https://doi.org/10.1111/JTH.13302.
Cahill, G.F. et al. (1966). Hormone-fuel interrelationships during fasting. *The Journal of clinical investigation*, 45 (11), 1751–1769. Available from https://doi.org/10.1172/JCI105481.
Cahill, G.F.J. and Veech, R.L. (2003). Ketoacids? Good medicine? *Transactions of the American Clinical and Climatological Association*, 114, 149–161.
Cai, Z. et al. (2012). Roles of AMP-activated protein kinase in Alzheimer's disease. *NeuroMolecular Medicine*, 14 (1), 1–14. Available from https://doi.org/10.1007/s12017-012-8173-2.
Calder, P.C. et al. (2017). Health relevance of the modification of low grade inflammation in ageing (inflammageing) and the role of nutrition. *Ageing Research Reviews*, 40, 95–119. Available from https://doi.org/10.1016/j.arr.2017.09.001.

Calvo-Rodriguez, M. and Bacskai, B.J. (2021). Mitochondria and calcium in Alzheimer's disease: From cell signaling to neuronal cell death. *Trends in Neurosciences*, 44 (2), 136–151. Available from https://doi.org/10.1016/j.tins.2020.10.004.

Camilleri, M., Malhi, H. and Acosta, A. (2017). Gastrointestinal complications of obesity. *Gastroenterology*, 152 (7), 1656. Available from https://doi.org/10.1053/J.GASTRO.2016.12.052.

Cantley, L.C. (2002). The phosphoinositide 3-kinase pathway. *Science*, 296 (5573), 1655–1657. Available from https://doi.org/10.1126/science.296.5573.1655.

Cantó, C. et al. (2009). AMPK regulates energy expenditure by modulating NAD + metabolism and SIRT1 activity. *Nature*, 458 (7241), 1056–1060. Available from https://doi.org/10.1038/nature07813.

Cao, J. and Yee, D. (2021). Disrupting insulin and IGF receptor function in cancer. *International Journal of Molecular Sciences 2021, Vol. 22, Page 555*, 22 (2), 555. Available from https://doi.org/10.3390/IJMS22020555.

Carabotti, M., Annibale, B. and Lahner, E. (2021). Common pitfalls in the management of patients with micronutrient deficiency: Keep in mind the stomach. *Nutrients 2021, Vol. 13, Page 208*, 13 (1), 208. Available from https://doi.org/10.3390/NU13010208.

Carrer, A. and Wellen, K.E. (2015). Metabolism and epigenetics: A link cancer cells exploit. *Current Opinion in Biotechnology*, 34, 23–29. Available from https://doi.org/10.1016/j.copbio.2014.11.012.

Casals, N. et al. (1992). Regulation of the expression of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene. Its role in the control of ketogenesis. *Biochemical Journal*, 283 (1), 261–264. Available from https://doi.org/10.1042/bj2830261.

Cerqueira, N.M.F.S.A. et al. (2016). Cholesterol biosynthesis: A mechanistic overview. *Biochemistry*, 55 (39), 5483–5506. Available from https://doi.org/10.1021/acs.biochem.6b00342.

Chadt, A. and Al-Hasani, H. (2020). Glucose transporters in adipose tissue, liver, and skeletal muscle in metabolic health and disease. *Pflügers Archiv - European Journal of Physiology 2020 472:9*, 472 (9), 1273–1298. Available from https://doi.org/10.1007/S00424-020-02417-X.

Chan, O.T.M. et al. (2017). Association of MMP-2, RB and PAI-1 with decreased recurrence-free survival and overall survival in bladder cancer patients. *Oncotarget*, 8 (59), 99707–99721. Available from https://doi.org/10.18632/ONCOTARGET.20686.

Chen, C. et al. (2017). Human beta cell mass and function in diabetes: recent advances in knowledge and technologies to understand disease pathogenesis. *Molecular Metabolism*, 6 (9), 943–957. Available from https://doi.org/10.1016/j.molmet.2017.06.019.

Chen, M. et al. (2021). AMPK: A bridge between diabetes mellitus and Alzheimer's disease. *Behavioural Brain Research*, 400, 113043. Available from https://doi.org/10.1016/j.bbr.2020.113043.

Chen, Y. et al. (2011). Tumour suppressor SIRT3 deacetylates and activates manganese superoxide dismutase to scavenge ROS. *EMBO Reports*, 12 (6), 534–541. Available from https://doi.org/10.1038/embor.2011.65.

Chen, Y. et al. (2020). Mitochondrial fusion and fission in neuronal death induced by cerebral ischemia-reperfusion and its clinical application: A mini-review. *Medical Science Monitor*, 26, e928651-1. Available from https://doi.org/10.12659/MSM.928651.

Cho, J.H. et al. (2011). β-cell mass in people with type 2 diabetes. *Journal of Diabetes Investigation*, 2 (1), 6–17. Available from https://doi.org/10.1111/j.2040-1124.2010.00072.x.

Choi, S.M. et al. (2010). Insulin regulates adipocyte lipolysis via an Akt-independent signaling pathway. *Molecular and Cellular Biology*, 30 (21), 5009. Available from https://doi.org/10.1128/MCB.00797-10.

Civitarese, A.E. et al. (2006). Role of adiponectin in human skeletal muscle bioenergetics. *Cell Metabolism*, 4 (1), 75–87. Available from https://doi.org/10.1016/j.cmet.2006.05.002.

Colman, R.J. et al. (2014). Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. *Nature Communications*, 5. Available from https://doi.org/10.1038/ncomms4557.

Cooper, I. and Bell, D. (2024). Biohacking to Better Health ⋆ Brownstone Institute. *Brownstone Insitute*. Available from https://brownstone.org/articles/biohacking-to-better-health/ [Accessed 15 December 2024].

Cooper, I.D. et al. (2020). Relationships between hyperinsulinaemia, magnesium, vitamin D, thrombosis and COVID-19: Rationale for clinical management. *Open Heart*, 7 (2), e001356. Available from https://doi.org/10.1136/openhrt-2020-001356.

Cooper, I.D. et al. (2021). Metabolic phenotypes and step by step evolution of type 2 diabetes: A new paradigm. *Biomedicines 2021, Vol. 9, Page 800*, 9 (7), 800. Available from https://doi.org/10.3390/BIOMEDICINES9070800.

Cooper, I.D., Brookler, K.H. and Crofts, C.A.P. (2021). Rethinking fragility fractures in type 2 diabetes: The link between hyperinsulinaemia and osteofragilitas. *Biomedicines 2021, Vol. 9, Page 1165*, 9 (9), 1165. Available from https://doi.org/10.3390/BIOMEDICINES9091165.

Cooper, I.D., Kyriakidou, Y., Petagine, L., et al. (2023). Bio-hacking better health—Leveraging metabolic biochemistry to maximise healthspan. *Antioxidants 2023, Vol. 12, Page 1749*, 12 (9), 1749. Available from https://doi.org/10.3390/ANTIOX12091749.

Cooper, I.D., Kyriakidou, Y., Edwards, K., et al. (2023). Ketosis Suppression and Ageing (KetoSAge): The effects of suppressing ketosis in long term keto-adapted non-athletic females. *International Journal of Molecular Sciences 2023, Vol. 24, Page 15621*, 24 (21), 15621. Available from https://doi.org/10.3390/IJMS242115621.

Cooper, I.D., Sanchez-Pizarro, C., Norwitz, N.G., et al. (2023). Thyroid markers and body composition predict LDL-cholesterol change in lean healthy women on a ketogenic diet: experimental support for the lipid energy model. *Frontiers in Endocrinology*, 14, 1326768. Available from https://doi.org/10.3389/FENDO.2023.1326768.

Cooper, I.D. et al. (2024). Ketosis Suppression and Ageing (KetoSAge) Part 2: The Effect of Suppressing Ketosis on Biomarkers Associated with Ageing, HOMA-IR, Leptin, Osteocalcin, and GLP-1, in Healthy Females. *Biomedicines*, 12 (7), 1553. Available from https://doi.org/10.3390/biomedicines12071553.

Crawley, D.J. et al. (2014). Serum glucose and risk of cancer: A meta-analysis. *BMC Cancer*, 14 (1), 1–11. Available from https://doi.org/10.1186/1471-2407-14-985/FIGURES/2.

Crofts, C. et al. (2016). Identifying hyperinsulinaemia in the absence of impaired glucose tolerance: An examination of the Kraft database. *Diabetes Research and Clinical Practice*, 118, 50–57. Available from https://doi.org/10.1016/j.diabres.2016.06.007.

Crofts, C. et al. (2019). Determining a diagnostic algorithm for hyperinsulinaemia. *Journal of Insulin Resistance*, 4 (1), 1–7. Available from https://doi.org/10.4102/jir.v4i1.49.

Crofts, C.A.P. et al. (2015). Hyperinsulinemia: A unifying theory of chronic disease? *Diabesity*, 1 (4), 34. Available from https://doi.org/10.15562/diabesity.2015.19.
Cunnane, S.C. et al. (2016). Can ketones help rescue brain fuel supply in later life? Implications for cognitive health during aging and the treatment of alzheimer's disease. *Frontiers in Molecular Neuroscience*, 9 (JUL), 53. Available from https://doi.org/10.3389/fnmol.2016.00053.
Curry, D.W. et al. (2018). Targeting AMPK signaling as a neuroprotective strategy in Parkinson's disease. *Journal of Parkinson's Disease*, 8 (2), 161–181. Available from https://doi.org/10.3233/JPD-171296.
da Silva, L.A. et al. (2017). Relation between diabetes mellitus, thyroid hormones and caffeine. *Journal of Applied Pharmaceutical Science*, 7, (3), 212–216. Available from https://doi.org/10.7324/JAPS.2017.70334.
Danan, A. et al. (2022). The ketogenic diet for refractory mental illness: A retrospective analysis of 31 inpatients. *Frontiers in Psychiatry*, 13, 951376. Available from https://doi.org/10.3389/FPSYT.2022.951376/BIBTEX.
Dandona, P. et al. (1996). Oxidative damage to DNA in diabetes mellitus. *Lancet*, 347 (8999), 444–445. Available from https://doi.org/10.1016/S0140-6736(96)90013-6.
Daryabor, G. et al. (2020). The effects of type 2 diabetes mellitus on organ metabolism and the immune system. *Frontiers in Immunology*, 11. Available from https://doi.org/10.3389/FIMMU.2020.01582/BIBTEX.
De Maré, A., D'haese, P.C. and Verhulst, A. (2020). The role of sclerostin in bone and ectopic calcification. *International Journal of Molecular Sciences*, 21 (9). Available from https://doi.org/10.3390/ijms21093199.
Dede, A.D. et al. (2014). Type 2 diabetes mellitus and fracture risk. *Metabolism: Clinical and Experimental*, 63 (12), 1480–1490. Available from https://doi.org/10.1016/j.metabol.2014.09.002.
DemantMia et al. (2018). Determinants of fasting hyperglucagonemia in patients with type 2 diabetes and nondiabetic control subjects. *https://home.liebertpub.com/met*. Available from https://doi.org/10.1089/MET.2018.0066.
Denis McGarry, J. (2002). Dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes*, 51 (1), 7–18. Available from https://doi.org/10.2337/diabetes.51.1.7.
Deshmane, S.L. et al. (2009). Monocyte chemoattractant protein-1 (MCP-1): An overview. *Journal of Interferon & Cytokine Research*, 29 (6), 313. Available from https://doi.org/10.1089/JIR.2008.0027.
Devy, L. et al. (2002). The pro- or antiangiogenic effect of plasminogen activator inhibitor 1 is dose dependent. *The FASEB Journal*, 16 (2), 147–154. Available from https://doi.org/10.1096/FJ.01-0552COM.
Dibble, C.C. and Cantley, L.C. (2015). Regulation of mTORC1 by PI3K signaling. *Trends in Cell Biology*, 25 (9), 545–555. Available from https://doi.org/10.1016/j.tcb.2015.06.002.
Diebold, L. and Chandel, N.S. (2016). Mitochondrial ROS regulation of proliferating cells. *Free Radical Biology and Medicine*, 100, 86–93. Available from https://doi.org/10.1016/j.freeradbiomed.2016.04.198.
Dikalova, A.E. et al. (2017). Sirt3 impairment and SOD2 hyperacetylation in vascular oxidative stress and hypertension. *Circulation Research*, 121 (5), 564–574. Available from https://doi.org/10.1161/CIRCRESAHA.117.310933.
Dilliraj, L.N. et al. (2022). The evolution of ketosis: Potential impact on clinical conditions.

Nutrients, 14 (17), 3613. Available from https://doi.org/10.3390/NU14173613/S1.
DiNicolantonio, J.J. et al. (2017). Postprandial insulin assay as the earliest biomarker for diagnosing pre-diabetes, type 2 diabetes and increased cardiovascular risk. *Open Heart*, 4 (2), e000656. Available from https://doi.org/10.1136/openhrt-2017-000656.
Dinkova-Kostova, A.T. and Abramov, A.Y. (2015). The emerging role of Nrf2 in mitochondrial function. *Free Radical Biology and Medicine*, 88 (Part B), 179–188. Available from https://doi.org/10.1016/j.freeradbiomed.2015.04.036.
Dorsey, E.R. and Bloem, B.R. (2018). The Parkinson pandemic - A call to action. *JAMA Neurology*, 75 (1), 9–10. Available from https://doi.org/10.1001/jamaneurol.2017.3299.
Drago, S.R., Binaghi, M.J. and Valencia, M.E. (2005). Effect of gastric digestion pH on Iron, zinc, and calcium dialyzability from preterm and term starting infant formulas. *Journal of Food Science*, 70 (2), S107–S112. Available from https://doi.org/10.1111/J.1365-2621.2005.TB07113.X.
Draznin, B. (2010). Mitogenic action of insulin: friend, foe or 'frenemy'? *Diabetologia*, 53 (2), 229–233. Available from https://doi.org/10.1007/s00125-009-1558-6.
Draznin, B. (2011). Mechanism of the mitogenic influence of hyperinsulinemia. Available from https://doi.org/10.1186/1758-5996-3-10.
Draznin, B. et al. (2000). Effects of insulin on prenylation as a mechanism of potentially detrimental influence of hyperinsulinemia. *Endocrinology*, 141 (4), 1310–1316. Available from https://doi.org/10.1210/ENDO.141.4.7411.
Drozdz, R. et al. (1998). γ-glutamyltransferase dependent generation of reactive oxygen species from a glutathione/transferrin system. *Free Radical Biology and Medicine*, 25 (7), 786–792. Available from https://doi.org/10.1016/S0891-5849(98)00127-0.
Drucker, D.J. (2006). The biology of incretin hormones. *Cell Metabolism*, 3 (3), 153–165. Available from https://doi.org/10.1016/J.CMET.2006.01.004.
Drucker, D.J. et al. (1987). Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proceedings of the National Academy of Sciences of the United States of America*, 84 (10), 3434. Available from https://doi.org/10.1073/PNAS.84.10.3434.
Du, Z. et al. (2016). Oxidative damage induces MCP-1 secretion and macrophage aggregation in age-related macular degeneration (AMD). *Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie*, 254 (12), 2469–2476. Available from https://doi.org/10.1007/S00417-016-3508-6.
Duffy, M.J. et al. (2016). Validated biomarkers: The key to precision treatment in patients with breast cancer. *Breast*, 29, 192–201. Available from https://doi.org/10.1016/j.breast.2016.07.009.
Dyńka, D. et al. (2023). The ketogenic diet and cardiovascular diseases. *Nutrients 2023, Vol. 15, Page 3368*, 15 (15), 3368. Available from https://doi.org/10.3390/NU15153368.
Ebeling, P., Koistinen, H.A. and Koivisto, V.A. (1998). Insulin-independent glucose transport regulates insulin sensitivity. *FEBS Letters*, 436 (3), 301–303. Available from https://doi.org/10.1016/S0014-5793(98)01149-1.
Ebou, M.H. et al. (2016). Glucocorticoids inhibit basal and hormone-induced serotonin synthesis in pancreatic beta cells. *PLoS ONE*, 11 (2), 149343. Available from https://doi.org/10.1371/journal.pone.0149343.
Elfawy, H.A. and Das, B. (2019). Crosstalk between mitochondrial dysfunction, oxidative

stress, and age related neurodegenerative disease: Etiologies and therapeutic strategies. *Life Sciences*, 218, 165–184. Available from https://doi.org/10.1016/j.lfs.2018.12.029.

Elliott, R.L., Jiang, X.P. and Head, J.F. (2012). Mitochondria organelle transplantation: Introduction of normal epithelial mitochondria into human cancer cells inhibits proliferation and increases drug sensitivity. *Breast Cancer Research and Treatment*, 136 (2), 347–354. Available from https://doi.org/10.1007/s10549-012-2283-2.

Ellulu, M.S. et al. (2017). Obesity and inflammation: the linking mechanism and the complications. *Archives of Medical Science : AMS*, 13 (4), 851. Available from https://doi.org/10.5114/AOMS.2016.58928.

Escudero, C.A. et al. (2017). Pro-angiogenic role of insulin: From physiology to pathology. *Frontiers in Physiology*, 8 (APR), 224224. Available from https://doi.org/10.3389/FPHYS.2017.00204/BIBTEX.

Fainsod-Levi, T. et al. (2017). Hyperglycemia impairs neutrophil mobilization leading to enhanced metastatic seeding. *CellReports*, 21, 2384–2392. Available from https://doi.org/10.1016/j.celrep.2017.11.010.

Fareed, A. and Hussain, A. (2023). The expanding role of GLP-1: From diabetes management to cancer treatment. *Clinical Medicine Insights. Endocrinology and Diabetes*, 16. Available from https://doi.org/10.1177/11795514231213566.

Ferron, M. (2018). Endocrine functions of bone. *Belfiore A., LeRoith D. (eds) Principles of Endocrinology and Hormone Action*, 1–27. Available from https://doi.org/10.1007/978-3-319-27318-1_21-1.

Ferron, M. and Lacombe, J. (2014). Regulation of energy metabolism by the skeleton: Osteocalcin and beyond. *Archives of Biochemistry and Biophysics*, 561, 137–146. Available from https://doi.org/10.1016/j.abb.2014.05.022.

Ferron, M. et al. (2008). Osteocalcin differentially regulates β cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. *Proceedings of the National Academy of Sciences of the United States of America*, 105 (13), 5266–5270. Available from https://doi.org/10.1073/pnas.0711119105.

Filardo, S. et al. (2022). The potential role of hypochlorhydria in the development of duodenal dysbiosis: A preliminary report. *Frontiers in Cellular and Infection Microbiology*, 12, 854904. Available from https://doi.org/10.3389/FCIMB.2022.854904/BIBTEX.

Fine, E.J. et al. (2012). Targeting insulin inhibition as a metabolic therapy in advanced cancer: A pilot safety and feasibility dietary trial in 10 patients. *Nutrition*, 28 (10), 1028–1035. Available from https://doi.org/10.1016/j.nut.2012.05.001.

Fiordelisi, A. et al. (2019). NfkappaB is a key player in the crosstalk between inflammation and cardiovascular diseases. *International Journal of Molecular Sciences*, 20 (7). Available from https://doi.org/10.3390/ijms20071599.

Flood, P.M. et al. (2011). Transcriptional factor NF-κb as a target for therapy in Parkinson's disease. *Parkinson's Disease*, 2011. Available from https://doi.org/10.4061/2011/216298.

Flores-López, L.A. et al. (2016). High glucose and insulin enhance uPA expression, ROS formation and invasiveness in breast cancer-derived cells. *Cellular Oncology*, 39 (4), 365–378. Available from https://doi.org/10.1007/S13402-016-0282-8/FIGURES/7.

Fontana, L. and Partridge, L. (2015). Promoting health and longevity through diet: from model organisms to humans. *Cell*, 161 (1), 106–118. Available from

https://doi.org/10.1016/j.cell.2015.02.020.
Foresta, C. et al. (2010). Evidence for osteocalcin production by adipose tissue and its role in human metabolism. *The Journal of Clinical Endocrinology & Metabolism*, 95 (7), 3502–3506. Available from https://doi.org/10.1210/JC.2009-2557.
Forrester, S.J. et al. (2018). Reactive oxygen species in metabolic and inflammatory signaling. *Circulation Research*, 122 (6), 877–902. Available from https://doi.org/10.1161/CIRCRESAHA.117.311401.
Frezza, C. and Gottlieb, E. (2009). Mitochondria in cancer: Not just innocent bystanders. *Seminars in Cancer Biology*, 19 (1), 4–11. Available from https://doi.org/10.1016/j.semcancer.2008.11.008.
Fruehauf, J.P. and Meyskens, F.L. (2007). Reactive oxygen species: A breath of life or death? *Clinical Cancer Research*, 13 (3), 789–794. Available from https://doi.org/10.1158/1078-0432.CCR-06-2082.
Fruman, D.A. et al. (2017). The PI3K pathway in human disease. *Cell*, 170 (4), 605–635. Available from https://doi.org/10.1016/j.cell.2017.07.029.
Fu, S.P. et al. (2015). Anti-inflammatory effects of BHBA in both in vivo and in vitro Parkinson's disease models are mediated by GPR109A-dependent mechanisms. *Journal of Neuroinflammation*, 12 (1), 9. Available from https://doi.org/10.1186/s12974-014-0230-3.
Gao, F., Chen, J. and Zhu, H. (2018). A potential strategy for treating atherosclerosis: Improving endothelial function via amp-activated protein kinase. *Science China Life Sciences*, 61 (9), 1024–1029. Available from https://doi.org/10.1007/s11427-017-9285-1.
Gao, J. et al. (2018). SIRT3/SOD2 maintains osteoblast differentiation and bone formation by regulating mitochondrial stress. *Cell Death and Differentiation*, 25 (2), 229–240. Available from https://doi.org/10.1038/cdd.2017.144.
Georgiadou, E. and Rutter, G.A. (2020). Control by Ca2+ of mitochondrial structure and function in pancreatic β-cells. *Cell Calcium*, 91. Available from https://doi.org/10.1016/j.ceca.2020.102282.
Gershuni, V.M., Yan, S.L. and Medici, V. (2018). Nutritional ketosis for weight management and reversal of metabolic syndrome. *Current Nutrition Reports*, 7 (3), 97–106. Available from https://doi.org/10.1007/s13668-018-0235-0.
Ghosh, A. et al. (2017). Insulin inhibits Nrf2 gene expressionvia heterogeneous nuclear ribonucleoprotein F/K in diabetic mice. *Endocrinology*, 158 (4), 903–919. Available from https://doi.org/10.1210/en.2016-1576.
Ghosh, A. et al. (2019). Insulin Inhibits Nrf2 Gene Expression via Heterogeneous Nuclear Ribonucleoprotein F/K in Diabetic Mice. *Endocrinology*, 158 (4), 903–919. Available from https://doi.org/10.1210/en.2016-1576.
Giacomello, M. et al. (2020). The cell biology of mitochondrial membrane dynamics. *Nature Reviews Molecular Cell Biology*, 21 (4), 204–224. Available from https://doi.org/10.1038/s41580-020-0210-7.
Giovannucci, E. et al. (2010). Diabetes and cancer: A consensus report. *Diabetes Care*, 33 (7), 1674–1685. Available from https://doi.org/10.2337/dc10-0666.
Goalstone, M. et al. (1997). Insulin stimulates the phosphorylation and activity of farnesyltransferase via the Ras-Mitogen-Activated Protein Kinase Pathway*. *Endocrinology*, 138 (12), 5119–5124. Available from https://doi.org/10.1210/endo.138.12.5621.

Goalstone, M. et al. (1998). Effect of insulin on farnesyltransferase. Specificity of insulin action and potentiation of nuclear effects of insulin-like growth factor-1, epidermal growth factor, and platelet-derived growth factor. *Journal of Biological Chemistry*, 273 (37), 23892–23896. Available from https://doi.org/10.1074/jbc.273.37.23892.

Goalstone, M.L. and Draznin, B. (1998). What does insulin do to Ras? *Cellular Signalling*, 10 (5), 297–301. Available from https://doi.org/10.1016/S0898-6568(97)00132-0.

Goalstone, M.L. et al. (1998). Effect of insulin on farnesyltransferase. *Journal of Biological Chemistry*, 273 (37), 23892–23896. Available from https://doi.org/10.1074/jbc.273.37.23892.

Gohel, M.G. and Chacko, A.N. (2013). Serum GGT activity and hsCRP level in patients with type 2 diabetes mellitus with good and poor glycemic control: An evidence linking oxidative stress, inflammation and glycemic control. *Journal of Diabetes and Metabolic Disorders*, 12 (1). Available from https://doi.org/10.1186/2251-6581-12-56.

Goldberg, R. et al. (2019). Regulation of heparanase in diabetes-associated pancreatic carcinoma. *Frontiers in Oncology*, 9, 494758. Available from https://doi.org/10.3389/FONC.2019.01405/BIBTEX.

Goldenshluger, A. et al. (2021). Effect of dietary strategies on respiratory quotient and its association with clinical parameters and organ fat loss: A randomized controlled trial. *Nutrients*, 13 (7). Available from https://doi.org/10.3390/NU13072230.

Golovchenko, I. et al. (2000). Hyperinsulinemia enhances transcriptional activity of nuclear factor-κB induced by angiotensin II, hyperglycemia, and advanced glycosylation end products in vascular smooth muscle cells. *Circulation Research*, 87 (9), 746–752. Available from https://doi.org/10.1161/01.RES.87.9.746.

Gonzalez, E. et al. (2011). Hyperinsulinemia leads to uncoupled insulin regulation of the GLUT4 glucose transporter and the FoxO1 transcription factor. *Proceedings of the National Academy of Sciences of the United States of America*, 108 (25), 10162–10167. Available from https://doi.org/10.1073/pnas.1019268108.

Gonzalo, J.A. et al. (1998). The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *The Journal of Experimental Medicine*, 188 (1), 157. Available from https://doi.org/10.1084/JEM.188.1.157.

Gosmanov, A.R. and Nematollahi, L.R. (2020). Diabetic ketoacidosis - Symptoms, diagnosis and treatment | BMJ Best Practice US. *BMJ Best Practic*. Available from https://bestpractice.bmj.com/topics/en-us/162 [Accessed 4 August 2020].

Gössl, M. et al. (2008). Osteocalcin expression by circulating endothelial progenitor cells in patients with coronary atherosclerosis. *Journal of the American College of Cardiology*, 52 (16), 1314. Available from https://doi.org/10.1016/J.JACC.2008.07.019.

Gottlieb, S. and Ruvkun, G. (1994). daf-2, daf-16 and daf-23: Genetically interacting genes controlling dauer formation in Caenorhabditis elegans. *Genetics*, 137 (1), 107–120. Available from https://doi.org/10.1093/genetics/137.1.107.

Grabacka, M. et al. (2016). Regulation of ketone body metabolism and the role of PPARα. *International Journal of Molecular Sciences*, 17 (12). Available from https://doi.org/10.3390/ijms17122093.

Granic, I. et al. (2009). Inflammation and NF-κB in Alzheimer's disease and diabetes. *Journal of Alzheimer's Disease*, 16 (4), 809–821. Available from https://doi.org/10.3233/JAD-2009-0976.

Greer, E.L. et al. (2007). The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. *Journal of Biological Chemistry*, 282 (41),

30107–30119. Available from https://doi.org/10.1074/jbc.M705325200.
Gross, D.N., Van Den Heuvel, A.P.J. and Birnbaum, M.J. (2008). The role of FoxO in the regulation of metabolism. *Oncogene*, 27 (16), 2320–2336. Available from https://doi.org/10.1038/onc.2008.25.
Grundy, S.M. (2007). Gamma-glutamyl transferase: Another biomarker for metabolic syndrome and cardiovascular risk. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 27 (1), 4–7. Available from https://doi.org/10.1161/01.ATV.0000253905.13219.4b.
Guney, G. et al. (2019a). Assessment of the relationship between serum vitamin D and osteocalcin levels with metabolic syndrome in non-osteoporotic postmenopausal women. *Geburtshilfe und Frauenheilkunde*, 79 (03), 293–299. Available from https://doi.org/10.1055/a-0767-6572.
Guney, G. et al. (2019b). Assessment of the Relationship between Serum Vitamin D and Osteocalcin Levels with Metabolic Syndrome in Non-Osteoporotic Postmenopausal Women. *Geburtshilfe und Frauenheilkunde*, 79 (3), 293–299. Available from https://doi.org/10.1055/a-0767-6572.
Gunter, M.J. et al. (2009). Insulin, insulin-like growth factor-I, and risk of breast cancer in postmenopausal women. *Journal of the National Cancer Institute*, 101 (1), 48–60. Available from https://doi.org/10.1093/jnci/djn415.
Gupta, A. et al. (2004). Coagulation and inflammatory markers in Alzheimer's and vascular dementia. *International Journal of Clinical Practice*, 59 (1), 52–57. Available from https://doi.org/10.1111/j.1742-1241.2004.00143.x.
Gureev, A.P., Shaforostova, E.A. and Popov, V.N. (2019). Regulation of mitochondrial biogenesis as a way for active longevity: Interaction between the Nrf2 and PGC-1α signaling pathways. *Frontiers in Genetics*, 10 (MAY). Available from https://doi.org/10.3389/fgene.2019.00435.
Hale, L.J. et al. (2013). Insulin directly stimulates VEGF-A production in the glomerular podocyte. *J Physiol Renal Physiol*, 305, 182–188. Available from https://doi.org/10.1152/ajprenal.00548.2012.-Podocytes.
Hallberg, S.J. et al. (2018). Effectiveness and safety of a novel care model for the management of type 2 diabetes at 1 Year: An open-label, non-randomized, controlled study. *Diabetes Therapy*, 9 (2), 583–612. Available from https://doi.org/10.1007/s13300-018-0373-9.
Hamanaka, R.B. and Chandel, N.S. (2010). Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. *Trends in Biochemical Sciences*, 35 (9), 505–513. Available from https://doi.org/10.1016/j.tibs.2010.04.002.
Han, L. et al. (2011). High glucose promotes pancreatic cancer cell proliferation via the induction of EGF expression and transactivation of EGFR. *PLOS ONE*, 6 (11), e27074. Available from https://doi.org/10.1371/JOURNAL.PONE.0027074.
Hanahan, D. and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. *Cell*, 144, 646–674. Available from https://doi.org/10.1016/j.cell.2011.02.013.
Hang, L. et al. (2019). AMP kinase activation is selectively disrupted in the ventral midbrain of mice deficient in parkin or PINK1 expression. *NeuroMolecular Medicine*, 21 (1), 25–32. Available from https://doi.org/10.1007/s12017-018-8517-7.
Hansen, M.E. et al. (2014). Insulin increases ceramide synthesis in skeletal muscle. *Journal of Diabetes Research*, 2014. Available from https://doi.org/10.1155/2014/765784.
Harbeck, N. et al. (2004). Urokinase-type plasminogen activator (uPA) and its inhibitor PAI-I: Novel tumor-derived factors with a high prognostic and predictive impact in breast

cancer. *Thrombosis and Haemostasis*, 92 (REV.), 47–53. Available from https://doi.org/10.1160/TH03-12-0798/ID/JR0798-7/BIB.
Harman, D. (1955). *Aging: A theory based on free radical and radiation chemistry*.
Harman, Denham. (1956). Aging: A theory based on free radical and radiation chemistry. *Journal of Gerontology*, 11 (3), 298–300. Available from https://doi.org/10.1093/geronj/11.3.298.
Harman, Dehnam. (1956). Aging: A theory based on free radical and radiation chemistry. *Journal of Gerontology*, 11 (3), 298–300. Available from https://doi.org/10.1093/geronj/11.3.298.
Harman, Denham. (1957). Atherosclerosis: Possible ill-effects of the use of highly unsaturated fats to lower serum-cholesterol levels. *The Lancet*, 270 (7005), 1116–1117. Available from https://doi.org/10.1016/S0140-6736(57)90137-X.
Harman, Dehnam. (1957). Prolongation of the normal life span by radiation protection chemicals. *Journal of Gerontology*, 12, 257–263. Available from https://doi.org/10.1093/geronj/12.3.257.
Harman, Denham. (1991). The aging process: Major risk factor for disease and death. *Proceedings of the National Academy of Sciences of the United States of America*, 88 (12), 5360–5363. Available from https://doi.org/10.1073/pnas.88.12.5360.
Harman, D. (1991). The aging process: Major risk factor for disease and death. *Proceedings of the National Academy of Sciences of the United States of America*, 88 (12), 5360–5363. Available from https://doi.org/10.1073/pnas.88.12.5360.
Harman, D. (2006). Free radical theory of aging: An update: Increasing the functional life span. *Annals of the New York Academy of Sciences*, 1067 (1), 10–21. Available from https://doi.org/10.1196/annals.1354.003.
Harman, D. (2009). Origin and evolution of the free radical theory of ageing: A brief personal history, 1954–2009. *Biogerontology*, 10 (6), 773–781. Available from https://doi.org/10.1007/s10522-009-9234-2.
Hauschka, P. V. et al. (1989). Osteocalcin and matrix Gla protein: vitamin K-dependent proteins in bone. *https://doi.org/10.1152/physrev.1989.69.3.990*, 69 (3), 990–1047. Available from https://doi.org/10.1152/PHYSREV.1989.69.3.990.
Hawley, S.A. et al. (2014). Phosphorylation by Akt within the ST loop of AMPK-α1 down-regulates its activation in tumour cells. *Biochemical Journal*, 459 (2), 275–287. Available from https://doi.org/10.1042/BJ20131344.
Hayflick, L. and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. *Experimental Cell Research*, 25 (3), 585–621. Available from https://doi.org/10.1016/0014-4827(61)90192-6.
Heidelbaugh, J.J. (2013). Proton pump inhibitors and risk of vitamin and mineral deficiency: evidence and clinical implications. *Therapeutic Advances in Drug Safety*, 4 (3). Available from https://doi.org/10.1177/2042098613482484.
Hernlund, E. et al. (2013). Osteoporosis in the European Union: Medical management, epidemiology and economic burden: A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). *Archives of Osteoporosis*, 8 (1–2). Available from https://doi.org/10.1007/s11657-013-0136-1.
Herst, P.M. et al. (2017). Functional mitochondria in health and disease. *Frontiers in Endocrinology*, 8 (NOV), 296. Available from https://doi.org/10.3389/fendo.2017.00296.

Herszényi, L. et al. (2008). Tumor marker utility and prognostic relevance of cathepsin B, cathepsin L, urokinase-type plasminogen activator, plasminogen activator inhibitor type-1, CEA and CA 19-9 in colorectal cancer. *BMC Cancer*, 8 (1), 1–12. Available from https://doi.org/10.1186/1471-2407-8-194/FIGURES/2.

Hill, D.J. and Milner, R.D.G. (1985). Insulin as a growth factor. *International Pediatric Research Foundation, Inc*, 19 (9).

Hill, H.. et al. (2014). Carboxylated and uncarboxylated forms of osteocalcin directly modulate the glucose transport system and inflammation in adipocytes. *Hormone and Metabolic Research*, 46 (5), 341–347. Available from https://doi.org/10.1055/s-0034-1368709.

Hill, P.A. and Tumber, A. (2010). Ceramide-induced cell death/survival in murine osteoblasts. *Journal of Endocrinology*, 206 (2), 225–233. Available from https://doi.org/10.1677/JOE-10-0068.

Hill, S. and Van Remmen, H. (2014). Mitochondrial stress signalling in longevity: A new role for mitochondrial function in ageing. *Redox Biology*, 2 (1), 936–944. Available from https://doi.org/10.1016/j.redox.2014.07.005.

Hinokio, Y. et al. (1999). Oxidative DNA damage in diabetes mellitus: Its association with diabetic complications. *Diabetologia*, 42 (8), 995–998. Available from https://doi.org/10.1007/s001250051258.

Hodson, A.E., Tippetts, T.S. and Bikman, B.T. (2015). Insulin treatment increases myocardial ceramide accumulation and disrupts cardiometabolic function. *Cardiovascular Diabetology*, 14 (1), 1–11. Available from https://doi.org/10.1186/S12933-015-0316-Y/FIGURES/8.

Holzenberger, M. et al. (2002). IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature 2003 421:6919*, 421 (6919), 182–187. Available from https://doi.org/10.1038/nature01298.

Hong, C.T. et al. (2020). Insulin resistance promotes Parkinson's disease through aberrant expression of α-synuclein, mitochondrial dysfunction, and deregulation of the polo-like kinase 2 signaling. *Cells*, 9 (3). Available from https://doi.org/10.3390/cells9030740.

Hopkins, B.D. et al. (2018). Suppression of insulin feedback enhances the efficacy of PI3K inhibitors. *Nature 2018 560:7719*, 560 (7719), 499–503. Available from https://doi.org/10.1038/s41586-018-0343-4.

Hopkins, B.D., Goncalves, M.D. and Cantley, L.C. (2020). Insulin–PI3K signalling: An evolutionarily insulated metabolic driver of cancer. *Nature Reviews Endocrinology*, 16 (5), 276–283. Available from https://doi.org/10.1038/s41574-020-0329-9.

Hou, Y. et al. (2017). High glucose levels promote the proliferation of breast cancer cells through GTPases Breast Cancer-Targets and Therapy Dovepress High glucose levels promote the proliferation of breast cancer cells through GTPases. Available from https://doi.org/10.2147/BCTT.S135665.

Hunot, S. et al. (1997). Nuclear translocation of NF-κb is increased in dopaminergic neurons of patients with Parkinson disease. *Proceedings of the National Academy of Sciences of the United States of America*, 94 (14), 7531–7536. Available from https://doi.org/10.1073/pnas.94.14.7531.

Hyde, P.N. et al. (2017). Pleiotropic effects of nutritional ketosis: Conceptual framework for keto-adaptation as a breast cancer therapy. *Cancer Treatment and Research Communications*, 12, 32–39. Available from https://doi.org/10.1016/j.ctarc.2017.06.001.

Iglesies-Grau, J. et al. (2023). Early insulin resistance in normoglycemic low-risk individuals is associated with subclinical atherosclerosis. *Cardiovascular Diabetology*, 22 (1), 1–12. Available from https://doi.org/10.1186/S12933-023-02090-1/FIGURES/5.

Irrcher, I., Ljubicic, V. and Hood, D.A. (2009). Interactions between ROS and AMP kinase activity in the regulation of PGC-1α transcription in skeletal muscle cells. *American Journal of Physiology - Cell Physiology*, 296 (1). Available from https://doi.org/10.1152/ajpcell.00267.2007.

Iwadate, Y. et al. (2008). High serum level of plasminogen activator inhibitor-1 predicts histological grade of intracerebral gliomas. *Anticancer Research*, 28 (1B), 415–418.

İyikesici, M.S. et al. (2017). Efficacy of metabolically supported chemotherapy combined with ketogenic diet, hyperthermia, and hyperbaric oxygen therapy for stage IV triple-negative breast cancer. *Cureus*, 9 (7). Available from https://doi.org/10.7759/cureus.1445.

Jag, U.R., Zavadil, J. and Stanley, F.M. (2009). Insulin acts through FOXO3a to activate transcription of plasminogen activator inhibitor type 1. *Molecular Endocrinology*, 23 (10), 1587–1602. Available from https://doi.org/10.1210/me.2008-0421.

Jansen, L.T. et al. (2022). Prolonged glycemic adaptation following transition from a low- to high-carbohydrate diet: A randomized controlled feeding trial. *Diabetes Care*, 45 (3), 576–584. Available from https://doi.org/10.2337/DC21-1970.

Janssen, J.A.M.J.L. and Street, E. (2021). Hyperinsulinemia and its pivotal role in aging, obesity, type 2 diabetes, cardiovascular disease and cancer. *International Journal of Molecular Sciences 2021, Vol. 22, Page 7797*, 22 (15), 7797. Available from https://doi.org/10.3390/IJMS22157797.

Janus, A. et al. (2016). Insulin resistance and endothelial dysfunction constitute a common therapeutic target in cardiometabolic disorders. *Mediators of Inflammation*, 2016. Available from https://doi.org/10.1155/2016/3634948.

Jayedi, A. et al. (2022). Dose-dependent effect of carbohydrate restriction for type 2 diabetes management: a systematic review and dose-response meta-analysis of randomized controlled trials. *The American Journal of Clinical Nutrition*, 116 (1), 40–56. Available from https://doi.org/10.1093/AJCN/NQAC066.

Jeon, S.M. (2016). Regulation and function of AMPK in physiology and diseases. *Experimental & molecular medicine*, 48 (7), e245. Available from https://doi.org/10.1038/emm.2016.81.

Jevrić, M. et al. (2019). Association of uPA and PAI-1 tumor levels and 4G/5G variants of PAI-1 gene with disease outcome in luminal HER2-negative node-negative breast cancer patients treated with adjuvant endocrine therapy. *BMC Cancer*, 19 (1), 1–13. Available from https://doi.org/10.1186/S12885-018-5255-Z/TABLES/7.

Jha, N.K. et al. (2019). Nuclear factor-kappa β as a therapeutic target for Alzheimer's disease. *Journal of Neurochemistry*, 150 (2), 113–137. Available from https://doi.org/10.1111/jnc.14687.

Jiang, C.S. et al. (2023). Aging, plasminogen activator inhibitor 1, brain cell senescence, and Alzheimer's Disease. *Aging and disease*, 14 (2), 515–528. Available from https://doi.org/10.14336/AD.2022.1220.

Jiang, Z. et al. (2022). Effects of ketogenic diet on neuroinflammation in neurodegenerative diseases. *Aging and Disease*, 13 (4), 1146. Available from https://doi.org/10.14336/AD.2021.1217.

Jo, U. and Park, K. (2023). Carbohydrate-based diet may increase the risk of cardiovascular

disease: A pooled analysis of two prospective cohort studies. *Clinical Nutrition*, 42 (8), 1301–1307. Available from https://doi.org/10.1016/j.clnu.2023.06.013.
Johnson, T.E. (1987). Aging can be genetically dissected into component processes using long-lived lines of Caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America*, 84 (11), 3777–3781. Available from https://doi.org/10.1073/pnas.84.11.3777.
Jones, S.V. and Kounatidis, I. (2017). Nuclear factor-kappa B and Alzheimer disease, unifying genetic and environmental risk factors from cell to humans. *Frontiers in Immunology*, 8 (DEC), 1805. Available from https://doi.org/10.3389/fimmu.2017.01805.
Joshi, A.U. et al. (2018). Drp1/Fis1 interaction mediates mitochondrial dysfunction, bioenergetic failure and cognitive decline in Alzheimer's disease. *Oncotarget*, 9 (5), 6128–6143. Available from https://doi.org/10.18632/oncotarget.23640.
Ju Hwang, C. et al. (2017). NF-κB as a key mediator of brain inflammation in Alzheimer's disease. *CNS & Neurological Disorders - Drug Targets*, 18 (1), 3–10. Available from https://doi.org/10.2174/1871527316666170807130011.
Juhan-Vague, I. et al. (2003). Plasminogen activator inhibitor-1, inflammation, obesity, insulin resistance and vascular risk. *Journal of Thrombosis and Haemostasis*, 1 (7), 1575–1579. Available from https://doi.org/10.1046/J.1538-7836.2003.00279.X.
Jujić, A. et al. (2023). Endogenous incretin levels and risk of first incident cancer: a prospective cohort study. *Scientific Reports*, 13 (1). Available from https://doi.org/10.1038/S41598-023-27509-3.
Kadlec, A.O. et al. (2016). Role of PGC-1α in vascular regulation: Implications for atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 36 (8), 1467–1474. Available from https://doi.org/10.1161/ATVBAHA.116.307123.
Kaludercic, N. and Di Lisa, F. (2020). Mitochondrial ROS formation in the pathogenesis of diabetic cardiomyopathy. *Frontiers in Cardiovascular Medicine*, 7, 12. Available from https://doi.org/10.3389/fcvm.2020.00012.
Kanazawa, I. et al. (2009). Serum osteocalcin level Is associated with glucose metabolism and atherosclerosis parameters in type 2 diabetes mellitus. *J Clin Endocrinol Metab*, 94 (1), 45–49. Available from https://doi.org/10.1210/jc.2008-1455.
Kanda, H. et al. (2006). MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *The Journal of Clinical Investigation*, 116 (6), 1494–1505. Available from https://doi.org/10.1172/JCI26498.
Kandimalla, R., Thirumala, V. and Reddy, P.H. (2017). Is Alzheimer's disease a type 3 diabetes? A critical appraisal. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 1863 (5), 1078–1089. Available from https://doi.org/10.1016/j.bbadis.2016.08.018.
Kaneko, N. et al. (2019). The role of interleukin-1 in general pathology. *Inflammation and Regeneration 2019 39:1*, 39 (1), 1–16. Available from https://doi.org/10.1186/S41232-019-0101-5.
Kaneto, H. et al. (2010). Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis. *Mediators of Inflammation*, 2010, 11. Available from https://doi.org/10.1155/2010/453892.
Kapeller, R. and Cantley, L.C. (1994). Phosphatidylinositol 3-kinase. *BioEssays*, 16 (8), 565–576. Available from https://doi.org/10.1002/bies.950160810.
Kautzky-Willer, A., Harreiter, J. and Pacini, G. (2016). Sex and gender differences in risk, pathophysiology and complications of type 2 diabetes mellitus. *Endocrine Reviews*, 37

(3), 278–316. Available from https://doi.org/10.1210/ER.2015-1137.
Keck, K.M. and Pemberton, L.F. (2012). Histone chaperones link histone nuclear import and chromatin assembly. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, 1819 (3–4), 277–289. Available from https://doi.org/10.1016/j.bbagrm.2011.09.007.
Kellerer, T. et al. (2021). Fatty acid esters of hydroxy fatty acids (FAHFAs) are associated with diet, BMI, and age. *Frontiers in Nutrition*, 8, 691401. Available from https://doi.org/10.3389/FNUT.2021.691401/BIBTEX.
Kelly, T., Unwin, D. and Finucane, F. (2020). Low-carbohydrate diets in the management of obesity and type 2 diabetes: A review from clinicians using the approach in practice. *International Journal of Environmental Research and Public Health*, 17 (7), 2557. Available from https://doi.org/10.3390/ijerph17072557.
Kempf, A.M. et al. (2006). Leptin as a marker of body fat and hyperinsulinemia in college students. *Journal of American College Health*, 55 (3), 175–180. Available from https://doi.org/10.3200/JACH.55.3.175-180.
Kenyon, C. et al. (1993). A C. elegans mutant that lives twice as long as wild type. *Nature*, 366 (6454), 461–464. Available from https://doi.org/10.1038/366461a0.
Khokhlov, A.N. (2013). Evolution of the term 'Cellular Senescence' and its impact on the current cytogerontological research. *Moscow University Biological Sciences Bulletin*, 68 (4), 158–161. Available from https://doi.org/10.3103/S0096392513040123.
Kimura, K.D. et al. (1997). Daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. *Science*, 277 (5328), 942–946. Available from https://doi.org/10.1126/science.277.5328.942.
Kinnaird, A. et al. (2016). Metabolic control of epigenetics in cancer. *Nature Reviews Cancer*, 16 (11), 694–707. Available from https://doi.org/10.1038/nrc.2016.82.
Kinzig, K.P., Honors, M.A. and Hargrave, S.L. (2010). Insulin sensitivity and glucose tolerance are altered by maintenance on a ketogenic diet. *Endocrinology*, 151 (7), 3105–3114. Available from https://doi.org/10.1210/en.2010-0175.
Kirk, B. et al. (2020). Muscle, bone, and fat crosstalk: The biological role of myokines, osteokines, and adipokines. *Current Osteoporosis Reports*, 18 (4), 388–400. Available from https://doi.org/10.1007/s11914-020-00599-y.
Klotz, L.O. et al. (2015). Redox regulation of FoxO transcription factors. *Redox Biology*, 6, 51–72. Available from https://doi.org/10.1016/j.redox.2015.06.019.
Knopp, J.L., Holder-Pearson, L. and Chase, J.G. (2019). Insulin units and conversion factors: A story of truth, boots, and faster half-truths. *Journal of diabetes science and technology*, 13 (3), 597–600. Available from https://doi.org/10.1177/1932296818805074.
Kobayashi, K. et al. (2015). Mitochondrial superoxide in osteocytes perturbs canalicular networks in the setting of age-related osteoporosis. *Scientific Reports*, 5 (1), 1–11. Available from https://doi.org/10.1038/srep09148.
Koensgen, D. et al. (2018). Expression, intracellular localization, and prognostic value of plasminogen activator inhibitor 1 and PAI-1 RNA-binding Protein 1 in primary and recurrent ovarian cancer: A study of the tumor bank ovarian cancer network. *Gynecologic and Obstetric Investigation*, 83 (5), 508–514. Available from https://doi.org/10.1159/000479027.
Koh, S., Dupuis, N. and Auvin, S. (2020). Ketogenic diet and neuroinflammation. *Epilepsy Research*, 167, 106454. Available from https://doi.org/10.1016/J.EPLEPSYRES.2020.106454.
Kolb, H. et al. (2020). Insulin: Too much of a good thing is bad. *BMC Medicine*, 18 (1), 224.

Available from https://doi.org/10.1186/s12916-020-01688-6.
Konopka, A.R. and Sreekumaran Nair, K. (2013). Mitochondrial and skeletal muscle health with advancing age. *Molecular and Cellular Endocrinology*, 379 (1–2), 19–29. Available from https://doi.org/10.1016/j.mce.2013.05.008.
Kreymann, B. et al. (1987). Glucagon-like peptide-1 7-36: A physiological incretin in man. *The Lancet*, 330 (8571), 1300–1304. Available from https://doi.org/10.1016/S0140-6736(87)91194-9.
Krishna, M. and Narang, H. (2008). The complexity of mitogen-activated protein kinases (MAPKs) made simple. *Cellular and Molecular Life Sciences*, 65 (22), 3525–3544. Available from https://doi.org/10.1007/s00018-008-8170-7.
Kuga, G.K. et al. (2017). Hippocampal insulin signaling and neuroprotection mediated by physical exercise in Alzheimeŕs Disease. *Motriz. Revista de Educacao Fisica*, 23 (SPE). Available from https://doi.org/10.1590/S1980-6574201700SI0008.
Kuhn, W. et al. (1999). Prognostic significance of urokinase (uPA) and its inhibitor PAI-1 for survival in advanced ovarian carcinoma stage FIGO IIIc. *British Journal of Cancer 1999 79:11*, 79 (11), 1746–1751. Available from https://doi.org/10.1038/sj.bjc.6690278.
Kumpatla, S. et al. (2021). Hyperglucagonemia and impaired insulin sensitivity are associated with development of prediabetes and type 2 diabetes - A study from South India. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, 15 (4), 102199. Available from https://doi.org/10.1016/J.DSX.2021.102199.
Kushnir, M.M. et al. (2004). Cortisol and cortisone analysis in serum and plasma by atmospheric pressure photoionization tandem mass spectrometry. *Clinical Biochemistry*, 37 (5), 357–362. Available from https://doi.org/10.1016/J.CLINBIOCHEM.2004.01.005.
Kwak, J., Seo, I.H. and Lee, Y.J. (2023). Serum γ-glutamyltransferase level and incidence risk of metabolic syndrome in community dwelling adults: longitudinal findings over 12 years. *Diabetology & metabolic syndrome*, 15 (1). Available from https://doi.org/10.1186/S13098-023-01000-5.
Kyriakidou, Y. et al. (2021). Preliminary investigations into the effect of exercise-induced muscle damage on systemic extracellular vesicle release in trained younger and older men. *Frontiers in Physiology*, 12, 723931. Available from https://doi.org/10.3389/FPHYS.2021.723931/FULL.
Lage, R. et al. (2008). AMPK: a metabolic gauge regulating whole-body energy homeostasis. *Trends in Molecular Medicine*, 14 (12), 539–549. Available from https://doi.org/10.1016/j.molmed.2008.09.007.
Lan, Y.L. et al. (2019). Update on the synergistic effect of HSL and insulin in the treatmentof metabolic disorders. *Therapeutic Advances in Endocrinology and Metabolism*, 10, 1–10. Available from https://doi.org/10.1177/2042018819877300.
Lana, A. et al. (2017). Serum leptin concentration is associated with incident frailty in older adults. *Aging and disease*, 8 (2), 240–249. Available from https://doi.org/10.14336/AD.2016.0819.
Lee, D.H., Blomhoff, R. and Jacobs, D.R. (2004). Is serum gamma glutamyltransferase a marker of oxidative stress? *Free radical research*, 38 (6), 535–539. Available from https://doi.org/10.1080/10715760410001694026.
Lee, D.Y. et al. (2018). Impact of systemic inflammation on the relationship between insulin resistance and all-cause and cancer-related mortality. *Metabolism: clinical and experimental*, 81, 52–62. Available from

https://doi.org/10.1016/J.METABOL.2017.11.014.
Lee, S.-H. and Min, K.-J. (2013). Caloric restriction and its mimetics. *BMB Reports*, 46 (4), 181–187. Available from https://doi.org/10.5483/BMBRep.2013.46.4.033.
Lee, S. and Lee, D.Y. (2017). Glucagon-like peptide-1 and glucagon-like peptide-1 receptor agonists in the treatment of type 2 diabetes. *Annals of Pediatric Endocrinology & Metabolism*, 22 (1), 15. Available from https://doi.org/10.6065/apem.2017.22.1.15.
Lee, S.C. and Chan, J.C.N. (2015). Evidence for DNA damage as a biological link between diabetes and cancer. *Chinese Medical Journal*, 128 (11), 1543–1548. Available from https://doi.org/10.4103/0366-6999.157693.
Lei, H. et al. (2022). Association between osteocalcin, a pivotal marker of bone metabolism, and secretory function of islet beta cells and alpha cells in Chinese patients with type 2 diabetes mellitus: an observational study. *Diabetology & Metabolic Syndrome*, 14 (1), 160. Available from https://doi.org/10.1186/S13098-022-00932-8.
Leissner, P. et al. (2006). Prognostic significance of urokinase plasminogen activator and plasminogen activator inhibitor-1 mRNA expression in lymph node- and hormone receptor-positive breast cancer. *BMC Cancer*, 6 (1), 1–9. Available from https://doi.org/10.1186/1471-2407-6-216/FIGURES/6.
Leitner, J.W. et al. (1997). Hyperinsulinemia potentiates activation of p21Ras by growth factors. *Endocrinology*, 138 (5), 2211–2214.
Li, F. et al. (2007). Regulation of HIF-1α stability through S-nitrosylation. *Molecular Cell*, 26 (1), 63–74. Available from https://doi.org/10.1016/j.molcel.2007.02.024.
Li, J. et al. (2015). Pharmacological activation of AMPK prevents Drp1-mediated mitochondrial fission and alleviates endoplasmic reticulum stress-associated endothelial dysfunction. *Journal of Molecular and Cellular Cardiology*, 86, 62–74. Available from https://doi.org/10.1016/j.yjmcc.2015.07.010.
Li, X. et al. (2007). Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1α transcription coactivator. *Nature*, 447 (7147), 1012–1016. Available from https://doi.org/10.1038/nature05861.
Liang, S. et al. (2020). Metabolic regulation of innate immunity. *Advances in Immunology*, 145, 129–157. Available from https://doi.org/10.1016/BS.AI.2019.11.005.
Liao, Z., Chua, D. and Tan, N.S. (2019). Reactive oxygen species: A volatile driver of field cancerization and metastasis. *Molecular Cancer*, 18 (1), 65. Available from https://doi.org/10.1186/s12943-019-0961-y.
Liberti, M. V. and Locasale, J.W. (2016). The Warburg Effect: How does it benefit cancer cells? *Trends in biochemical sciences*, 41 (3), 211.
Liguori, I. et al. (2018). Oxidative stress, aging, and diseases. *Clinical Interventions in Aging*, 13, 757. Available from https://doi.org/10.2147/CIA.S158513.
Lim, J.H. et al. (2010). Sirtuin 1 modulates cellular responses to hypoxia by deacetylating hypoxia-inducible factor 1α. *Molecular Cell*, 38 (6), 864–878. Available from https://doi.org/10.1016/j.molcel.2010.05.023.
Lin, K. et al. (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science*, 278 (5341), 1319–1322. Available from https://doi.org/10.1126/science.278.5341.1319.
Lin, S.J., Defossez, P.A. and Guarente, L. (2000). Requirement of NAD and SIR2 for life-span extension by calorie restriction in saccharomyces cerevisiae. *Science*, 289 (5487), 2126–2128. Available from https://doi.org/10.1126/science.289.5487.2126.
Lineweaver, C.H., Davies, P.C.W.W. and Vincent, M.D. (2014). Targeting cancer's

weaknesses (not its strengths): Therapeutic strategies suggested by the atavistic model. *BioEssays*, 36 (9), 827–835. Available from https://doi.org/10.1002/bies.201400070.

Liu, H.Y. et al. (2009). Hepatic autophagy is suppressed in the presence of insulin resistance and hyperinsulinemia. Inhibition of FoxO1-dependent expression of key autophagy genes by insulin. *Journal of Biological Chemistry*, 284 (45), 31484–31492. Available from https://doi.org/10.1074/jbc.M109.033936.

Liu, T. et al. (2017). NF-κB Signaling in Inflammation. *Signal Transduction and Targeted Therapy*, 2, 17023. Available from https://doi.org/10.1038/sigtrans.2017.23.

Liu, Y. et al. (2018). Critical role of FOXO3a in carcinogenesis. *Molecular Cancer*, 17 (1). Available from https://doi.org/10.1186/s12943-018-0856-3.

Lizcano, J.M. and Alessi, D.R. (2002). The insulin signalling pathway. *Current Biology*, 12 (7), R236–R238. Available from https://doi.org/10.1016/S0960-9822(02)00777-7.

Loh, W.J. et al. (2010). Insulin resistance-related biomarker clustering and subclinical inflammation as predictors of cancer mortality during 21.5 years of follow-up. *Cancer Causes and Control*, 21 (5), 709–718. Available from https://doi.org/10.1007/S10552-009-9499-4/TABLES/4.

Long, Y.C. et al. (2014). The biochemistry and cell biology of ageing: metabolic regulation through mitochondrial signalling. *American Journal of Physiology-Endocrinology and Metabolism*, 306 (6), E581–E591. Available from https://doi.org/10.1152/ajpendo.00665.2013.

Longo, V.D. and Panda, S. (2016). Fasting, circadian rhythms, and time-restricted feeding in healthy lifespan. *Cell Metabolism*, 23 (6), 1048–1059. Available from https://doi.org/10.1016/j.cmet.2016.06.001.

Lopez-Castejon, G. and Brough, D. (2011). Understanding the mechanism of IL-1β secretion. *Cytokine & Growth Factor Reviews*, 22 (4), 189. Available from https://doi.org/10.1016/J.CYTOGFR.2011.10.001.

López-Otín, C. et al. (2013). The hallmarks of aging. *Cell*, 153 (6), 1194–1217. Available from https://doi.org/10.1016/J.CELL.2013.05.039.

Lopez, R. et al. (2013). Hyperglycemia enhances the proliferation of non-tumorigenic and malignant mammary epithelial cells through increased leptin/IGF1R signaling and activation of AKT/mTOR. *PLOS ONE*, 8 (11), e79708. Available from https://doi.org/10.1371/JOURNAL.PONE.0079708.

Luchsinger, J.A. et al. (2004). Hyperinsulinemia and risk of Alzheimer disease. *Neurology*, 63 (7), 1187–1192. Available from https://doi.org/10.1212/01.WNL.0000140292.04932.87.

Luengo, A. et al. (2021). Increased demand for NAD+ relative to ATP drives aerobic glycolysis. *Molecular Cell*, 81 (4), 691-707.e6. Available from https://doi.org/10.1016/j.molcel.2020.12.012.

Luong, N. et al. (2006). Activated FOXO-mediated insulin resistance is blocked by reduction of TOR activity. *Cell Metabolism*, 4 (2), 133–142. Available from https://doi.org/10.1016/j.cmet.2006.05.013.

Lustig, Y. et al. (2011). Separation of the gluconeogenic and mitochondrial functions of pgc-1α through s6 kinase. *Genes and Development*, 25 (12), 1232–1244. Available from https://doi.org/10.1101/gad.2054711.

Ma, Q. (2013). Role of Nrf2 in oxidative stress and toxicity. *Annual Review of Pharmacology and Toxicology*, 53, 401–426. Available from https://doi.org/10.1146/annurev-

pharmtox-011112-140320.
Ma, X. et al. (2020). The roles of FoxO transcription factors in regulation of bone cells function. *International Journal of Molecular Sciences*, 21 (3), 692. Available from https://doi.org/10.3390/ijms21030692.
Mabeta, P. and Steenkamp, V. (2022). The VEGF/VEGFR axis revisited: Implications for cancer therapy. *International Journal of Molecular Sciences 2022, Vol. 23, Page 15585*, 23 (24), 15585. Available from https://doi.org/10.3390/IJMS232415585.
Mahmood, N., Mihalcioiu, C. and Rabbani, S.A. (2018). Multifaceted role of the urokinase-type plasminogen activator (uPA) and its receptor (uPAR): diagnostic, prognostic, and therapeutic applications. *Frontiers in Oncology*, 8 (FEB), 24. Available from https://doi.org/10.3389/FONC.2018.00024.
Malhotra, A., Kamepalli, R. and Bamrah, J.S. (2020). Poor metabolic health is a major issue for increased COVID-19 mortality in BAME patient groups. *The Physician*, 6 (2), 1–4. Available from https://doi.org/10.38192/1.6.2.4.
Manczak, M., Calkins, M.J. and Reddy, P.H. (2011). Impaired mitochondrial dynamics and abnormal interaction of amyloid beta with mitochondrial protein Drp1 in neurons from patients with Alzheimer's disease: Implications for neuronal damage. *Human Molecular Genetics*, 20 (13), 2495–2509. Available from https://doi.org/10.1093/hmg/ddr139.
Manful, C.F. et al. (2022). Assessing the fate of fatty acid esters of hydroxy fatty acids, diglycerides and monoacetyldiacylglycerides in grilled ruminant meats marinated with unfiltered beer-based marinades. *Journal of Food and Drug Analysis*, 30 (2), 234. Available from https://doi.org/10.38212/2224-6614.3406.
Manoharan, S. et al. (2016). The role of reactive oxygen species in the pathogenesis of Alzheimer's disease, Parkinson's disease, and Huntington's disease: A mini review. *Oxidative Medicine and Cellular Longevity*, 2016. Available from https://doi.org/10.1155/2016/8590578.
Manolopoulos, K.N. et al. (2010). Linking Alzheimer's disease to insulin resistance: The FoxO response to oxidative stress. *Molecular Psychiatry*, 15 (11), 1046–1052. Available from https://doi.org/10.1038/mp.2010.17.
Marchi, S. et al. (2012). Mitochondria-Ros crosstalk in the control of cell death and aging. *Journal of Signal Transduction*, 2012, 1–17. Available from https://doi.org/10.1155/2012/329635.
Margaritelis, N. V. et al. (2016). Going retro: oxidative stress biomarkers in modern redox biology. *Free Radical Biology and Medicine*, 98, 2–12. Available from https://doi.org/10.1016/j.freeradbiomed.2016.02.005.
Marin, T.L. et al. (2017). AMPK promotes mitochondrial biogenesis and function by phosphorylating the epigenetic factors DNMT1, RBBP7, and HAT1. *Science Signaling*, 10 (464). Available from https://doi.org/10.1126/scisignal.aaf7478.
Marliss, E. et al. (1970). Hormones and substrates in the regulation of gluconeogenesis in fasting man. *Advances in Enzyme Regulation*, 8 (C), 3–11. Available from https://doi.org/10.1016/0065-2571(70)90005-1.
Martinez, B. and Ortiz, R.M. (2017). Thyroid hormone regulation and insulin resistance: Insights from animals naturally adapted to fasting. *Physiology*, 32 (2), 141–151. Available from https://doi.org/10.1152/PHYSIOL.00018.2016/ASSET/IMAGES/LARGE/PHY0021703640002.JPEG.
Mattison, J.A. et al. (2012). Impact of caloric restriction on health and survival in rhesus

monkeys from the NIA study. *Nature*, 489 (7415), 318–321. Available from https://doi.org/10.1038/nature11432.
Mattson, M.P. (2005). The need for controlled studies of the effects of meal frequency on health. *Lancet*, 365 (9475), 1978–1980. Available from https://doi.org/10.1016/S0140-6736(05)66667-6.
Maxhimer, J.B. et al. (2005). Heparanase-1 gene expression and regulation by high glucose in renal epithelial cells. *Diabetes*, 54 (7), 2172–2178. Available from https://doi.org/10.2337/DIABETES.54.7.2172.
McCarty, M.F., DiNicolantonio, J.J. and O'Keefe, J.H. (2015). Ketosis may promote brain macroautophagy by activating Sirt1 and hypoxia-inducible factor-1. *Medical Hypotheses*, 85 (5), 631–639. Available from https://doi.org/10.1016/j.mehy.2015.08.002.
McCay, C.M., Crowell, M.F. and Maynard, L.A. (1989). The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935. *The Journal of Nutrition (Burbank, Los Angeles County, Calif.)*, 5 (3), 155–171; discussion 172. Available from https://doi.org/10.1093/jn/10.1.63.
McHugh, D. and Gil, J. (2018). Senescence and aging: Causes, consequences, and therapeutic avenues. *Journal of Cell Biology*, 217 (1), 65–77. Available from https://doi.org/10.1083/jcb.201708092.
Meidenbauer, J.J., Mukherjee, P. and Seyfried, T.N. (2015). The glucose ketone index calculator: A simple tool to monitor therapeutic efficacy for metabolic management of brain cancer. *Nutrition and Metabolism*, 12 (1), 1–7. Available from https://doi.org/10.1186/S12986-015-0009-2/TABLES/2.
Menke, A. et al. (2015). Prevalence of and trends in diabetes among adults in the united states, 1988-2012. *JAMA - Journal of the American Medical Association*, 314 (10), 1021–1029. Available from https://doi.org/10.1001/jama.2015.10029.
Mercken, E.M. et al. (2012). Of mice and men: The benefits of caloric restriction, exercise, and mimetics. *Ageing Research Reviews*, 11 (3), 390–398. Available from https://doi.org/10.1016/j.arr.2011.11.005.
Miele, C. et al. (2000). Insulin and Insulin-like Growth Factor-I Induce Vascular Endothelial Growth Factor mRNA Expression via Different Signaling Pathways. *Journal of Biological Chemistry*, 275 (28), 21695–21702. Available from https://doi.org/10.1074/JBC.M000805200.
Milder, J. and Patel, M. (2012). Modulation of oxidative stress and mitochondrial function by the ketogenic diet. *Epilepsy Research*, 100 (3), 295–303. Available from https://doi.org/10.1016/j.eplepsyres.2011.09.021.
Miller, V.J., Villamena, F.A. and Volek, J.S. (2018). Nutritional ketosis and mitohormesis: potential implications for mitochondrial function and human health. *Journal of Nutrition and Metabolism*, 2018. Available from https://doi.org/10.1155/2018/5157645.
Min, S.H. et al. (2018). Degree of ketonaemia and its association with insulin resistance after dapagliflozin treatment in type 2 diabetes. *Diabetes and Metabolism*, 44 (1), 73–76. Available from https://doi.org/10.1016/j.diabet.2017.09.006.
Miyauchi, T. et al. (2019). Up-regulation of FOXO1 and reduced inflammation by β-hydroxybutyric acid are essential diet restriction benefits against liver injury. *Proceedings of the National Academy of Sciences of the United States of America*, 116 (27), 13533–13542. Available from https://doi.org/10.1073/PNAS.1820282116/-

/DCSUPPLEMENTAL.
Mizokami, A. et al. (2013). Osteocalcin induces release of glucagon-like peptide-1 and thereby stimulates insulin secretion in mice. *PLoS ONE*, 8 (2), 57375. Available from https://doi.org/10.1371/journal.pone.0057375.
Mizokami, A. et al. (2016). An extract from pork bones containing osteocalcin improves glucose metabolism in mice by oral administration. *Bioscience, Biotechnology, and Biochemistry*, 80 (11), 2176–2183. Available from https://doi.org/10.1080/09168451.2016.1214530.
Mizokami, A. et al. (2020). GLP-1 signaling is required for improvement of glucose tolerance by osteocalcin. *Journal of Endocrinology*, 244 (2), 285–296. Available from https://doi.org/10.1530/JOE-19-0288.
Molavian, H. et al. (2015). The synergetic coupling among the cellular antioxidants glutathione peroxidase/peroxiredoxin and other antioxidants and its effect on the concentration of H2O2. *Scientific Reports*, 5 (1), 1–8. Available from https://doi.org/10.1038/srep13620.
Moloudizargari, M. et al. (2017). Autophagy, its mechanisms and regulation: Implications in neurodegenerative diseases. *Ageing Research Reviews*, 40, 64–74. Available from https://doi.org/10.1016/j.arr.2017.09.005.
Monsalve, M. and Olmos, Y. (2011). The complex biology of FOXO. *Current Drug Targets*, 12 (9), 1322–1350. Available from https://doi.org/10.2174/138945011796150307.
Montagnani, M. et al. (2002). Inhibition of phosphatidylinositol 3-kinase enhances mitogenic actions of insulin in endothelial cells. *Journal of Biological Chemistry*, 277 (3), 1794–1799. Available from https://doi.org/10.1074/jbc.M103728200.
Mookerjee, S.A. et al. (2010). Mitochondrial uncoupling and lifespan. *Mechanisms of Ageing and Development*, 131 (7–8), 463–472. Available from https://doi.org/10.1016/j.mad.2010.03.010.
Moore, A.R. et al. (2021). Reliability and diagnostic performance of a new blood ketone and glucose meter in humans. *Journal of the International Society of Sports Nutrition*, 18 (1), 1–9. Available from https://doi.org/10.1186/S12970-020-00404-2/TABLES/2.
Mora, S. et al. (2021). Association of lipid, inflammatory, and metabolic biomarkers with age at onset for incident coronary heart disease in women. *JAMA Cardiology*, 6 (4), 437–447. Available from https://doi.org/10.1001/jamacardio.2020.7073.
Moreno-Santos, I. et al. (2016). Type 2 diabetes is associated with decreased PGC1α expression in epicardial adipose tissue of patients with coronary artery disease. *Journal of Translational Medicine*, 14 (1), 243. Available from https://doi.org/10.1186/s12967-016-0999-1.
Morris, B.J. et al. (2015). FOXO3: A major gene for human longevity - a mini-r eview. *Gerontology*, 61 (6), 515–525. Available from https://doi.org/10.1159/000375235.
Morrison, D.K. (2012). MAP kinase pathways. *Cold Spring Harbor Perspectives in Biology*, 4 (11). Available from https://doi.org/10.1101/cshperspect.a011254.
Moser, S.C. and van der Eerden, B.C.J. (2019). Osteocalcin—A versatile bone-derived hormone. *Frontiers in Endocrinology*, 9 (JAN), 794. Available from https://doi.org/10.3389/fendo.2018.00794.
Mulholland, B.S. et al. (2023). MCP-1 expression in breast cancer and its association with distant relapse. *Cancer Medicine*, 12 (15), 16221–16230. Available from https://doi.org/10.1002/CAM4.6284.
Müller, T.D. et al. (2019). Glucagon-like peptide 1 (GLP-1). *Molecular Metabolism*, 30, 72–

130. Available from https://doi.org/10.1016/J.MOLMET.2019.09.010.
Muoio, D.M. (2014). Metabolic inflexibility: When mitochondrial indecision leads to metabolic gridlock. *Cell*, 159 (6), 1253–1262. Available from https://doi.org/10.1016/j.cell.2014.11.034.
Nabulsi, A.A. et al. (1995). Fasting hyperinsulinemia and cardiovascular disease risk factors in nondiabetic adults: Stronger associations in lean versus obese subjects. *Metabolism*, 44 (7), 914–922. Available from https://doi.org/10.1016/0026-0495(95)90245-7.
Nadal, A., Marrero, P.F. and Haro, D. (2002). Down-regulation of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by insulin: the role of the forkhead transcription factor FKHRL1. *The Biochemical journal*, 366 (Pt 1), 289–297. Available from https://doi.org/10.1042/BJ20020598.
Nakamura, M., Imaoka, M. and Takeda, M. (2020). Interaction of bone and brain: Osteocalcin and cognition. *International Journal of Neuroscience*. Available from https://doi.org/10.1080/00207454.2020.1770247.
Nakamura, S. and Yoshimori, T. (2018). Autophagy and longevity. *Molecules and Cells*, 41 (1), 65–72. Available from https://doi.org/10.14348/molcells.2018.2333.
Nakatsuka, E. et al. (2017). Plasminogen activator inhibitor-1 is an independent prognostic factor of ovarian cancer and IMD-4482, a novel plasminogen activator inhibitor-1 inhibitor, inhibits ovarian cancer peritoneal dissemination. *Oncotarget*, 8 (52), 89887. Available from https://doi.org/10.18632/ONCOTARGET.20834.
Napoleão, A. et al. (2021). Effects of calorie restriction on health span and insulin resistance: classic calorie restriction diet vs. ketosis-inducing diet. *Nutrients*, 13 (4). Available from https://doi.org/10.3390/NU13041302.
Narasimhan, S.D., Yen, K. and Tissenbaum, H.A. (2009). Converging pathways in lifespan regulation. *Current Biology*, 19 (15), R657. Available from https://doi.org/10.1016/j.cub.2009.06.013.
Navale, A.M. and Paranjape, A.N. (2016). Glucose transporters: physiological and pathological roles. *Biophysical Reviews*, 8 (1), 5–9. Available from https://doi.org/10.1007/S12551-015-0186-2/TABLES/1.
Nawaz, S.S. and Siddiqui, K. (2022). Plasminogen activator inhibitor-1 mediate downregulation of adiponectin in type 2 diabetes patients with metabolic syndrome. *Cytokine: X*, 4 (1), 100064. Available from https://doi.org/10.1016/J.CYTOX.2022.100064.
Negro, R. (2008). Selenium and thyroid autoimmunity. *Biologics : Targets & Therapy*, 2 (2), 265. Available from https://doi.org/10.2147/BTT.S2746.
Newman, J.C. and Verdin, E. (2014a). Ketone bodies as signaling metabolites. *Trends in Endocrinology and Metabolism*, 25 (1), 42–52. Available from https://doi.org/10.1016/j.tem.2013.09.002.
Newman, J.C. and Verdin, E. (2014b). β-hydroxybutyrate: Much more than a metabolite. *Diabetes Research and Clinical Practice*, 106 (2), 173–181. Available from https://doi.org/10.1016/j.diabres.2014.08.009.
Newman, J.C. and Verdin, E. (2017). β-hydroxybutyrate: A signaling metabolite. *Annual Review of Nutrition*, 37 (1), 51–76. Available from https://doi.org/10.1146/annurev-nutr-071816-064916.
Nio, Y. et al. (2012). Monocyte chemoattractant protein-1 (MCP-1) deficiency enhances alternatively activated M2 macrophages and ameliorates insulin resistance and fatty liver in lipoatrophic diabetic A-ZIP transgenic mice. *Diabetologia*, 55 (12), 3350–3358.

Available from https://doi.org/10.1007/S00125-012-2710-2/FIGURES/7.
Nishida, C., Ko, G.T. and Kumanyika, S. (2009). Body fat distribution and noncommunicable diseases in populations: overview of the 2008 WHO expert consultation on waist circumference and waist–hip ratio. *European Journal of Clinical Nutrition 2010 64:1*, 64 (1), 2–5. Available from https://doi.org/10.1038/ejcn.2009.139.
Noch, E.K. et al. (2023). Insulin feedback is a targetable resistance mechanism of PI3K inhibition in glioblastoma. *Neuro-Oncology*. Available from https://doi.org/10.1093/NEUONC/NOAD117.
Norwitz, N.G., Hu, M.T. and Clarke, K. (2019). The mechanisms by which the ketone body D-β-hydroxybutyrate may improve the multiple cellular pathologies of Parkinson's disease. *Frontiers in Nutrition*, 6, 63. Available from https://doi.org/10.3389/fnut.2019.00063.
Norwitz, N.G. et al. (2023). Animal-based ketogenic diet puts severe anorexia nervosa into multi-year remission: A case series. *Journal of Metabolic Health*, 6 (1), 8. Available from https://doi.org/10.4102/JIR.V6I1.84.
Nourshahi, M. et al. (2012). Mitochondrial biogenesis in skeletal muscle: exercise and aging. *Skeletal Muscle - From Myogenesis to Clinical Relations*. Available from https://doi.org/10.5772/47292.
O'Connor, D. et al. (2023). Sustaining an ageing population: the role of micronutrients in frailty and cognitive impairment. *Proceedings of the Nutrition Society*, 82 (3), 315–328. Available from https://doi.org/10.1017/S0029665123002707.
O'Flanagan, C.H. et al. (2017). When less may be more: Calorie restriction and response to cancer therapy. *BMC Medicine*, 15 (1). Available from https://doi.org/10.1186/s12916-017-0873-x.
O'Neill, B.T. et al. (2019). FOXO transcription factors are critical regulators of diabetes-related muscle atrophy. *Diabetes*, 68 (3), 556–570. Available from https://doi.org/10.2337/db18-0416.
Ogilvie, R.F. (1933). The islands of langerhans in 19 cases of obesity. *The Journal of Pathology and Bacteriology*, 37 (3), 473–481. Available from https://doi.org/10.1002/path.1700370314.
Oh, J. et al. (2014). Plasminogen activator inhibitor-1 as an early potential diagnostic marker for Alzheimer's disease. *Experimental Gerontology*, 60, 87–91. Available from https://doi.org/10.1016/j.exger.2014.10.004.
ONS. (2023). Death registration summary statistics, England and Wales - Office for National Statistics. *ONS Website*. Available from https://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriages/deaths/articles/deathregistrationsummarystatisticsenglandandwales/2022 [Accessed 14 September 2023].
Ostheimer, C. et al. (2018). Prognostic implications of the co-detection of the urokinase plasminogen activator system and osteopontin in patients with non-small-cell lung cancer undergoing radiotherapy and correlation with gross tumor volume. *Strahlentherapie und Onkologie*, 194 (6), 539–551. Available from https://doi.org/10.1007/s00066-017-1255-1.
Ott, B. et al. (2018). Short-term overfeeding with dairy cream does not modify gut permeability, the fecal microbiota, or glucose metabolism in young healthy men. *The Journal of Nutrition*, 148 (1), 77–85. Available from https://doi.org/10.1093/JN/NXX020.

Ottens, F., Franz, A. and Hoppe, T. (2021). Build-UPS and break-downs: metabolism impacts on proteostasis and aging. *Cell Death & Differentiation 2021 28:2*, 28 (2), 505–521. Available from https://doi.org/10.1038/s41418-020-00682-y.

Owen, O.E. et al. (1967). Brain metabolism during fasting. *The Journal of clinical investigation*, 46 (10), 1589–1595. Available from https://doi.org/10.1172/JCI105650.

Pamukcu, B., Lip, G.Y.H. and Shantsila, E. (2011). The nuclear factor - Kappa B pathway in atherosclerosis: a potential therapeutic target for atherothrombotic vascular disease. *Thrombosis Research*, 128 (2), 117–123. Available from https://doi.org/10.1016/j.thromres.2011.03.025.

Pan, H. et al. (2018). Role of plasminogen activator inhibitor-1 in the diagnosis and prognosis of patients with Parkinson's disease. *Experimental and Therapeutic Medicine*, 15 (6), 5517–5522. Available from https://doi.org/10.3892/etm.2018.6076.

Pan, K. et al. (2020). Insulin resistance and cancer-specific and all-cause mortality in postmenopausal women: The Women's Health Initiative. *JNCI: Journal of the National Cancer Institute*, 112 (2), 170–178. Available from https://doi.org/10.1093/JNCI/DJZ069.

Pandey, M., Loskutoff, D.J. and Samad, F. (2005). Molecular mechanisms of tumor necrosis factor-alpha-mediated plasminogen activator inhibitor-1 expression in adipocytes. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 19 (10), 1317–1319. Available from https://doi.org/10.1096/FJ.04-3459FJE.

Paoli, A.; et al. (2023). The effects of ketogenic diet on insulin sensitivity and weight loss, which came first: The chicken or the egg? *Nutrients 2023, Vol. 15, Page 3120*, 15 (14), 3120. Available from https://doi.org/10.3390/NU15143120.

Paoli, A. et al. (2013). Beyond weight loss: A review of the therapeutic uses of very-low-carbohydrate (ketogenic) diets. *European Journal of Clinical Nutrition*, 67 (8), 789–796. Available from https://doi.org/10.1038/ejcn.2013.116.

Paradies, G. et al. (2014). Functional role of cardiolipin in mitochondrial bioenergetics. *Biochimica et Biophysica Acta - Bioenergetics*, 1837 (4), 408–417. Available from https://doi.org/10.1016/j.bbabio.2013.10.006.

Paradies, G. et al. (2019). Role of cardiolipin in mitochondrial function and dynamics in health and disease: molecular and pharmacological aspects. *Cells*, 8 (7), 728. Available from https://doi.org/10.3390/cells8070728.

Parker, B.A. et al. (2018). β-hydroxybutyrate elicits favorable mitochondrial changes in skeletal muscle. *International Journal of Molecular Sciences*, 19 (8). Available from https://doi.org/10.3390/ijms19082247.

Patsouris, D. et al. (2014). Insulin resistance is associated with MCP1-mediated macrophage accumulation in skeletal muscle in mice and humans. *PLOS ONE*, 9 (10), e110653. Available from https://doi.org/10.1371/JOURNAL.PONE.0110653.

Perkins, J.M. et al. (2015). Acute effects of hyperinsulinemia and hyperglycemia on vascular inflammatory biomarkers and endothelial function in overweight and obese humans. *American Journal of Physiology-Endocrinology and Metabolism*, 309 (2), E168–E176. Available from https://doi.org/10.1152/ajpendo.00064.2015.

Perseghin, G. et al. (2012). Insulin resistance/hyperinsulinemia and cancer mortality: the Cremona study at the 15th year of follow-up. *Acta diabetologica*, 49 (6), 421–428. Available from https://doi.org/10.1007/S00592-011-0361-2.

Petagine, L., Zariwala, M.G. and Patel, V.B. (2023). Non-alcoholic fatty liver disease:

Immunological mechanisms and current treatments. *World journal of gastroenterology*, 29 (32), 4831–4850. Available from https://doi.org/10.3748/wjg.v29.i32.4831.
PHE. (2016). *Government recommendations for energy and nutrients for males and females aged 1-18 years and 19+ years*. Available from https://doi.org/PHE publications gateway number: 2016202.
Pi, M., Nishimoto, S.K. and Quarles, L.D. (2017). GPRC6A: Jack of all metabolism (or master of none). *Molecular Metabolism*, 6 (2), 185–193. Available from https://doi.org/10.1016/j.molmet.2016.12.006.
Picca, A. et al. (2020). Mitochondrial dysfunction, oxidative stress, and neuroinflammation: Intertwined roads to neurodegeneration. *Antioxidants*, 9 (8), 1–21. Available from https://doi.org/10.3390/antiox9080647.
Pierre, K. and Pellerin, L. (2005). Monocarboxylate transporters in the central nervous system: distribution, regulation and function. *Journal of Neurochemistry*, 94 (1), 1–14. Available from https://doi.org/10.1111/j.1471-4159.2005.03168.x.
Pignatti, C. et al. (2020). Nutrients and pathways that regulate health span and life span. *Geriatrics 2020, Vol. 5, Page 95*, 5 (4), 95. Available from https://doi.org/10.3390/GERIATRICS5040095.
Pinto, A. et al. (2018). Anti-oxidant and anti-inflammatory activity of ketogenic diet: new perspectives for neuroprotection in Alzheimer's disease. *Antioxidants*, 7 (5), 63. Available from https://doi.org/10.3390/antiox7050063.
Pizzino, G. et al. (2017). Oxidative stress: Harms and benefits for human health. *Oxidative Medicine and Cellular Longevity*, 2017. Available from https://doi.org/10.1155/2017/8416763.
Ploumi, C., Daskalaki, I. and Tavernarakis, N. (2016). Mitochondrial biogenesis and clearance: a balancing act. *Federation of European Biochemical Societies Journal*, (284), 183–195. Available from https://doi.org/10.1111/febs.13820.
Pluta, W., Dudzińska, W. and Lubkowska, A. (2022). Metabolic obesity in people with normal body weight (MONW)—review of diagnostic criteria. *International Journal of Environmental Research and Public Health*, 19 (2), 624. Available from https://doi.org/10.3390/IJERPH19020624.
Poff, A. et al. (2019). Targeting the Warburg effect for cancer treatment: Ketogenic diets for management of glioma. *Seminars in Cancer Biology*, 56, 135–148. Available from https://doi.org/10.1016/j.semcancer.2017.12.011.
Pointer, S.D. et al. (2016). Dietary carbohydrate intake, insulin resistance and gastro-oesophageal reflux disease: a pilot study in European- and African-American obese women. *Alimentary Pharmacology & Therapeutics*, 44 (9), 976–988. Available from https://doi.org/10.1111/APT.13784.
Pollak, M. (2008). Insulin and insulin-like growth factor signalling in neoplasia. *Nature Reviews Cancer*, 8 (12), 915–928. Available from https://doi.org/10.1038/nrc2536.
Popko, J. et al. (2018). Decreased levels of circulating carboxylated osteocalcin in children with low energy fractures: A pilot study. *Nutrients*, 10 (6). Available from https://doi.org/10.3390/nu10060734.
Popoviciu, M.S. et al. (2023). Emerging role of GLP-1 agonists in obesity: A comprehensive review of randomised controlled trials. *International Journal of Molecular Sciences*, 24 (13). Available from https://doi.org/10.3390/IJMS241310449.
Powell, E.S., Smith-Taillie, L.P. and Popkin, B.M. (2016). Added sugars intake across the

distribution of US children and adult consumers: 1977–2012. *Journal of the Academy of Nutrition and Dietetics*, 116 (10), 1543. Available from https://doi.org/10.1016/J.JAND.2016.06.003.

Puchalska, P. and Crawford, P.A. (2017). Multi-dimensional roles of ketone bodies in fuel metabolism, signaling, and therapeutics. *Cell Metabolism*, 25 (2), 262–284. Available from https://doi.org/10.1016/j.cmet.2016.12.022.

Puig, J. et al. (2016). Lower serum osteocalcin concentrations are associated with brain microstructural changes and worse cognitive performance. *Clinical Endocrinology*, 84 (5), 756–763. Available from https://doi.org/10.1111/CEN.12954.

Qiu, W.Q. and Folstein, M.F. (2006). Insulin, insulin-degrading enzyme and amyloid-β peptide in Alzheimer's disease: Review and hypothesis. *Neurobiology of Aging*, 27 (2), 190–198. Available from https://doi.org/10.1016/j.neurobiolaging.2005.01.004.

Qiu, X. et al. (2010). Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. *Cell Metabolism*, 12 (6), 662–667. Available from https://doi.org/10.1016/j.cmet.2010.11.015.

Qu, C. et al. (2021). The ketogenic diet as a therapeutic intervention strategy in mitochondrial disease. *The International Journal of Biochemistry & Cell Biology*, 138, 106050. Available from https://doi.org/10.1016/J.BIOCEL.2021.106050.

Quoc, Q.L. et al. (2023). Contribution of monocyte and macrophage extracellular traps to neutrophilic airway inflammation in severe asthma. *Allergology International*. Available from https://doi.org/https://doi.org/10.1016/j.alit.2023.06.004.

Rabinovitch, R.C. et al. (2017). AMPK maintains cellular metabolic homeostasis through regulation of mitochondrial reactive oxygen species. *Cell Reports*, 21 (1), 1–9. Available from https://doi.org/10.1016/j.celrep.2017.09.026.

Rahman, M. et al. (2014). The b-hydroxybutyrate receptor HCA 2 activates a neuroprotective subset of macrophages. *Nature Communications*, 5 (1), 1–11. Available from https://doi.org/10.1038/ncomms4944.

Rahmani, J. et al. (2022). Association between IGF-1 levels ranges and all-cause mortality: A meta-analysis. *Aging Cell*, 21 (2). Available from https://doi.org/10.1111/ACEL.13540.

Ramirez, A. et al. (2015). Elevated HbA1c is associated with increased risk of incident dementia in primary care patients. *Journal of Alzheimer's disease : JAD*, 44 (4), 1203–1212. Available from https://doi.org/10.3233/JAD-141521.

Ramos-Jiménez, A. et al. (2008). The respiratory exchange ratio is associated with fitness indicators both in trained and untrained men: A possible application for people with reduced exercise tolerance. *Clinical medicine. Circulatory, respiratory and pulmonary medicine*, 2. Available from https://doi.org/https://doi.org/10.4137/CCRPM.S449.

Ramracheya, R. et al. (2018). GLP-1 suppresses glucagon secretion in human pancreatic alpha-cells by inhibition of P/Q-type Ca 2+ channels. *Physiological Reports*, 6 (17), e13852. Available from https://doi.org/10.14814/phy2.13852.

Rannem, T. et al. (1998). Selenium depletion in patients with gastrointestinal diseases: Are there any predictive factors? *Scandinavian Journal of Gastroenterology*, 33 (10), 1057–1061. Available from https://doi.org/10.1080/003655298750026750.

Razny, U. et al. (2017). Carboxylated and undercarboxylated osteocalcin in metabolic complications of human obesity and prediabetes. *Diabetes/Metabolism Research and Reviews*, 33 (3). Available from https://doi.org/10.1002/dmrr.2862.

Reddy, P.H. et al. (2011). Dynamin-related protein 1 and mitochondrial fragmentation in neurodegenerative diseases. *Brain Research Reviews*, 67 (1–2), 103–118. Available

from https://doi.org/10.1016/j.brainresrev.2010.11.004.
Reilly, M.P. et al. (2004). Plasma leptin levels are associated with coronary atherosclerosis in type 2 diabetes. *The Journal of Clinical Endocrinology & Metabolism*, 89 (8), 3872–3878. Available from https://doi.org/10.1210/JC.2003-031676.
Reyes-Reveles, J. et al. (2017). Phagocytosis-dependent ketogenesis in retinal pigment epithelium. *Journal of Biological Chemistry*, 292 (19), 8038–8047. Available from https://doi.org/10.1074/jbc.M116.770784.
Reynolds, R.M. et al. (2010). Elevated fasting plasma cortisol is associated with ischaemic heart disease and its risk factors in people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. *The Journal of clinical endocrinology and metabolism*, 95 (4), 1602. Available from https://doi.org/10.1210/JC.2009-2112.
Ricciarelli, R. and Fedele, E. (2017). The amyloid cascade hypothesis in Alzheimer's disease: It's time to change our mind. *Current Neuropharmacology*, 15 (6), 926. Available from https://doi.org/10.2174/1570159x15666170116143743.
Riquelme-Gallego, B. et al. (2020). Circulating undercarboxylated osteocalcin as estimator of cardiovascular and type 2 diabetes risk in metabolic syndrome patients. *Scientific Reports*, 10 (1). Available from https://doi.org/10.1038/s41598-020-58760-7.
Ristow, M. and Zarse, K. (2010). How increased oxidative stress promotes longevity and metabolic health: The concept of mitochondrial hormesis (mitohormesis). *Experimental Gerontology*, 45 (6), 410–418. Available from https://doi.org/10.1016/j.exger.2010.03.014.
Robb, E.L. et al. (2018). Control of mitochondrial superoxide production by reverse electron transport at complex I. *Journal of Biological Chemistry*, 293 (25), 9869–9879. Available from https://doi.org/10.1074/jbc.RA118.003647.
Roberts, M.N. et al. (2017). A ketogenic diet extends longevity and healthspan in adult mice. *Cell Metabolism*, 26 (3), 539-546.e5. Available from https://doi.org/10.1016/j.cmet.2017.08.005.
Robinson, A.M. and Williamson, D.H. (1980). Physiological roles of ketone bodies as substrates and signals in mammalian tissues. *Physiological Reviews*, 60 (1), 143–187. Available from https://doi.org/10.1152/physrev.1980.60.1.143.
Robling, A.G. and Bonewald, L.F. (2020). The osteocyte: New insights. *Annual Review of Physiology*, 82, 485–506. Available from https://doi.org/10.1146/annurev-physiol-021119-034332.
Rojas-Morales, P., Tapia, E. and Pedraza-Chaverri, J. (2016). β-hydroxybutyrate: A signaling metabolite in starvation response? *Cellular Signalling*, 28 (8), 917–923. Available from https://doi.org/10.1016/j.cellsig.2016.04.005.
Rollo, F. et al. (2002). Ötzi's last meals: DNA analysis of the intestinal content of the neolithic glacier mummy from the Alps. *Proceedings of the National Academy of Sciences of the United States of America*, 99 (20), 12594–12599. Available from https://doi.org/10.1073/PNAS.192184599/SUPPL_FILE/1845FIG7.PDF.
Roy, A. et al. (2015). Glioma-derived plasminogen activator inhibitor-1 (PAI-1) regulates the recruitment of LRP1 positive mast cells. *Oncotarget*, 6 (27), 23647–23661. Available from https://doi.org/10.18632/ONCOTARGET.4640.
Ruderman, N. et al. (1998). The metabolically obese, normal-weight individual revisited. *Diabetes*, 47 (5), 699–713. Available from https://doi.org/10.2337/DIABETES.47.5.699.
Ruderman, N.B., Schneider, S.H. and Berchtold, P. (1981). The 'metabolically-obese,' normal-weight individual. *The American journal of clinical nutrition*, 34 (8), 1617–1621.

Available from https://doi.org/10.1093/AJCN/34.8.1617.
Ruderman, N.B. et al. (2013). AMPK, insulin resistance, and the metabolic syndrome. *Journal of Clinical Investigation*, 123 (7), 2764–2772. Available from https://doi.org/10.1172/JCI67227.
Rushmore, T.H., Morton, M.R. and Pickett, C.B. (1991). The antioxidant responsive element: Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *Journal of Biological Chemistry*, 266 (18), 11632–11639. Available from https://doi.org/10.1016/s0021-9258(18)99004-6.
Russell, S.J. and Kahn, C.R. (2007). Endocrine regulation of ageing. *Nature Reviews Molecular Cell Biology*, 8 (9), 681–691. Available from https://doi.org/10.1038/nrm2234.
Saiki, S. et al. (2011). Caffeine induces apoptosis by enhancement of autophagy via PI3K/Akt/mTOR/p70S6K inhibition. *Autophagy*, 7 (2), 176–187. Available from https://doi.org/10.4161/auto.7.2.14074.
Saini, M. et al. (2021). Bacterial gamma-glutamyl transpeptidase, an emerging biocatalyst: Insights into structure–function relationship and its biotechnological applications. *Frontiers in Microbiology*, 12, 641251. Available from https://doi.org/10.3389/FMICB.2021.641251/BIBTEX.
Saleem, U., Mosley, T.H. and Kullo, I.J. (2010). Serum osteocalcin is associated with measures of insulin resistance, adipokine levels, and the presence of metabolic syndrome. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 30 (7), 1474–1478. Available from https://doi.org/10.1161/ATVBAHA.110.204859.
Salvatore, T. et al. (2022). Current knowledge on the pathophysiology of lean/normal-weight type 2 diabetes. *International Journal of Molecular Sciences 2023, Vol. 24, Page 658*, 24 (1), 658. Available from https://doi.org/10.3390/IJMS24010658.
Samad, F. et al. (2000). Insulin continues to induce plasminogen activator inhibitor 1 gene expression in insulin-resistant mice and adipocytes. *Molecular medicine (Cambridge, Mass.)*, 6 (8), 680–692. Available from https://doi.org/10.1007/BF03402048/TABLES/1.
Sampson, M. et al. (2017). β-hydroxybutyrate improves β-cell mitochondrial function and survival. *Journal of Insulin Resistance*, 1 (1). Available from https://doi.org/10.4102/jir.v2i1.25.
Sandhu, M.S., Dunger, D.B. and Giovannucci, E.L. (2002). Insulin, insulin-like growth factor-I (IGF-I), IGF binding proteins, their biologic interactions, and colorectal cancer. *JNCI: Journal of the National Cancer Institute*, 94 (13), 972–980. Available from https://doi.org/10.1093/JNCI/94.13.972.
Santo, E.E. et al. (2013). FOXO3a is a major target of inactivation by PI3K/AKT signaling in aggressive neuroblastoma. *Cancer Research*, 73 (7), 2189–2198. Available from https://doi.org/10.1158/0008-5472.CAN-12-3767.
Sarkar, S. et al. (2017). Mitochondrial impairment in microglia amplifies NLRP3 inflammasome proinflammatory signaling in cell culture and animal models of Parkinson's disease. *npj Parkinson's Disease*, 3 (1). Available from https://doi.org/10.1038/s41531-017-0032-2.
Sartipy, P. and Loskutoff, D.J. (2003). Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America*, 100 (12), 7265. Available from https://doi.org/10.1073/PNAS.1133870100.
Sato, K. et al. (1995). Insulin, ketone bodies, and mitochondrial energy transduction. *The FASEB Journal*, 9 (8), 651–658. Available from

https://doi.org/10.1096/fasebj.9.8.7768357.
Savage, M.W. et al. (2010). *The management of diabetic ketoacidosis in adults*. Wells Lawrence House, 126 Back Church Lane London E1 1FH.
Sawdey, M.S. and Loskutoff, D.J. (1991). Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo. Tissue specificity and induction by lipopolysaccharide, tumor necrosis factor-alpha, and transforming growth factor-beta. *Journal of Clinical Investigation*, 88 (4), 1346. Available from https://doi.org/10.1172/JCI115440.
Saxton, R.A. and Sabatini, D.M. (2017). mTOR signaling in growth, metabolism, and disease. *Cell*, 168 (6), 960–976. Available from https://doi.org/10.1016/j.cell.2017.02.004.
Scheibye-Knudsen, M. et al. (2014). A high-fat diet and NAD+ activate sirt1 to rescue premature aging in cockayne syndrome. *Cell Metabolism*, 20 (5), 840–855. Available from https://doi.org/10.1016/j.cmet.2014.10.005.
Schiavi, A. et al. (2013). Autophagy induction extends lifespan and reduces lipid content in response to frataxin silencing in C. elegans. *Experimental Gerontology*, 48 (2), 191–201. Available from https://doi.org/10.1016/j.exger.2012.12.002.
Schieber, M. and Chandel, N.S. (2014). ROS function in redox signaling and oxidative stress. *Current Biology*, 24 (10), R453. Available from https://doi.org/10.1016/j.cub.2014.03.034.
Schild, L. et al. (2012). Composition of molecular cardiolipin species correlates with proliferation of lymphocytes. *Experimental Biology and Medicine*, 237 (4), 372–379. Available from https://doi.org/10.1258/ebm.2011.011311.
Schutz, Y. and Ravussin, E. (1980). Respiratory quotients lower than 0.70 in ketogenic diets. *The American Journal of Clinical Nutrition*, 33 (6), 1317–1319. Available from https://doi.org/10.1093/AJCN/33.6.1317.
Seal, S. V. and Turner, J.D. (2021). The 'Jekyll and Hyde' of gluconeogenesis: Early life adversity, later life stress, and metabolic disturbances. *International Journal of Molecular Sciences 2021, Vol. 22, Page 3344*, 22 (7), 3344. Available from https://doi.org/10.3390/IJMS22073344.
Sedlák, E. and Robinson, N.C. (1999). Phospholipase A2 digestion of cardiolipin bound to bovine cytochrome c oxidase alters both activity and quaternary structure. *Biochemistry*, 38 (45), 14966–14972. Available from https://doi.org/10.1021/bi9914053.
Seetho, I.W. and Wilding, J.P.H. (2014). The clinical management of diabetes mellitus. *Clinical Biochemistry: Metabolic and Clinical Aspects: Third Edition*, 305–332. Available from https://doi.org/10.1016/B978-0-7020-5140-1.00016-X.
Semenza, G.L. (2009). Regulation of cancer cell metabolism by hypoxia-inducible factor 1. *Seminars in Cancer Biology*, 19 (1), 12–16. Available from https://doi.org/10.1016/j.semcancer.2008.11.009.
Senyilmaz-Tiebe, D. et al. (2018). Dietary stearic acid regulates mitochondria in vivo in humans. *Nature Communications*, 9 (1). Available from https://doi.org/10.1038/S41467-018-05614-6.
Seyfried, T.N. (2015). Cancer as a mitochondrial metabolic disease. *Frontiers in Cell and Developmental Biology*, 3 (JUL), 43. Available from https://doi.org/10.3389/fcell.2015.00043.
Seyfried, T.N. et al. (2014). Cancer as a metabolic disease: Implications for novel therapeutics. *Carcinogenesis*, 001 (13). Available from https://doi.org/10.1093/carcin/bgt480.

Seyfried, T.N. et al. (2019). Provocative question: Should ketogenic metabolic therapy become the standard of care for glioblastoma? *Neurochemical Research*, 44 (10), 2392–2404. Available from https://doi.org/10.1007/s11064-019-02795-4.

Shah, S. et al. (2019). Ras and Rap1: A tale of two GTPases. *Seminars in Cancer Biology*, 54, 29–39. Available from https://doi.org/10.1016/j.semcancer.2018.03.005.

Shao, D. and Tian, R. (2015). Glucose transporters in cardiac metabolism and hypertrophy. *Comprehensive Physiology*, 6 (1), 331. Available from https://doi.org/10.1002/CPHY.C150016.

Shen, S. et al. (2017). Waist-to-height ratio is an effective indicator for comprehensive cardiovascular health. *Scientific Reports 2017 7:1*, 7 (1), 1–7. Available from https://doi.org/10.1038/srep43046.

Sherwani, S.I. et al. (2016). Significance of HbA1c test in diagnosis and prognosis of diabetic patients. *Biomarker Insights*, 11, 95. Available from https://doi.org/10.4137/BMI.S38440.

Shih, Y.L. et al. (2022). Relationship between leptin and insulin resistance among community—dwelling middle-aged and elderly populations in Taiwan. *Journal of Clinical Medicine 2022, Vol. 11, Page 5357*, 11 (18), 5357. Available from https://doi.org/10.3390/JCM11185357.

Shimazu, T. et al. (2013). Suppression of oxidative stress by β-hydroxybutyrate, an endogenous histone deacetylase inhibitor. *Science*, 339 (6116), 211–214. Available from https://doi.org/10.1126/science.1227166.

Short, K.R. et al. (2001). T3 increases mitochondrial ATP production in oxidative muscle despite increased expression of UCP2 and -3. *American Journal of Physiology - Endocrinology and Metabolism*, 280 (5 43-5). Available from https://doi.org/10.1152/AJPENDO.2001.280.5.E761/ASSET/IMAGES/LARGE/H10510354006.JPEG.

Shukla, S.K. et al. (2014). Metabolic reprogramming induced by ketone bodies diminishes pancreatic cancer cachexia. *Cancer & Metabolism 2014 2:1*, 2 (1), 1–19. Available from https://doi.org/10.1186/2049-3002-2-18.

Siegel, R.L., Miller, K.D. and Jemal, A. (2020). Cancer statistics, 2020. *CA: A Cancer Journal for Clinicians*, 70 (1), 7–30. Available from https://doi.org/10.3322/caac.21590.

Sillen, M. and Declerck, P.J. (2021). A narrative review on plasminogen activator inhibitor-1 and Its (patho)physiological role: To target or not to target? *International Journal of Molecular Sciences 2021, Vol. 22, Page 2721*, 22 (5), 2721. Available from https://doi.org/10.3390/IJMS22052721.

Singh, S., Anshita, D. and Ravichandiran, V. (2021). MCP-1: Function, regulation, and involvement in disease. *International Immunopharmacology*, 101, 107598. Available from https://doi.org/10.1016/J.INTIMP.2021.107598.

Skow, S.L. and Jha, R.K. (2022). A ketogenic diet is effective in improving insulin sensitivity in individuals with type 2 diabetes. *Current Diabetes Reviews*, 19 (6). Available from https://doi.org/10.2174/1573399818666220425093535.

Skurk, C. et al. (2005). The FOXO3a transcription factor regulates cardiac myocyte size downstream of AKT signaling. *Journal of Biological Chemistry*, 280 (21), 20814–20823. Available from https://doi.org/10.1074/jbc.M500528200.

Sliwinska, A. et al. (2008). In vitro effect of gliclazide on DNA damage and repair in patients with type 2 diabetes mellitus (T2DM). *Chemico-Biological Interactions*, 173 (3), 159–165. Available from https://doi.org/10.1016/j.cbi.2008.03.017.

Smith, M.E. et al. (2013). Mitochondrial fission mediates ceramide-induced metabolic disruption in skeletal muscle. *Biochemical Journal*, 456 (3), 427–439. Available from https://doi.org/10.1042/BJ20130807.
Sobocan, M. et al. (2020). The value of uPA and PAI-1 levels in triple negative breast cancer. *European Journal of Cancer*, 138, S73–S74. Available from https://doi.org/10.1016/s0959-8049(20)30724-3.
Somogyi, M. (1941). Effect of insulin upon the production of ketone bodies. *Journal of Biological Chemistry*, 141 (1), 219–227.
Song, L. (2017). Calcium and bone metabolism indices. *Advances in Clinical Chemistry*, 82, 1–46. Available from https://doi.org/10.1016/bs.acc.2017.06.005.
Sonnenschein, C. and Soto, A.M. (2016). Carcinogenesis explained within the context of a theory of organisms. *Progress in Biophysics and Molecular Biology*, 122 (1), 70–76. Available from https://doi.org/10.1016/j.pbiomolbio.2016.07.004.
Stalder, T. and Kirschbaum, C. (2020). Cortisol. *Encyclopedia of Behavioral Medicine*, 561–567. Available from https://doi.org/10.1007/978-3-030-39903-0_171.
Stegenga, M.E. et al. (2006). Hyperglycemia stimulates coagulation, whereas hyperinsulinemia impairs fibrinolysis in healthy humans. *Diabetes*, 55 (6), 1807–1812. Available from https://doi.org/10.2337/db05-1543.
Stein, L.R. and Imai, S.I. (2012). The dynamic regulation of NAD metabolism in mitochondria. *Trends in Endocrinology and Metabolism*, 23 (9), 420–428. Available from https://doi.org/10.1016/j.tem.2012.06.005.
Steinberg, G.R. and Kemp, B.E. (2009). AMPK in health and disease. *Physiological Reviews*, 89 (3), 1025–1078. Available from https://doi.org/10.1152/physrev.00011.2008.
Steller, M.A., Delgado, C.H. and Zou, Z. (1995). Insulin-like growth factor II mediates epidermal growth factor-induced mitogenesis in cervical cancer cells. *Proceedings of the National Academy of Sciences*, 92 (26), 11970–11974. Available from https://doi.org/10.1073/PNAS.92.26.11970.
Stephens, E. et al. (2001). Fetal hyperinsulinemia increases farnesylation of p21 Ras in fetal tissues. *American Journal of Physiology - Endocrinology and Metabolism*, 281 (2 44-2). Available from https://doi.org/10.1152/ajpendo.2001.281.2.e217.
Su, B. et al. (2009). Ectopic localization of FOXO3a protein in Lewy bodies in Lewy body dementia and Parkinson's disease. *Molecular Neurodegeneration*, 4 (1), 1–5. Available from https://doi.org/10.1186/1750-1326-4-32.
Swenson, S.A. et al. (2020). From synthesis to utilization: The ins and outs of mitochondrial heme. *Cells*, 9 (3), 579. Available from https://doi.org/10.3390/cells9030579.
Tan-Shalaby, J. (2017). Ketogenic diets and cancer: Emerging evidence. *Federal Practitioner*, 34 (Suppl 1), 37S.
Tan, Z. et al. (2016). The role of PGC1α in cancer metabolism and its therapeutic implications. *Molecular Cancer Therapeutics*, 15 (5), 774–782. Available from https://doi.org/10.1158/1535-7163.MCT-15-0621.
Tate, S.S. and Meister, A. (1981). γ-Glutamyl transpeptidase: catalytic, structural and functional aspects. *The Biological Effects of Glutamic Acid and Its Derivatives*, 357–368. Available from https://doi.org/10.1007/978-94-009-8027-3_23.
Taylor, M.K., Swerdlow, R.H. and Sullivan, D.K. (2019). Dietary neuroketotherapeutics for Alzheimer's disease: An evidence update and the potential role for diet quality. *Nutrients*, 11 (8). Available from https://doi.org/10.3390/nu11081910.
Thakur, C. and Chen, F. (2019). Connections between metabolism and epigenetics in

cancers. *Seminars in Cancer Biology*, 57, 52–58. Available from https://doi.org/10.1016/j.semcancer.2019.06.006.

The Emerging Risk Factors Collaboration. (2011). Diabetes mellitus, fasting glucose, and risk of cause-specific death. *New England Journal of Medicine*, 364 (9), 829–841. Available from https://doi.org/10.1056/NEJMOA1008862/SUPPL_FILE/NEJMOA1008862_DISCLOSURES.PDF.

Thomas, & C.R. (no date). Genotype, obesity and cardiovascular disease-has technical and social advancement outstripped evolution? Available from https://doi.org/10.1046/j.1365-2796.2003.01170.x.

Tieu, K. et al. (2003). D-β-Hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease. *Journal of Clinical Investigation*, 112 (6), 892–901. Available from https://doi.org/10.1172/jci18797.

Toker, A. and Cantley, L.C. (1997). Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature*, 387 (6634), 673–676. Available from https://doi.org/10.1038/42648.

Tsatsoulis, A. (2018). The role of insulin resistance/hyperinsulinism on the rising Trend of Thyroid and adrenal nodular disease in the current environment. *Journal of Clinical Medicine 2018, Vol. 7, Page 37*, 7 (3), 37. Available from https://doi.org/10.3390/JCM7030037.

Tschopp, J. (2011). Mitochondria: sovereign of inflammation? *European Journal of Immunology*, 41 (5), 1196–1202. Available from https://doi.org/10.1002/eji.201141436.

Tsuchida, A. et al. (2004). Insulin/Foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity. *Journal of Biological Chemistry*, 279 (29), 30817–30822. Available from https://doi.org/10.1074/jbc.M402367200.

Tsujimoto, T., Kajio, H. and Sugiyama, T. (2017). Association between hyperinsulinemia and increased risk of cancer death in nonobese and obese people: A population-based observational study. *International Journal of Cancer*, 141 (1), 102–111. Available from https://doi.org/10.1002/ijc.30729.

Tu, K.N. et al. (2018). Osteoporosis: A review of treatment options. *P & T : a peer-reviewed journal for formulary management*, 43 (2), 92–104.

Tyson, J. et al. (2020). Mechanisms of the osteogenic switch of smooth muscle cells in vascular calcification: WNT signaling, BMPs, mechanotransduction, and EndMT. *Bioengineering 2020, Vol. 7, Page 88*, 7 (3), 88. Available from https://doi.org/10.3390/BIOENGINEERING7030088.

UN. (2019). World population ageing 2019: Highlights. *of Economic, Department Affairs, Social Division, Population*. Available from https://www.un.org/en/development/desa/population/publications/pdf/ageing/WorldPopulationAgeing2019-Report.pdf [Accessed 13 September 2023].

Unwin, D. et al. (2021). Renal function in patients following a low carbohydrate diet for type 2 diabetes: a review of the literature and analysis of routine clinical data from a primary care service over 7 years. *Current Opinion in Endocrinology, Diabetes and Obesity*, 28 (5), 469–479. Available from https://doi.org/10.1097/MED.0000000000000658.

Unwin, D.J. et al. (2015). A pilot study to explore the role of a low-carbohydrate intervention to improve GGT levels and HbA 1c. *Diabesity in Practice*, 4.

van de Ven, R.A.H., Santos, D. and Haigis, M.C. (2017). Mitochondrial sirtuins and molecular mechanisms of aging. *Trends in Molecular Medicine*, 23 (4), 320–331. Available from https://doi.org/10.1016/j.molmed.2017.02.005.
Vassilopoulos, A. et al. (2011). The human sirtuin family: evolutionary divergences and functions. *Human Genomics*, 5 (5), 485–496. Available from https://doi.org/10.1186/1479-7364-5-5-485.
Vaughan, D.E. et al. (2017). PAI-1 is a marker and a mediator of senescence. *Arteriosclerosis, thrombosis, and vascular biology*, 37 (8), 1446. Available from https://doi.org/10.1161/ATVBAHA.117.309451.
Veech, R.L. (2004). The therapeutic implications of ketone bodies: The effects of ketone bodies in pathological conditions: Ketosis, ketogenic diet, redox states, insulin resistance, and mitochondrial metabolism. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 70 (3), 309–319. Available from https://doi.org/10.1016/j.plefa.2003.09.007.
Veech, R.L. et al. (2017). Ketone bodies mimic the life span extending properties of caloric restriction. *IUBMB Life*, 69 (5), 305–314. Available from https://doi.org/10.1002/iub.1627.
Verdin, E. (2015). NAD+ in aging, metabolism, and neurodegeneration. *Science*, 350 (6265), 1208–1213. Available from https://doi.org/10.1126/science.aac4854.
Versele, R. et al. (2020). Ketone bodies promote amyloid-β1–40 clearance in a human in vitro blood–brain barrier model. *International Journal of Molecular Sciences*, 21 (3). Available from https://doi.org/10.3390/ijms21030934.
Veyrat-Durebex, C. et al. (2018). How can a ketogenic diet improve motor function? *Frontiers in Molecular Neuroscience*, 11. Available from https://doi.org/10.3389/FNMOL.2018.00015.
Visscher, T.L.S. and Seidell, J.C. (2003). The public health impact of obesity. *https://doi.org/10.1146/annurev.publhealth.22.1.355*, 22, 355–375. Available from https://doi.org/10.1146/ANNUREV.PUBLHEALTH.22.1.355.
Vögtle, T. et al. (2019). Heparan sulfates are critical regulators of the inhibitory megakaryocyte-platelet receptor G6B-B. *eLife*, 8. Available from https://doi.org/10.7554/ELIFE.46840.
Volek, J.S. et al. (2015). Metabolic characteristics of keto-adapted ultra-endurance runners. *Metabolism: Clinical and Experimental*, 65 (3), 100–110. Available from https://doi.org/10.1016/j.metabol.2015.10.028.
Volpe, C.M.O. et al. (2018). Cellular death, reactive oxygen species (ROS) and diabetic complications review-Article. *Cell Death and Disease*, 9 (2), 1–9. Available from https://doi.org/10.1038/s41419-017-0135-z.
Vowels, J.J. and Thomas, J.H. (1992). Genetic analysis of chemosensory control of dauer formation in Caenorhabditis elegans. *Genetics*, 130 (1), 105–123. Available from https://doi.org/10.1093/genetics/130.1.105.
Wallace, D.C. (2005). A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: A dawn for evolutionary medicine. *Annual Review of Genetics*, 39, 359–407. Available from https://doi.org/10.1146/annurev.genet.39.110304.095751.
Wang, J.S., Mazur, C.M. and Wein, M.N. (2021). Sclerostin and osteocalcin: Candidate bone-produced hormones. *Frontiers in Endocrinology*, 12, 584147. Available from https://doi.org/10.3389/FENDO.2021.584147/BIBTEX.
Wang, L., Chen, P. and Xiao, W. (2021). β-hydroxybutyrate as an anti-aging metabolite.

Nutrients, 13 (10). Available from https://doi.org/10.3390/NU13103420.
Wang, L. et al. (2022). MCP-1 targeting: Shutting off an engine for tumor development. *Oncology Letters*, 23 (1). Available from https://doi.org/10.3892/OL.2021.13144.
Wang, P. et al. (2015). Micro-RNA-155 is induced by K-Ras oncogenic signal and promotes ROS stress in pancreatic cancer. *Oncotarget*, 6 (25), 21148–21158. Available from https://doi.org/10.18632/oncotarget.4125.
Wang, W. et al. (2020). Mitochondria dysfunction in the pathogenesis of Alzheimer's disease: Recent advances. *Molecular Neurodegeneration*, 15 (1), 1–22. Available from https://doi.org/10.1186/s13024-020-00376-6.
Wang, X. et al. (2009). Impaired balance of mitochondrial fission and fusion in Alzheimer's disease. *Journal of Neuroscience*, 29 (28), 9090–9103. Available from https://doi.org/10.1523/JNEUROSCI.1357-09.2009.
Wang, Yun et al. (2021). Relationship between serum gamma-glutamyl transferase level and impaired fasting glucose among chinese community-dwelling adults: A follow-up observation of 6 years. *Metabolic syndrome and related disorders*, 19 (2), 100–106. Available from https://doi.org/10.1089/MET.2020.0032.
Wang, Z., Du, Z. and Zhu, F. (2020). Glycosylated hemoglobin is associated with systemic inflammation, hypercoagulability, and prognosis of COVID-19 patients. *Diabetes Research and Clinical Practice*, 164, 108214. Available from https://doi.org/10.1016/j.diabres.2020.108214.
Webb, A.E. and Brunet, A. (2014). FOXO transcription factors: key regulators of cellular quality control. *Trends in biochemical sciences*, 39 (4), 159. Available from https://doi.org/10.1016/J.TIBS.2014.02.003.
Webb, A.E., Kundaje, A. and Brunet, A. (2016). Characterization of the direct targets of FOXO transcription factors throughout evolution. *Aging Cell*, 15 (4), 673–685. Available from https://doi.org/10.1111/acel.12479.
Weber, D.D. et al. (2020). Ketogenic diet in the treatment of cancer – Where do we stand? *Molecular Metabolism*, 33, 102. Available from https://doi.org/10.1016/J.MOLMET.2019.06.026.
Wei, J. and Karsenty, G. (2015). An overview of the metabolic functions of osteocalcin. *Reviews in Endocrine and Metabolic Disorders*, 16 (2), 93–98. Available from https://doi.org/10.1007/s11154-014-9307-7.
Wei, S. et al. (2022). β-Hydroxybutyrate in cardiovascular diseases : A minor metabolite of great expectations. *Frontiers in Molecular Biosciences*, 9. Available from https://doi.org/10.3389/FMOLB.2022.823602.
Weindruch, R. and Walford, R.L. (1982). Dietary restriction in mice beginning at 1 year of age: Effect on life-span and spontaneous cancer incidence. *Science*, 215 (4538), 1415–1418. Available from https://doi.org/10.1126/science.7063854.
Westerbacka, J. et al. (2008). Insulin regulation of MCP-1 in human adipose tissue of obese and lean women. *American Journal of Physiology - Endocrinology and Metabolism*, 294 (5), 841–845. Available from https://doi.org/10.1152/AJPENDO.00653.2006/ASSET/IMAGES/LARGE/ZH1004085284 0003.JPEG.
White, M.C. et al. (2014). Age and cancer risk: A potentially modifiable relationship. *American Journal of Preventive Medicine*, 46 (3 SUPPL. 1). Available from https://doi.org/10.1016/j.amepre.2013.10.029.
WHO. (2011). Waist Circumference and Waist–Hip Ratio. *WHO Expert*, 64 (1), 2–5.

WHO. (2019). Global health estimates: Leading causes of death. Available from https://www.who.int/data/gho/data/themes/mortality-and-global-health-estimates/ghe-leading-causes-of-death [Accessed 13 September 2023].
WHO. (2023). Breast cancer. *World Health Organisation*. Available from https://www.who.int/news-room/fact-sheets/detail/breast-cancer#:~:text=In 2020%2C there were 2.3,the world%27s most prevalent cancer. [Accessed 19 September 2023].
Wiebe, N., Muntner, P. and Tonelli, M. (2022). Associations of body mass index, fasting insulin, and inflammation with mortality: a prospective cohort study. *International Journal of Obesity 2022 46:12*, 46 (12), 2107–2113. Available from https://doi.org/10.1038/s41366-022-01211-2.
Wilcox, G. (2005). Insulin and insulin resistance. *The Clinical biochemist. Reviews*, 26 (May), 19–39. Available from https://doi.org/10.1016/S0025-7125(03)00128-7.
Willcox, B.J. et al. (2008). FOXO3A genotype is strongly associated with human longevity. *Proceedings of the National Academy of Sciences of the United States of America*, 105 (37), 13987–13992. Available from https://doi.org/10.1073/pnas.0801030105.
Wolfrum, C. et al. (2003). Insulin regulates the activity of forkhead transcription factor Hnf-3β/Foxa-2 by Akt-mediated phosphorylation and nuclear/cytosolic localization. *Proceedings of the National Academy of Sciences of the United States of America*, 100 (20), 11624–11629. Available from https://doi.org/10.1073/pnas.1931483100.
Wright, E., Scism-Bacon, J.L. and Glass, L.C. (2006). Oxidative stress in type 2 diabetes: The role of fasting and postprandial glycaemia. *International Journal of Clinical Practice*, 60 (3), 308–314. Available from https://doi.org/10.1111/j.1368-5031.2006.00825.x.
Wu, K.L. et al. (2018). The effect of dietary carbohydrate on gastroesophageal reflux disease. *Journal of the Formosan Medical Association*, 117 (11), 973–978. Available from https://doi.org/10.1016/J.JFMA.2017.11.001.
Wu, S. and Zou, M.H. (2020). AMPK, mitochondrial function, and cardiovascular disease. *International Journal of Molecular Sciences*, 21 (14), 1–34. Available from https://doi.org/10.3390/ijms21144987.
Xing, M. et al. (2022). Characteristics of peripheral blood Gamma-glutamyl transferase in different liver diseases. *Medicine*, 101 (1), E28443. Available from https://doi.org/10.1097/MD.0000000000028443.
Yaktapour, N. et al. (2013). Insulin-like growth factor-1 receptor (IGF1R) as a novel target in chronic lymphocytic leukemia key points. Available from https://doi.org/10.1182/blood-2013-02.
Yamasaki, T. et al. (2018). The changing epidemiology of gastroesophageal reflux disease: Are patients getting younger? *Journal of Neurogastroenterology and Motility*, 24 (4), 559. Available from https://doi.org/10.5056/JNM18140.
Yan, Y.X. et al. (2016). Investigation of the relationship between chronic stress and Insulin resistance in a chinese population. *Journal of Epidemiology*, 26 (7), 355–360. Available from https://doi.org/10.2188/JEA.JE20150183.
Yang, H. et al. (2019). Ketone bodies in neurological diseases: Focus on neuroprotection and underlying mechanisms. *Frontiers in Neurology*, 10 (JUN), 585. Available from https://doi.org/10.3389/fneur.2019.00585.
Yang, H. et al. (2023). HOMA-IR is positively correlated with biological age and advanced aging in the US adult population. *European Journal of Medical Research*, 28 (1), 1–14. Available from https://doi.org/10.1186/S40001-023-01448-1/TABLES/8.

Yang, Y. and Cao, Y. (2022). The impact of VEGF on cancer metastasis and systemic disease. *Seminars in Cancer Biology*, 86, 251–261. Available from https://doi.org/10.1016/J.SEMCANCER.2022.03.011.

Yarmolinsky, J. et al. (2016). Plasminogen activator inhibitor-1 and type 2 diabetes: a systematic review and meta-analysis of observational studies. *Scientific Reports 2016 6:1*, 6 (1), 1–13. Available from https://doi.org/10.1038/srep17714.

Yaylali, Y.T. et al. (2019). Osteocalcin and epicardial adipose tissue in obesity: new hints for epicardial adipose tissue–bone crosstalk. *Scandinavian Cardiovascular Journal*, 53 (6), 296–298. Available from https://doi.org/10.1080/14017431.2019.1659397.

Yore, M.M. et al. (2014). Discovery of a class of endogenous mammalian lipids with anti-diabetic and anti-inflammatory effects. *Cell*, 159 (2), 318–332. Available from https://doi.org/10.1016/j.cell.2014.09.035.

Younossi, Z.M. et al. (2021). From NAFLD to MAFLD: Implications of a Premature Change in Terminology. *Hepatology*, 73 (3), 1194–1198. Available from https://doi.org/10.1002/HEP.31420.

Yu, T., Robotham, J.L. and Yoon, Y. (2006). Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. *Proceedings of the National Academy of Sciences of the United States of America*, 103 (8), 2653–2658. Available from https://doi.org/10.1073/pnas.0511154103.

Yu, Yongsheng et al. (2018). Treatment with D-β-hydroxybutyrate protects heart from ischemia/reperfusion injury in mice. *European Journal of Pharmacology*, 829, 121–128. Available from https://doi.org/10.1016/J.EJPHAR.2018.04.019.

Yuan, X. et al. (2020). Effect of the ketogenic diet on glycemic control, insulin resistance, and lipid metabolism in patients with T2DM: a systematic review and meta-analysis. *Nutrition & Diabetes*, 10 (1). Available from https://doi.org/10.1038/S41387-020-00142-Z.

Zdrojewicz, Z. et al. (2017). TOFI phenotype - its effect on the occurrence of diabetes. *Pediatric endocrinology, diabetes, and metabolism*, 23 (2), 96–100. Available from https://doi.org/10.18544/PEDM-23.02.0079.

Zhang, A.M.Y. et al. (2021). Hyperinsulinemia in obesity, inflammation, and cancer. *Diabetes & Metabolism Journal*, 45 (3), 285–311. Available from https://doi.org/10.4093/DMJ.2020.0250.

Zhang, W.B. et al. (2020). Insulin-like growth factor-1 and IGF binding proteins predict all-cause mortality and morbidity in older adults. *Cells 2020, Vol. 9, Page 1368*, 9 (6), 1368. Available from https://doi.org/10.3390/CELLS9061368.

Zhang, Y. et al. (2011). Elevated insulin-like growth factor 1 receptor signaling induces antiestrogen resistance through the MAPK/ERK and PI3K/Akt signaling routes. *Breast Cancer Research*, 13 (3). Available from https://doi.org/10.1186/bcr2883.

Zhang, Z. et al. (2016). The essential role of Drp1 and its regulation by S-Nitrosylation of Parkin in dopaminergic neurodegeneration: Implications for Parkinson's disease. *Antioxidants and Redox Signaling*, 25 (11), 609–622. Available from https://doi.org/10.1089/ars.2016.6634.

Zhao, W. et al. (2015). High glucose promotes gastric cancer chemoresistance in vivo and in vitro. *Molecular Medicine Reports*, 12 (1), 843–850. Available from https://doi.org/10.3892/MMR.2015.3522/HTML.

Zhou, X. et al. (2023). Study on insulin resistance and ischemic cerebrovascular disease: A bibliometric analysis via CiteSpace. *Frontiers in Public Health*, 11, 1021378. Available

from https://doi.org/10.3389/FPUBH.2023.1021378/BIBTEX.
Zhu, D. et al. (2011). The appearance and modulation of osteocyte marker expression during calcification of vascular smooth muscle cells. *PLOS ONE*, 6 (5), e19595. Available from https://doi.org/10.1371/JOURNAL.PONE.0019595.
Zhu, H. et al. (2022). Ketogenic diet for human diseases: the underlying mechanisms and potential for clinical implementations. *Signal Transduction and Targeted Therapy 2022 7:1*, 7 (1), 1–21. Available from https://doi.org/10.1038/s41392-021-00831-w.
Zubac, D.P. et al. (2010). Type 1 plasminogen activator inhibitor (PAI-1) in clear cell renal cell carcinoma (CCRCC) and its impact on angiogenesis, progression and patient survival after radical nephrectomy. *BMC Urology*, 10, 20. Available from https://doi.org/10.1186/1471-2490-10-20.
Zupkovitz, G. et al. (2006). Negative and positive regulation of gene expression by mouse histone deacetylase 1. *Molecular and Cellular Biology*, 26 (21), 7913–7928. Available from https://doi.org/10.1128/mcb.01220-06.

APPENDIX A: Figure and Table Acknowledgements, and Copyright

Figure 1 Source: Cooper, Brookler and Crofts (2020). This work is licensed under CC BY 4.0. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license. To view a copy of this license, visit (https://creativecommons.org/licenses/by/4.0/). Created with BioRender.com.

Figure 2. Created in BioRender. Cooper, I. (2025) https://BioRender.com/n40n524

Figures 3 and 4 Source: Cooper et al., (2021). This work is licensed under CC BY 4.0. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license. To view a copy of this license, visit (https://creativecommons.org/licenses/by/4.0/). Created with BioRender.com.

Figure 5 Source: Cooper, Brookler and Crofts (2021). This work is licensed under CC BY 4.0. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license. To view a copy of this license, visit (https://creativecommons.org/licenses/by/4.0/). Created with BioRender.com.

Figures 7, 8, 9, 10 11 and 12 Source: Cooper, Kyriakidou, Edwards et al., (2023). This work is licensed under CC BY 4.0. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license. To view a copy of this license, visit (https://creativecommons.org/licenses/by/4.0/).

Tables 1, 2, 4, 5, 6 and 7 Source: Cooper, Kyriakidou, Edwards et al., (2023). This work is licensed under CC BY 4.0. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license. To view a copy of this license, visit (https://creativecommons.org/licenses/by/4.0/).

Table 3 and 8 Source: Cooper, Kyriakidou, Petagine et al., (2024). This work is licensed under CC BY 4.0. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license. To view a copy of this license, visit (https://creativecommons.org/licenses/by/4.0/).

Figures 6, 13,14, 15, 16, 17 and 18 Source: Cooper, Kyriakidou, Petagine et al., (2024). This work is licensed under CC BY 4.0. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license. To view a copy of this license, visit (https://creativecommons.org/licenses/by/4.0/). Figure 6 created with BioRender.com

Table 9 Source: Adapted from Cooper, Kyriakidou, Edwards et al., (2023). This work is licensed under CC BY 4.0. This article is an open access article distributed under the terms and conditions

APPENDIX B: Published version of Cooper, Kyriakidou, Edwards et al., (2023).

https://doi.org/ 10.3390/ijms242115621

International Journal of
Molecular Sciences

MDPI

Article

Ketosis Suppression and Ageing (KetoSAge): The Effects of Suppressing Ketosis in Long Term Keto-Adapted Non-Athletic Females

Isabella D. Cooper [1,*], Yvoni Kyriakidou [1], Kurtis Edwards [2], Lucy Petagine [1], Thomas N. Seyfried [3], Tomas Duraj [3], Adrian Soto-Mota [4,5], Andrew Scarborough [1], Sandra L. Jacome [1], Kenneth Brookler [6], Valentina Borgognoni [1], Vanusa Novaes [1], Rima Al-Faour [1] and Bradley T. Elliott [1]

1 Ageing Biology and Age-Related Diseases, School of Life Sciences, University of Westminster, 115 New Cavendish Street, London W1W 6UW, UK; y.kyriakidou@westminster.ac.uk (Y.K.); l.petagine@westminster.ac.uk (L.P.); andrewscarborough.research@gmail.com (A.S.); jacomenutrition@gmail.com (S.L.J.); valentina.borgognoni@gmail.com (V.B.); info@vanusanovaes.com (V.N.); rima.alfaour@gmail.com (R.A.-F.); b.elliott@westminster.ac.uk (B.T.E.)
2 Cancer Biomarkers and Mechanisms Group, School of Life Sciences, University of Westminster, London W1W 6UW, UK; k.edwards2@westminster.ac.uk
3 Biology Department, Boston College, Chestnut Hill, MA 02467, USA; thomas.seyfried@bc.edu (T.N.S.); durajto@gmail.com (T.D.)
4 Metabolic Diseases Research Unit, National Institute of Medical Sciences and Nutrition Salvador Zubiran, Mexico City 14080, Mexico; adrian.sotom@incmnsz.mx
5 Tecnologico de Monterrey, School of Medicine, Mexico City 14380, Mexico
6 Retired former Research Collaborator, Aerospace Medicine and Vestibular Research Laboratory, Mayo Clinic, Scottsdale, AZ 85259, USA; kennethhbrookler@mac.com
* Correspondence: isabella@bellamitochondria.com or i.cooper@westminster.ac.uk

Citation: Cooper, I.D.; Kyriakidou, Y.; Edwards, K.; Petagine, L.; Seyfried, T.N.; Duraj, T.; Soto-Mota, A.; Scarborough, A.; Jacome, S.L.; Brookler, K.; et al. Ketosis Suppression and Ageing (KetoSAge): The Effects of Suppressing Ketosis in Long Term Keto-Adapted Non-Athletic Females. *Int. J. Mol. Sci.* **2023**, *24*, 15621. https://doi.org/10.3390/ijms242115621

Academic Editors: HyeMee Kim and Byungyong Ahn

Received: 25 September 2023
Revised: 16 October 2023
Accepted: 23 October 2023
Published: 26 October 2023

Abstract: Most studies on ketosis have focused on short-term effects, male athletes, or weight loss. Hereby, we studied the effects of short-term ketosis suppression in healthy women on long-standing ketosis. Ten lean (BMI 20.5 ± 1.4), metabolically healthy, pre-menopausal women (age 32.3 ± 8.9) maintaining nutritional ketosis (NK) for > 1 year (3.9 years ± 2.3) underwent three 21-day phases: nutritional ketosis (NK; P1), suppressed ketosis (SuK; P2), and returned to NK (P3). Adherence to each phase was confirmed with daily capillary D-beta-hydroxybutyrate (BHB) tests (P1 = 1.9 ± 0.7; P2 = 0.1 ± 0.1; and P3 = 1.9 ± 0.6 pmol/L). Ageing biomarkers and anthropometrics were evaluated at the end of each phase. Ketosis suppression significantly increased: insulin, 1.78-fold from 33.60 (± 8.63) to 59.80 (± 14.69) pmol/L ($p = 0.0002$); IGF1, 1.83-fold from 149.30 (± 32.96) to 273.40 (± 85.66) µg/L ($p = 0.0045$); glucose, 1.17-fold from 78.6 (± 9.5) to 92.2 (± 10.6) mg/dL ($p = 0.0088$); respiratory quotient (RQ), 1.09-fold 0.66 (± 0.05) to 0.72 (± 0.06; $p = 0.0427$); and PAI-1, 13.34 (± 6.85) to 16.69 (± 6.26) ng/mL ($p = 0.0428$). VEGF, EGF, and monocyte chemotactic protein also significantly increased, indicating a pro-inflammatory shift. Sustained ketosis showed no adverse health effects, and may mitigate hyperinsulinemia without impairing metabolic flexibility in metabolically healthy women.

Keywords: ageing; beta-hydroxybutyrate; cancer; hyperinsulinaemia; insulin resistance; ketosis; type 2 diabetes mellitus

1. Introduction

The 21st century bears the hallmark of an ageing global population, in an estimated 8 billion people by 2023 [1]. By 2030, one in every six Europeans are expected to be aged over 60 years, and by 2040, a quarter of older adults will surpass 85 years of age [2]. This demographic shift gains paramount significance when viewed through the prism of health implications associated with ageing. In 2019 and 2022, the leading cause of death for females in England and Wales was Alzheimer's disease (AD) and other dementias [3], followed by

cardiovascular disease (CVD) and stroke, as well as cancers including: tracheae, bronchus, and lung; colon and rectum; prostate; breast; and lymphomas and multiple myeloma [4]. These diseases also top the leading causes of death in the United States, with CVD leading, closely followed by AD and cancers (WHO, 2019). Analysed data of 8721 participants from the National Health and Nutrition Examination Survey 2009–2016 showed that the proportion of metabolically healthy Americans decreased from 19.9% to 12.2%, which means 87.8% were metabolically unhealthy and on the hyperinsulinaemia spectrum [5,6]. Ageing is associated with increased risk and rates of non-communicable chronic diseases, including CVD, AD, hypertension, type 2 diabetes mellitus (T2DM), metabolic syndrome (MetS), non-alcoholic fatty liver disease (NAFLD), chronic inflammation, and cancer [6]. These conditions detrimentally affect quality of life, healthspan, and lifespan. Specifically, MetS emerges as a direct consequence of chronic hyperinsulinaemia, which is closely linked to inflammation [6–9].

Lifespan, healthspan, and cellular health are greatly influenced by nutrient availability. When the availability of nutrients is low, cells prioritise essential functions over cell division, which slows progression through their replicative cycles preserving their Hayflick limit [10]. Carbohydrate restriction (CR) and fasting have been shown to extend lifespan [11]. Cells are capable of sensing energy availability and nutrient types, activating intracellular signalling pathways to stimulate anabolic or catabolic processes which affect cellular health and longevity [12–14]. Glucose, fatty acids, D-beta-hydroxybutyrate (BHB), and protein metabolic substrates serve as indicators of the body's overall metabolic condition and nutrient availability. CR and fasting induce a metabolic phenotype called ketosis [8], characterised by decreased glucose and insulin levels and elevated BHB concentration; this state is termed nutritional ketosis (NK) when detectable plasma BHB concentration begins to exceed > 0.3 mmol/L and < 10 mmol/L, with endogenous insulin production below a level that inhibits ketogenesis [8].

Chronic insulin secretion and signalling, driven by dietary sources of glucose, leads to hyperinsulinaemia and/or insulin resistance, and consequently chronic diseases which decrease healthspan by accelerating cellular growth and division whilst impeding apoptosis and promoting production of inflammatory cytokines. Reducing insulin and insulin-like growth factor receptor signalling (IIS) as well as increasing BHB has been found to increase lifespan and healthspan in model organisms and animal studies [11,15–17]. Conversely, ketosis has been shown to increase healthspan and lifespan through mechanisms such as promoting transcription of longevity-related genes, increasing autophagy, mitophagy, and mitochondrial biogenesis, and enhancing antioxidant production [6,17–20]. Fasting mimicking diets (FMD), including ketogenic diets, upregulate beta-oxidation, ketogenesis and ketolysis, enhance mitophagy, increase mitochondrial biogenesis, and alter gene expression, promoting oxidative stress responses and cell survival [6,21–24].

Historical and emerging research demonstrates the positive impact of ketogenic metabolic therapy (KMT) in treating and preventing neurological diseases, CVD, cancer, T2DM, and chronic inflammation [25]. Insulin negatively regulates 3-hydroxy-3-methylglutaryl-COA (HMG-CoA) synthase, the rate-limiting enzyme for ketogenesis [19,26]. Dietary farinaceous and sucrose-rich foods are potent stimulators of bolus insulin secretion [8,27]. Repeated bolus glucose excursions chronically stimulate bolus insulin synthesis and release, and over time downregulate ketogenesis enzyme expression, leading to chronic hypoketonaemia [8,26,28]. There are a paucity of trials studying long-standing ketosis metabolically healthy individuals who sustain ketosis as their normal metabolic phenotype 1 lifestyle [8], and even fewer on active, yet non-athletic females. We therefore studied the effect of suppressing ketosis for 21 days in this demographic cohort. In order to suppress ketosis, participants followed the Standard U.K. (SUK) dietary guidelines, which recommend the daily consumption of at least 267 g of carbohydrate per day for women [29]. Following the intervention to suppress ketosis, participants returned to NK and were reassessed 21 days later to better understand if changes seen after suppression of ketosis for 21 days were due to the intervention, and to investigate metabolic flexibility.

2. Results

2.1. Suppression of Ketosis Increases BMI and Fat Mass

Following 21-days suppression of ketosis (SuK), phase 2 (P2), there were significant increases in both weight from baseline NK, phase 1 (P1) 52.99 kg (± 4.24) to 55.65 kg (± 4.10, P2; p = 0.0002), and BMI, from 20.52 (± 1.39, P1) to 21.54 (± 1.30, P2; $p < 0.0001$), in all participants, compared to NK; P1 (Table 1). Fat mass and TBW also increased from 14.21 kg (± 2.55, P1) to 15.88 kg (± 2.23, P2; p = 0.0008) and from 28.15 L (± 2.87, P1) to 29.15 L (± 2.96, P2; p = 0.0016), respectively (Table 1). Additionally, both waist-to-hip and waist-to-height ratios increased significantly in P2 compared to P1 (Table 1).

Table 1. Participants' characteristics. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK P3. Measurements were taken at 8 a.m. after a 12 h overnight fast; (n = 10).

	P1	P2	P3	ANOVA p Value	P1 vs. P2	P2 vs. P3	P1 vs. P3
Age (years)		32.30 (±8.97)					
Height (cm)		160.95 (±7.28)					
Weight (kg)	52.99 (±4.24)	55.65 (±4.10)	53.93 (±4.04)	<0.0001	0.0002	<0.0001	0.7888
BMI	20.52 (±1.39)	21.54 (±1.30)	20.82 (±1.46)	<0.0001	<0.0001	0.0025	0.0197
Waist/Hip	0.75 (±0.03)	0.77 (±0.03)	0.74 (±0.03)	<0.0001	0.0015	<0.0001	0.5361
Waist/Height	0.43 (±0.03)	0.45 (±0.03)	0.43 (±0.03)	<0.0001	0.0009	<0.0001	>0.9999
Fat mass (kg)	14.21 (±2.55)	15.88 (±2.23)	14.78 (±2.20)	<0.0001	0.0008	0.0057	0.1016
TBW (L)	28.15 (±2.87)	29.15 (±2.96)	28.42 (±3.15)	0.0005	0.0016	0.0262	0.3473
RQ	0.66 (±0.05)	0.72 (±0.06)	0.65 (±0.06)	0.0096	0.0427	0.0005	0.8606
Systole (mmHg)	103.25 (±6.24)	103.70 (±10.17)	100.00 (±9.54)	0.1455	0.9753	0.1746	0.2274
Diastole (mmHg)	70.75 (±4.91)	69.45 (±7.14)	68.15 (±7.36)	0.3227	0.8044	0.7147	0.1715

These trends then reversed after the removal of SuK at the end of 21-days, reverting to NK, phase 3 (P3), compared to P2. Both weight (53.93 kg ± 4.04; $p < 0.0001$, P3) and BMI (20.82 ± 1.46; p = 0.0025, P3) trended back towards baseline in all but one participant, where only a small increase in weight was observed (+0.2 kg). Concordantly, the decreases in weight between the phases were accompanied by significant decreases in fat mass (14.78 kg ± 2.20; p = 0.0057, P3) and TBW (28.42 L ± 3.15; p = 0.0026, P3). These changes were also accompanied by decreases in both waist-to-hip and waist-to-height ratios (Table 1).

RQ increased in most participants (80%) following P2 (0.66 ± 0.05, P1 to 0.72 ± 0.06, P2; p = 0.0427; Figure 1). After removal of the intervention at the end of P3, we observed a decrease in RQ in all participants, returning to their baseline (0.65 ± 0.06, P3; p = 0.0005; Figure 1). There were no changes in either systolic or diastolic blood pressure across all the study phases (Table 1).

2.2. Adherence

Based on the study protocol, participants were required to self-report 252 capillary BHB concentrations: 84 tests across each of the phases (Figure 7 in Section 6). The number of fulfilled tests and percentage of completed tests out of the possible 252 for all participants is shown in Table 2. The average percentage of successful tests was 99.37%, with four participants completing 100% of all 252 potential tests.

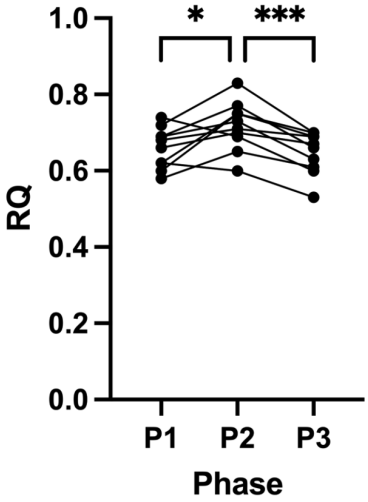

Figure 1. Respiratory quotient (RQ) measurements across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK P3; RQ was determined by indirect calorimetry. Measurements were taken at 8 a.m. after a 12 h overnight fast; (n = 10); * $p < 0.05$; *** $p < 0.001$.

Table 2. Summary of fulfilled capillary BHB testing for all study participants across all phases (P1–P3). Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK P3; (n = 10).

			Mean Capillary BHB Concentration (mmol/L)		
Participant	**No of Tests Taken**	**% of Tests Fulfilled Out of 252**	**P1**	**P2**	**P3**
1011	251	99.6	2.7	0.1	2.3
1021	252	100	2.8	0.1	2.2
1031	252	100	2.6	0.1	1.8
1041	252	100	1.5	0.2	1.6
1051	251	99.6	1.7	0	1.6
1061	245	97.22	0.7	0.1	0.8
1071	248	98.41	1.7	0.2	2.4
1081	250	99.21	2	0.1	1.2
1091	251	99.6	1.8	0.1	2.5
1101	252	100	1.5	0.1	2.4
Mean	250.4	99.37	1.9	0.1	1.9
±SD	2.15	0.85	0.7	0.1	0.6

The mean capillary BHB concentration significantly decreased from 1.9 mmol/L (± 0.7) in the baseline ketosis phase (P1) to 0.1 mmol/L (± 0.1) following the suppression of ketosis phase (P2; $p < 0.0001$). During P3, mean capillary BHB concentration increased significantly ($p < 0.0001$) and returned to baseline (1.9 ± 0.6 mmol/L). The maintenance of high mean capillary BHB concentrations (> 0.5 mmol/L) during P1 and P3 indicated that all participants adhered to the requirements to maintain ketosis during these phases. Similarly, the low levels of BHB during P2 indicated adherence to the study protocol, whereby participants effectively suppressed nutritional ketosis (Table 2).

There were variations in capillary BHB concentrations across the daily tests. The frequencies of tests which satisfied different cut-offs are summarised in Table 3. During P1 and P3, almost all reported capillary BHB concentrations were > 0.3 mmol/L or ≥ 0.5 mmol/L, which are generally considered the cut-off for ketosis or nutritional

ketosis, respectively [8,30]. There were very few tests meeting these thresholds in P2, compared to P1 and P3 (Table 3).

Table 3. Percentages of capillary BHB readings as categorised by different cut-offs across the study phases.

	Capillary BHB (mmol/L)											
	≥ 0.5			> 0.3			≤ 0.3			< 0.1		
Participant	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**	**P1**	**P2**	**P3**
1011	100.00	2.38	95.18	100.00	4.76	98.80	0.00	95.24	1.20	0.00	28.57	0.00
1021	100.00	2.38	88.10	100.00	2.38	94.05	0.00	97.62	5.95	0.00	60.71	0.00
1031	100.00	2.38	92.86	100.00	2.38	95.24	0.00	97.62	4.76	0.00	59.52	0.00
1041	98.81	0.00	100.00	100.00	8.33	100.00	0.00	91.67	0.00	0.00	13.10	0.00
1051	100.00	0.00	97.62	100.00	1.19	98.81	0.00	98.81	0.00	0.00	94.05	0.00
1061	94.05	0.00	82.93	97.62	0.00	90.24	2.38	100.00	9.76	0.00	37.97	0.00
1071	96.30	4.82	97.62	97.53	4.82	97.62	2.47	95.18	2.38	0.00	1.20	0.00
1081	96.39	0.00	90.48	98.80	1.20	95.24	1.20	98.80	4.76	0.00	33.73	0.00
1091	98.81	0.00	98.80	100.00	1.19	100.00	0.00	98.81	0.00	0.00	21.43	0.00
1101	96.43	0.00	98.81	100.00	0.00	100.00	0.00	100.00	0.00	0.00	54.76	0.00

During P1, 2/252 tests for two participants showed capillary BHB concentrations of 0.3 mmol/L, and 8/252 and 2/252 for the same two participants in P2 and P3, respectively (Table 2). There were no reported capillary BHB concentrations of < 0.1 mmol/L in either P1 or P3 (Table 3).

During P2, the majority of capillary BHB concentrations were < 0.3 mmol/L, with a significant proportion of readings < 0.1 mmol/L. There were also some instances of capillary BHB readings > 0.3 mmol/L or ≥0.5 mmol/L during P2 (Table 3). These tests were often early in the morning after an overnight fast and during the first days of SuK.

The high level of adherence to testing, coupled with the expected high concentrations of capillary BHB in P1 and P3, and low concentrations of capillary BHB in P2, indicated high levels of adherence to the protocol throughout the entire study.

2.3. Suppression of Ketosis Is Associated with Increases in Insulin, IGF-1, Glucose and T3

Following P2, fasting insulin significantly increased from 33.60 pmol/L (± 8.63, P1) to 59.80 pmol/L (± 14.69, P2; $p = 0.0002$; Figure 2A) and IGF-1 from 149.30 µg/L (± 32.96, P1) to 273.40 µg/L (± 85.66, P2; $p = 0.0045$; Figure 2B) compared to P1 (Table 4). This was accompanied by a significant increase in blood glucose from 4.36 (± 0.53) to 5.12 mmol/L (± 0.59, P2; $p = 0.0088$) (in mg/dL: 78.6 (± 9.5) to 92.3 (± 10.6)); Figure 2C) and decrease in BHB concentrations from 2.43 (± 1.28) to 0.18 mmol/L (± 0.13, P2; $p = 0.0012$); Figure 2D; Table 4). Free T3 also significantly increased from 3.81 pmol/L (± 0.28, P1) to 5.51 pmol/L (± 0.72, P2; $p = < 0.0001$; Figure 3B) following P2.

These trends reversed following P3, where we observed significant changes in the concentrations of insulin ($p < 0.0001$; Figure 2A), IGF-1 ($p = 0.0055$; Figure 2B), glucose ($p = 0.0177$; Figure 2B), BHB ($p < 0.0001$; Figure 2D), and free T3 ($p = 0.0015$; Figure 3B; Table 4), compared to P2.

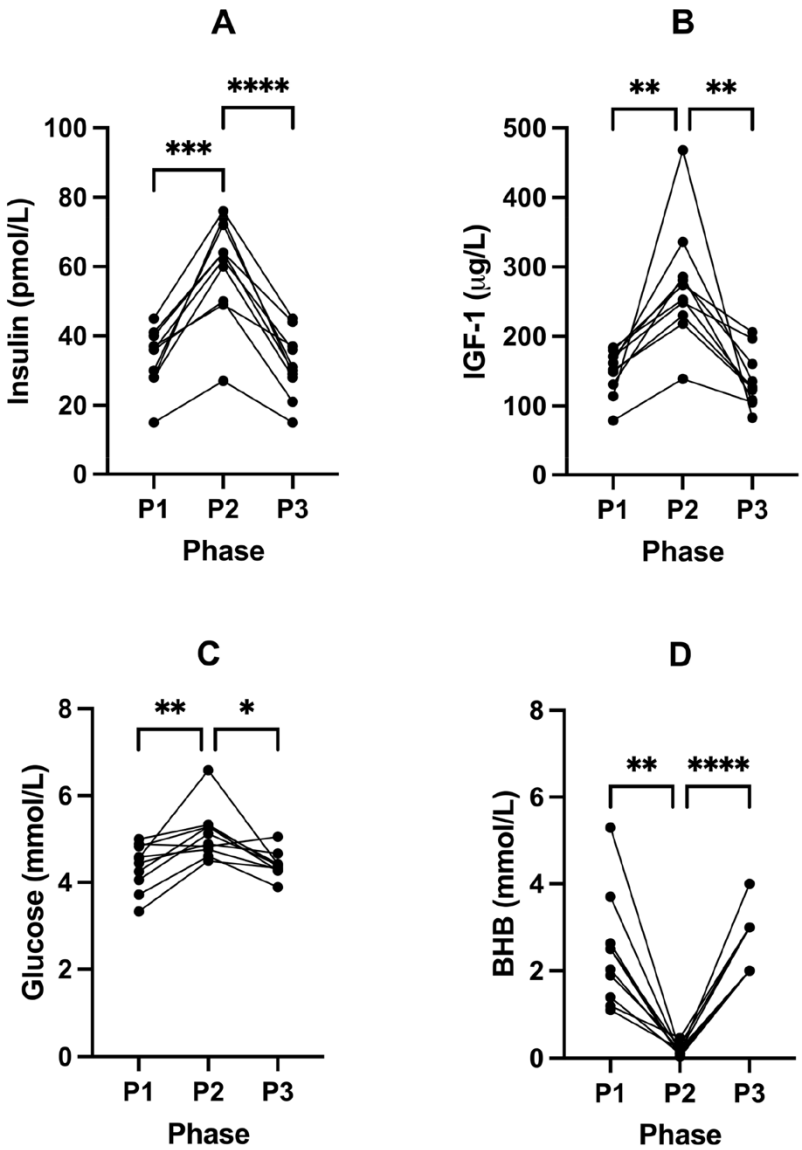

Figure 2. Changes in fasted blood insulin (**A**), IGF-1 (**B**), glucose (**C**), and BHB (**D**) concentrations across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3. Measurements were taken at 8 a.m. after a 12 h overnight fast; (n = 10). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Table 4. Fasted insulin, IGF-1, glucose, and BHB across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK P3. Measurements were taken at 8 a.m. after a 12 h overnight fast; (n = 10; † n = 5).

	P1	P2	P3	ANOVA *p* Value	P1 vs. P2	P2 vs. P3	P1 vs. P3
Insulin (pmol/L)	33.60 (± 8.63)	59.80 (± 14.69)	31.60 (± 9.38)	<0.0001	0.0002	<0.0001	0.5361
IGF-1 (µg/L)	149.30 (± 32.96)	273.40 (± 85.66)	136.90 (± 39.60)	0.0015	0.0045	0.0055	0.4124
Glucose (mmol/L)	4.36 (± 0.53)	5.12 (± 0.59)	4.41 (± 0.30)	0.0015	0.0088	0.0177	0.9469
BHB (mmol/L)	2.43 (± 1.28)	0.18 (± 0.13)	2.31 (± 0.71)	0.0001	0.0012	<0.0001	0.9854
IGFBP-3 (mg/mL)	3.69 (± 0.56)	4.41 (± 1.27)	3.67 (± 0.70)	0.2357	0.3621	0.4272	0.9361
IGF-1/IGFBP-3†	0.14 (± 0.03)	0.25 (± 0.08)	0.15 (± 0.04)	0.0584	0.0870	0.1554	0.9049
TSH (mU/L)	1.40 (± 0.74)	1.56 (± 0.75)	1.25 (± 0.81)	0.3065	0.2334	0.4498	0.7742
Free T3 (pmol/L)	3.81 (± 0.28)	5.51 (± 0.72)	4.05 (± 0.54)	<0.0001	<0.0001	0.0015	0.3040
Reverse T3 (nmol/L)	0.29 (± 0.09)	0.26 (± 0.10)	0.25 (± 0.09)	0.6039	0.7030	0.9674	0.6323
T4 (pmol/L)	13.51 (± 1.61)	13.24 (± 1.49)	12.65 (± 0.66)	0.2125	0.8795	0.3059	0.2099

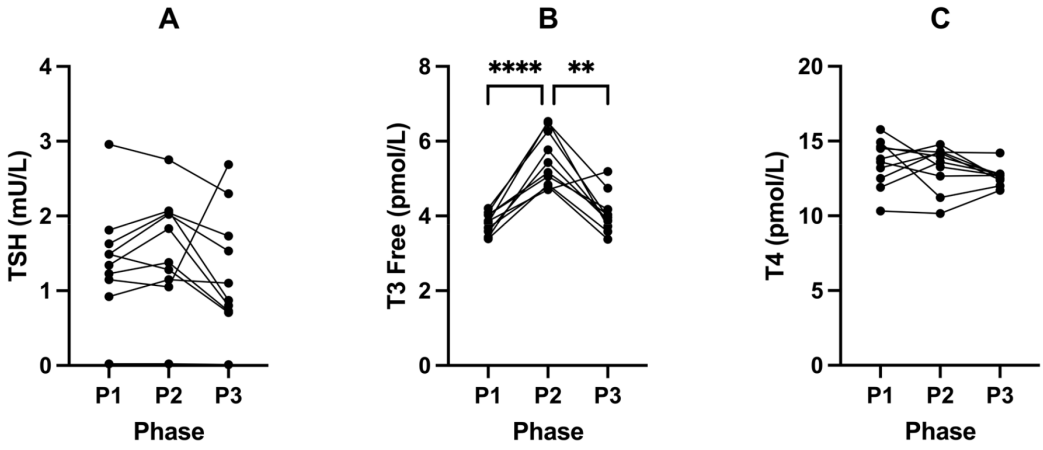

Figure 3. Changes in TSH (**A**), T3 Free (**B**), and T4 (**C**) concentrations across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK P3. Measurements were taken at 8 a.m. after a 12 h overnight fast; (n = 10). ** $p < 0.01$; **** $p < 0.0001$.

2.4. Oral Glucose Tolerance Tests

2.4.1. Between Phases (P1 vs. P2 vs. P3) OGTT Glucose Response

Basal values of blood glucose measured as part of the OGTT were lower in P1 and P3 (4.23 mmol/L ± 0.50 and 4.24 mmol/L ± 0.28, respectively) compared to P2 (5.01 mmol/L ± 0.70; Figure 4A). We found significant differences in mean glucose concentration amongst the three phases at baseline ($p = 0.0016$). Post hoc comparisons showed a significant difference in glucose concentration between P1 and P2 ($p = 0.0065$) and between P2 and P3 ($p = 0.0166$).

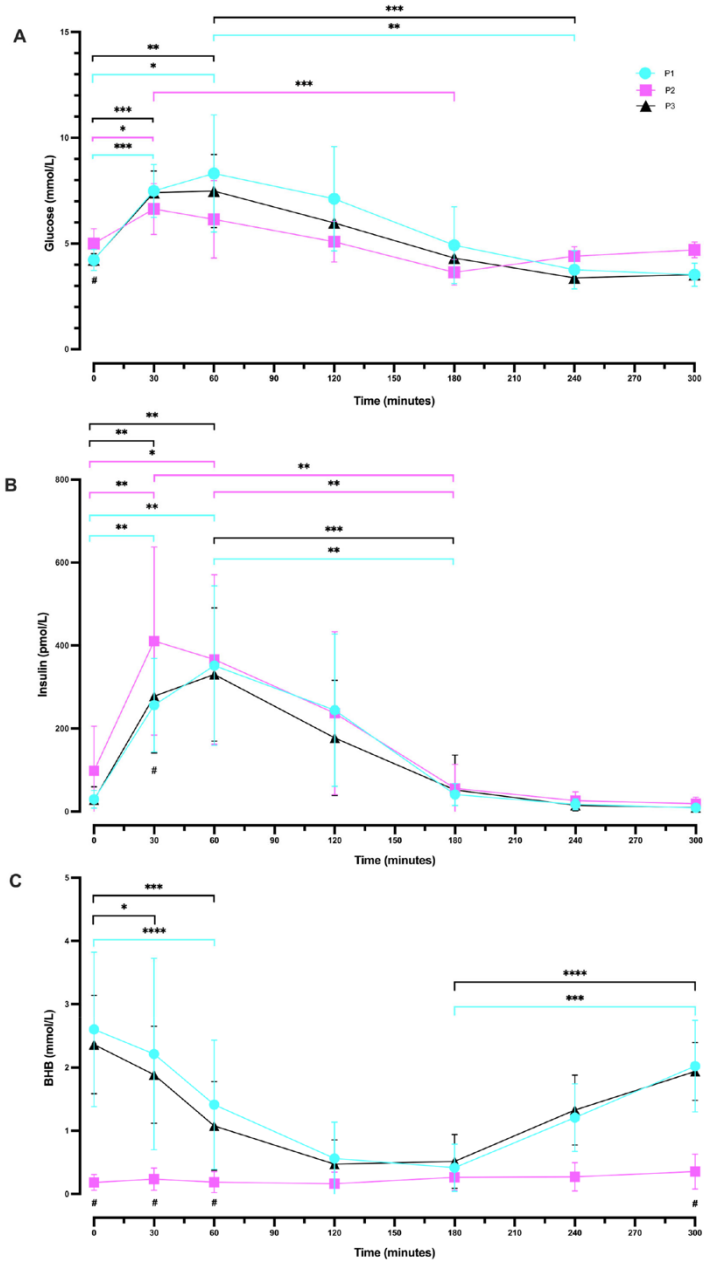

Figure 4. Differences in glucose (**A**), insulin (**B**), and BHB (**C**) in response to oral glucose tolerance tests across all study participants in all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1 (blue circles); intervention to suppress ketosis (SuK) P2 (pink squares); and removal of SuK returning to NK, P3 (black triangles); (n = 10). *The connected line indicates group means (± SD); * indicates significant difference within each phase across different time points; # indicates significant difference between phases at the time point as indicated. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

P1 and P3 showed a similar pattern in blood glucose response, whereby glucose peaked at 60 min (8.31 mmol/L ± 2.78 and 7.48 ± 1.73, respectively; $p = 0.4385$). However, in P2, glucose concentration reached a peak earlier, at 30 min (6.63 mmol/L ± 1.20). P1 and P3 showed a trough in glucose response at 240 min (3.76 mmol/L ± 0.91 and 3.38 mmol/L ± 0.20, respectively; $p = 0.4137$), whereas glucose concentration in P2 dropped earlier at 180 min (3.64 mmol/L ± 0.61). In P1 and 3, by minutes 240 and 300, glucose continued to trend down, whereas in P2, glucose trends up at these time points. By 300 min, values returned to their phase baselines.

2.4.2. Within-Phase Glucose Response during a 5 h OGTT

There were significant changes in glucose concentration across seven time points (0, 30, 60, 120, 180, 240, and 300 min) in P1 ($p < 0.0001$; Figure 4A). More specifically, there was a statistically significant increase in blood glucose change from 0 min (4.23 mmol/L ± 0.50) to 30 min (7.49 mmol/L ± 1.25; $p = 0.0009$) and to 60 min (8.31 mmol/L ± 2.78; $p = 0.0287$). There was also a significant difference between the peak of glucose at 60 min (8.31 mmol/L ± 2.78) and at 240 min (3.76 mmol/L ± 0.91; $p = 0.0070$), which continued to decrease at 300 min (3.52 mmol/L ± 0.55; $p = 0.0068$).

Following P2, there were significant changes in glucose concentration amongst all time points ($p = 0.0002$; Figure 4A). More precisely, glucose concentration was significantly increased from 0 min (5.01 mmol/L ± 0.70) to 30 min (6.63 mmol/L ± 1.20; $p = 0.0435$), but not between 0 and 60 min. There were also statistically significant differences between the peak at 30 min (6.63 mmol/L ± 1.20) and the trough at 180 min (3.64 mmol/L ± 0.61; $p = 0.0002$).

After returning to P3, significant changes were observed in glucose concentration amongst all time points ($p < 0.0001$; Figure 4A). There was a statistically significant increase from 0 min (4.24 mmol/L ± 0.28) to 30 min (7.41 mmol/L ± 1.02; $p = 0.0001$) and to 60 min (7.48 mmol/L ± 1.73; $p = 0.0059$). Similarly, to P1, there was also a significant difference between the peak of glucose at 60 min (7.48 mmol/L ± 1.73) and the trough at 240 min (3.38 mmol/L ± 0.20; $p = 0.0004$).

2.4.3. Following Plateau, Blood Glucose Concentration Increased during Ketosis Suppression

After 180 min in P2, glucose began to trend upwards, which was not observed in P1 or P3. At 240 min, whilst glucose concentration was higher in P2 (4.40 mmol/L ± 0.45) vs. P1 (3.76 mmol/L ± 0.90), this difference was not statistically significant ($p = 0.1342$). However, glucose concentration was significantly higher in P2 (4.40 mmol/L ± 0.45) compared to P3 (3.38 ± 0.20; $p = 0.0007$). Similarly, at 300 min glucose concentration was significantly higher in P2 (4.70 mmol/L ± 0.38) compared to P1 (3.52 ± 0.55; $p = 0.0001$) and to P3 (3.53 ± 0.13; $p < 0.0001$). There was no difference in the concentration of glucose at 240 min ($p = 0.4137$) or at 300 min ($p = 0.9988$) when comparing P1 and P3; Figure 4A.

2.4.4. Between Phases (P1 vs. P2 vs. P3) OGTT Insulin Response

Fasting insulin concentrations were also found to be lower in P1 and P3 (29.94 pmol/L ± 21.48 and 27.97 pmol/L ± 31.68, respectively) in comparison to P2 (98.19 pmol/L ± 107.69), although they were not significantly different (P1 vs. P2; $p = 0.0971$, and P2 vs. P3, $p = 0.0754$; Figure 4B). Insulin concentration peaked at 30 min in P2 (411.07 pmol/L ± 226.59) and at 60 min in P1 and P3 (351.94 pmol/L ± 192.36 and 330.27 pmol/L ± 160.56, respectively). However, insulin concentration returned to baseline values in all phases with a similar pattern at the end of the experimental period (300 min). We also analysed changes in insulin concentration at 30 min amongst the three phases. The data showed a significant difference between P1 (256.27 pmol/L ± 112.59) and P2 (411.07 pmol/L ± 226.59; $p = 0.0324$), and between P2 (411.07 pmol/L ± 226.59) and P3 (278.23 pmol/L ± 137.20; $p = 0.0161$).

2.4.5. Within-Phase Insulin Response during a 5 h OGTT

Following P1, repeated measures one-way ANOVA illustrated statistically significant changes in insulin concentration across all time points ($p < 0.0001$; Figure 4B). More precisely, there was a significant increase from 0 min (29.94 pmol/L ± 21.48) to 30 min (256.27 pmol/L ± 112.59; $p = 0.0013$) and to 60 min (351.94 pmol/L ± 192.36; $p = 0.0047$). There was also a significant difference between the peak at 60 min (351.94 pmol/L ± 192.36) and 180 min (41.08 pmol/L ± 26.62; $p = 0.0069$).

P2 also showed significant changes in insulin concentrations amongst all time points ($p < 0.0001$; Figure 4B). More specifically, there was a statistically significant increase from 0 min (98.19 pmol/L ± 107.69) to 30 min (411.07 pmol/L ± 226.59; $p = 0.0011$) and to 60 min (366.68 pmol/L ± 204.21; $p = 0.0291$). Additionally, there was a statistically significant difference between the peak at 30 min (411.07 pmol/L ± 226.59) and 180 min (55.95 pmol/L ± 57.99; $p = 0.0054$), and between that at 60 min (366.68 pmol/L ± 204.21) and 180 min (55.95 pmol/L ± 57.99; $p = 0.0028$).

Similarly, after returning to P3, insulin concentrations significantly changed across all time points ($p < 0.0001$; Figure 4B). Insulin levels were significantly increased from 0 min (27.97 pmol/L ± 31.68) to 30 min (278.23 pmol/L ± 137.20; $p = 0.0024$) and to 60 min (330.27 pmol/L ± 160.56; $p = 0.0015$). As in P1, there was a significant difference between the peak at 60 min (330.27 pmol/L ± 160.56) and 180 min (52.11 pmol/L ± 84.10; $p = 0.0005$).

Notably, in all three phases, insulin concentrations began to converge and trend significantly downwards at 180 min. At 240 and 300 min, the concentration of insulin began to plateau.

2.4.6. Between Phases (P1 vs. P2 vs. P3) OGTT BHB Response

Basal values of BHB were higher in P1 and P3 (2.60 mmol/L ± 1.22 and 2.36 mmol/L ± 0.78, respectively) than in P2 (0.18 mmol/L ± 0.12), and they significantly differed between P1 vs. P2 ($p = 0.0004$) and between P2 vs. P3 ($p < 0.0001$; Figure 4C).

A statistically significant difference was also observed amongst the three phases in mean BHB concentration at 30 min ($p = 0.0020$), at 60 min ($p = 0.0034$), and at 300 min ($p < 0.0001$). Post hoc testing indicated that BHB concentration significantly differed between P1 (2.22 mmol/L ± 1.51) and P2 (0.24 mmol/L ± 0.18; $p = 0.0078$) and between P2 (0.24 mmol/L ± 0.18) and P3 (1.89 mmol/L ± 0.77; $p = 0.0004$) at 30 min. In addition, BHB concentration at 60 min was significantly different between P1 (1.41 mmol/L ± 1.02) and P2 (0.19 mmol/L ± 0.17; $p = 0.0107$), and between P2 (0.19 mmol/L ± 0.17) and P3 (1.08 mmol/L ± 0.70; $p = 0.0039$). Further, there were significant differences between P1 (2.02 mmol/L ± 0.72) and P2 (0.36 mmol/L ± 0.28; $p < 0.0001$) and between P2 (0.36 mmol/L ± 0.28) and P3 (1.94 mmol/L ± 0.46; $p < 0.0001$) at 300 min.

2.4.7. Within-Phase BHB Response during a 5 h OGTT

There were statistically significant changes in BHB concentration across time points overall in P1 ($p = 0.0006$; Figure 4C), with no significant changes between 0 min (2.60 mmol/L ± 1.22) and 30 min (2.22 mmol/L ± 1.51; $p = 0.2056$). However, there was a significant decrease from 0 min (2.60 mmol/L ± 1.22) to 60 min (1.41 mmol/L ± 1.02; $p < 0.0001$). Whilst there were minimal changes in BHB concentration across all time points following P2 ($p = 0.0961$; Figure 4C), after returning to P3, there were significant changes across time points ($p < 0.0001$). More specifically, there was a significant decrease from 0 min (2.36 mmol/L ± 0.78) to 30 min (1.89 mmol/L ± 0.77; $p = 0.0444$), and to 60 min (1.08 mmol/L ± 0.70; $p = 0.0001$).

Following the 75 g glucose loaded drink, both P1 and P3 demonstrated a similar pattern response in BHB change, with BHB concentration showing a steady time-dependent decrease until 120 min. Subsequently returning in a linear recovery from 180 min (0.42 mmol/L ± 0.37, P1; 0.52 mmol/L ± 0.42, P3), with a significant increase until 300 min (2.02 mmol/L ± 0.72; $p = 0.0005$, P1; 1.94 mmol/L ± 0.46; $p < 0.0001$, P3). Conversely,

during SuK (P2), BHB concentration followed a similar response across time, whereby there were minimal changes throughout the experimental period (Figure 4C).

2.5. Suppression of Ketosis Is Associated with Increases in Inflammatory Liver Markers

Following P2, GGT concentrations increased significantly in all participants from 9.60 U/L (± 3.13) in P1 to 12.40 U/L (± 2.55) in P2 (p = 0.0087; Figure 5A). From P2 to P3, GGT levels were significantly reduced to 9.70 U/L (± 2.50; p = 0.0286; Figure 5A; Table 5). We also found that SuK (P2) significantly increased PAI-1 levels from 13.34 ng/mL (± 6.85, P1) to 16.69 ng/mL (± 6.26, P2; p = 0.0428). No changes in PAI-1 levels were observed following P3 (17.05 ng/mL ± 5.58) compared to P2 (p = 0.9483; Figure 5B; Table 5).

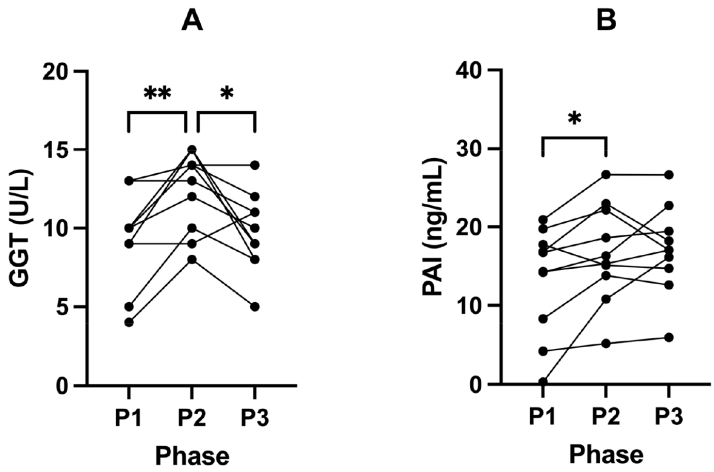

Figure 5. Changes in liver parameters GGT (**A**) and PAI-1 (**B**) in participants across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3. Measurements were taken at 8 a.m. after a 12 h overnight fast; (n = 10). * $p < 0.05$; ** $p < 0.01$.

Table 5. Concentrations of fasted liver markers measured across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3. Measurements were taken at 8 a.m. after a 12 h overnight fast; (n = 10; † P3, n = 9; § P1, P2, P3, n = 5).

	P1	P2	P3	ANOVA p Value	P1 vs. P2	P2 vs. P3	P1 vs. P3
Triglycerides (mg/dL)	66.80 (± 28.00)	66.10 (± 21.09)	79.30 (± 45.88)	0.5018	0.9972	0.6629	0.6270
Total cholesterol (mg/dL)	231.50 (± 62.42)	188.50 (± 30.28)	210.20 (± 43.44)	0.0335	0.0802	0.2132	0.1061
HDL cholesterol (mg/dL)	70.10 (± 10.37)	72.70 (± 13.59)	69.80 (± 11.84)	0.6231	0.7460	0.6762	0.9943
LDL cholesterol (mg/dL) †	4.46 (± 2.03)	3.13 (± 0.91)	3.96 (± 1.34)	0.0888	0.1798	0.3280	0.1498
Triglycerides/HDL (mmol/L)	1.01 (± 0.55)	0.95(± 0.38)	1.25 (± 0.90)	0.3804	0.9478	0.5358	0.5515
CRP (Ultra-Sensitive) (mg/L) §	1.00 (± 1.19)	1.16 (± 1.56)	1.35 (± 2.23)	0.7103	0.9938	0.7477	0.7728
Gamma-GT (U/L)	9.60 (± 3.13)	12.40 (± 2.55)	9.70 (± 2.50)	0.0029	0.0087	0.0286	0.9885
Cortisol (µg/dL)	12.62 (± 5.27)	11.27 (± 5.85)	13.19 (± 5.22)	0.3574	0.6886	0.4087	0.8258
PAI-1 (ng/mL)	13.34 (± 6.85)	16.69 (± 6.26)	17.05 (± 5.58)	0.0431	0.0428	0.9483	0.1373

CRP was found to be low or less than the lowest detectable limit of the assay in all participants across all study phases. CRP was therefore measured using a high-sensitivity assay (ultra-sensitive CRP) in five participants (Table 5). Despite this, no significant changes were determined from P1 (1.00 mg/L ± 1.19) to P2 (1.16 mg/L ± 1.56; p = 0.9938); or from P2 to P3 (1.35 mg/L ± 2.23; p = 0.7477). We found no statistically significant changes in all other liver or lipid markers across all phases of the study (Table 5).

2.6. Ketosis Maintains Lower Levels of EGF, VEGF and MCP-1

There were increases in EGF from 33.02 pg/mL (± 30.96) in P1 to 50.13 pg/mL (± 38.19) following P2 (p = 0.0450; Figure 6A; Table 6). VEGF also increased from 93.93 pg/mL (± 54.30) in P1 to 147.33 pg/mL (± 100.03) following P2 (p = 0.0314; Figure 6B; Table 6). MCP—1 significantly increased from 103.98 pg/mL (± 39.30) in P1 to 192.53 (± 84.73) following P2 (p = 0.0137; Figure 6C; Table 6).

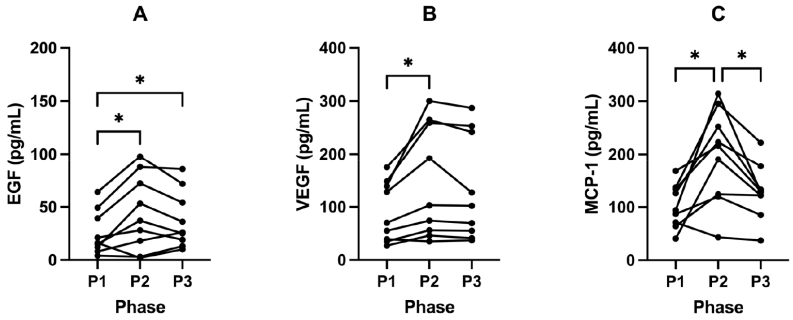

Figure 6. Fasted EGF (**A**), VEGF (**B**) and MCP-1 (**C**) in participants across all phases. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3. Measurements were taken at 8 a.m. after a 12 h overnight fast; *(A and B, n = 10; C, n = 9).* * $p < 0.05$.

Table 6. Concentrations of fasted growth factors and cytokines across the different phases of the study Measurements were taken following each of the study phases: baseline nutritional ketosis (NK) P1; intervention to suppress ketosis (SuK) P2; and removal of SuK returning to NK, P3. Measurements were taken at 8 a.m. after a 12 h overnight fast; (P1, P2, n = 10; P3, n = 9).

	P1	P2	P3	ANOVA p Value	P1 vs. P2	P2 vs. P3	P1 vs. P3
EGF (pg/mL)	33.02 (± 30.96)	50.13 (± 38.19)	37.82 (± 26.81)	0.0139	0.0450	0.3473	0.0478
VEGF (pg/mL)	93.93 (± 54.30)	147.33 (± 100.03)	134.80 (± 98.79)	0.0147	0.0314	0.2102	0.0801
Interferon-γ (pg/mL)	1.14 (± 2.64)	0.72 (± 1.05)	0.57 (± 0.90)	0.3755	0.7019	0.2452	0.6019
(MCP-1) (pg/mL)	103.98 (± 39.30)	192.53 (± 84.73)	128.52 (± 51.80)	0.0026	0.0137	0.0175	0.2622
TNF-α (pg/mL)	2.23 (± 1.75)	2.66 (± 1.26)	2.09 (± 0.97)	0.1387	0.3887	0.0785	0.8430
IL-1a (pg/mL)	0.30 (± 0.40)	0.26 (± 0.25)	0.26 (± 0.25)	0.3230	0.6266	0.5406	0.5104
IL-1b (pg/mL)	2.23 (± 3.42)	1.85 (± 2.02)	1.71 (± 2.04)	0.3090	0.7045	0.0381	0.4989
IL-2 (pg/mL)	1.92 (± 1.48)	1.71 (± 1.16)	1.94 (± 1.37)	0.2932	0.4409	0.7569	0.3809
IL-4 (pg/mL)	2.14 (± 0.80)	2.06 (± 0.99)	2.25 (± 1.17)	0.4635	0.5358	0.5138	0.9090
IL-6 (pg/mL)	0.95 (± 0.80)	1.22 (± 1.11)	0.84 (± 0.56)	0.5034	0.9238	0.5677	0.5771
IL-8 (pg/mL)	8.91 (± 9.56)	8.60 (± 5.93)	8.08 (± 6.30)	0.6738	0.9966	0.5725	0.8009
IL-10 (pg/mL)	0.61 (± 0.37)	0.68 (± 0.46)	0.53 (± 0.25)	0.4323	0.9084	0.4420	0.5573

Following P3, these growth factors and cytokines trended back to baseline and decreased significantly compared to P2. EGF (p = 0.3473; Figure 6A; Table 6) and VEGF (p = 0.2102; Figure 6B; Table 6) decreased to 37.82 pg/mL (± 26.81) and 134.80 pg/mL (± 98.79), respectively. Concentrations of MCP-1 also decreased significantly to 128.52 pg/mL (± 51.80) following P3 compared to P2 (p = 0.0175; Figure 6C; Table 6).

There were minimal changes in IL-1b following P2 (p = 0.7045). However, following P3, all participants had a significantly decreased expression of IL-1b (p = 0.0381; Table 6). Similarly, there were minimal changes following P2 in the expression of TNF-α (p = 0.3887); however, in P3, 86% of participants showed a decreased expression of TNF—α (p = 0.0785; Table 6). We found no change in all other cytokines and growth factors across all phases of the study (Table 6).

3. Discussion

There have been many studies investigating the effect of ketosis in humans; however, little is known about the physiological adaptations in individuals who have never had a metabolic illness and maintained long-term (>1 year) habitual ketosis for more than 80% of their year. Furthermore, prior work has primarily examined males. Our cohort self-reported to have sustained nutritional ketosis for an average of 3.9 years, with confirmed NK for at least 6 months in the lead-in period to the trial. Participants presented with healthy weights, BMI, waist-to-hip, waist-to-height, and blood pressure (Table 3). Molecular markers including lipid panels, liver enzymes (Table 5), and cytokines (Table 6) were also within healthy ranges. Thus, our data indicate that long term NK, euketonaemia, does not have a negative effect on health in this cohort.

Euketonaemia is defined as a state of ketosis that is not associated with any harmful effects. Although, in most cases, the term has been used to refer to a state of normal ketonemia in patients with diabetes, the term has also been used in a broader sense to refer to a state of normal ketonemia in healthy individuals [31,32]. Euketonaemia in adults has been associated with improved insulin sensitivity and euglycaemia [33–35]. Moreover, euketonaemia is associated with reduced inflammation in the brain [36,37], is consistent with evolutionary biology, and has a protective effect on mitochondria [38].

3.1. Macroscopic Changes/Anthropometrics

Throughout the intervention phase (P2), where participants were actively suppressing ketosis, participants often reported capillary BHB concentrations of > 0.3 mmol/L after the overnight fast, and even three hours after a carbohydrate-containing meal (Table 2). Together, these data indicate that the participants were indeed highly fat-adapted and, even with the introduction of carbohydrates into their diet, their bodies reverted to beta-oxidation and ketolysis during periods of fasting. Following P3, participants tracked back to the baseline level of ketosis as indicated by their capillary BHB levels (Figure 2D). The participants enrolled in this study were able to tolerate 21 days of suppression of ketosis and the consequent upregulation of glucose metabolism and still return to their baseline level of ketosis. This suggests that metabolic flexibility is maintained in long-term habitual ketosis in metabolically healthy individuals.

Across all three phases, in two different metabolic states (ketosis vs. glucose fuelling), participants' RQ values were indicative of individuals that were metabolically healthy; interestingly, their values were superior to observations previously made in high performance athletes [39–42]. Even after 21 days of SuK, a 12 h overnight fast still induced higher fat oxidation, as evidenced by their P2 RQ values (Figure 1; Table 1). However, surprisingly, there was still a significant difference between P2 compared to P1 and P3, even within the overall highly fat-adapted state that all the participants were in, in all three phases after an overnight fast (Figure 1). Given that the baseline RQ values were indicative of a high state of beta-oxidation, it was not expected that a 21-day of SuK would result in RQ measurements that were statistically inferior to those observed at baseline (P1).

Three weeks of SuK resulted in changes in body composition, with increases in weight and BMI. This was largely accounted for by increases in TBW and total body fat (Table 1). However, the increased body composition measurements taken following P2 (and indeed, P1 and P3) were still within normal ranges [43,44] and tracked back to baseline levels following P3.

3.2. Insulin, IGF-1, and Glucose

In the normative setting, the most influential pancreatic insulin secretagogue is dietary carbohydrate, whilst basal insulin release is regulated by a multitude of factors including hepatic glycogenolysis, which is further regulated by glucagon, osteocalcin, and other secretagogues [8]. It is interesting that with the increased repeated stimulation of bolus insulin during P2, fasting (basal) insulin and glucose subsequently increased (Figure 2). This is likely due to insulin's systemic effects, where enforced glucose fuelling results in increased

glucose demand. In addition, insulin's suppressive effect on beta-oxidation, where neither lipid provision for beta-oxidation nor ketogenesis is sufficient due to insulin also inhibiting insulin sensitive lipase, which is required to release lipids from adipocytes [45–47]. Therefore, the upregulation of hepatic glycogenolysis occurs in response to chronic insulin signalling.

Participants' habitual ketosis lifestyle also demonstrated significantly lower IGF-1 levels in P1 and P3 (Figure 3). IGF-1 is regulated by insulin on multiple fronts; regulating synthesis and bioavailability via IGF1-binding proteins [48], as well as amplification of signal transduction capacity [49–51]. Insulin and IGF-1 both transactivate each other's receptors, as well as form cross hybridised receptors [52]. Chronically elevated IGF-1, and/or increased IGF-1 bioavailability and sensitivity, receptor expression, and amount of Ras protein prenylation [51] are strongly implicated in neoplasia and ageing [48,53–56], whilst IGF-1 knockdown within in vivo models show improved longevity [56,57].

In observational studies, low levels of insulin and IGF-1 have also been associated with reduced levels of pathologies. For example, elevated IGF-1 has been shown to correspond to a 69% increase in colorectal cancer risk, a 49% increase in prostate cancer risk, 65% increase in breast cancer risk, and a 106% increase in lung cancer risk [58] (relative risks). Notably, a recent meta-analysis involving over 30,000 participants indicated that IGF-1 within the range of 120–160 ng/mL was the optimum range associated with the lowest risk of all-cause mortality [58]. The participants in the present study fell well within this range during the P1 and P3 phase; however, during SuK (P2), IGF-1 significantly increased, which may confer an increased risk of all-cause mortality. Conversely, the lower levels of insulin and IGF-1 during the P1 and P3 phases may be of health benefit given that higher levels of IGF-1 and insulin are significant risk factors for various diseases.

Insulin/IGF-1 signalling inhibits FOXO activity via increasing phosphorylation, causing cytosolic sequestration, and suppressing BHB action on FOXO expression and nuclear translocation, through the Akt signalling pathway [59]. FOXO is a transcription factor which regulates the expression of a vast number of genes with functions associated with longevity, including cell cycle arrest, autophagy, and DNA damage repair [60], as well as regulating metabolism and antioxidant defence [61]. In addition to being a metabolic substrate, BHB also acts as a signalling molecule, modulating intracellular activity in cells across the body, such as regulating gene expression through inhibition of class I histone deacetylases (HDACs) via competitive inhibition [62]. Specifically, BHB prevents histone acetylation at the *FOXO* gene regions [18], and 12 h fasted mice have significantly increased levels of FOXO protein expression in the liver [63]. Based on our understanding of these cellular and intracellular signalling and fuelling dynamics, we propose that the low levels of insulin and IGF-1 maintained by the participants in our study during P1 and P2, along with BHB $\geq$ 0.5 mmol/L, through their lifestyle habits, are a logical and potentially effective way to slow and/or reduce cellular ageing. It is unlikely that life-long sustained vs. suppressed ketosis human trials will ever happen, and these indirect comparisons are our next best option, as we see in whole of life animal trials, maintaining minimal insulin demand and IGF-1 levels consistently results in optimum longevity [54–56,64].

Considering that our cohort is exclusively female, it is imperative to recognise, for global population health, the pressing importance of focusing on diabetes, hyperinsulinaemia (insulin may be inside reference ranges; however, chronic hypoketonaemia may indicate an individual's hyperinsulinaemia threshold), obesity, and breast cancer. Given their widespread prevalence [65–68], understanding the intricate links between these conditions is critical in order to prevent occurrence and to improve outcomes. Our participant data show that long-term NK reduces fasting insulin, IGF-1, and glucose. This data adds to existing evidence that sustaining a lifestyle which promotes ketosis is an effective modality for the prevention and management of both type 1 and type 2 diabetes [69].

3.3. Thyroid—Free T3

Along with increased glucose and insulin concentrations, SuK (P2) resulted in increased levels of free T3 (fT3). Given fT3 is highly involved in the transcription and

translation of OXPHOS proteins, it would be expected that being in ketosis would come with higher levels of fT3 than a suppressed ketosis state. Being in ketosis is highly dependent on OXPHOS capacity. We found, in our healthy long-standing-ketosis-maintaining cohort, that their fT3 was significantly lower than after 21 days of suppressed ketosis. A plausible explanation is that ketosis, a fasting-mimicking metabolic state, reduces thyroid hormone (TH) demand due to less ROS damage on OXPHOS proteins and mt IMM lipids, such as cardiolipin [70], and may increase sensitivity, such as increasing mitochondrial fT3 receptors and/or increasing monocarboxylate transporter 8 [71]. In addition, BHB has an epigenetic regulatory role of its own, enabling increased transcription of OXPHOS proteins [20]. Short durations, such as 21 days which, evolutionarily, would be akin to a short summer/autumn, are within the thyroid's capacity to deal with. However, in possible similarity to the pancreatic beta cells, chronic demand of the thyroid to produce increased amounts of TH may result in mechanisms that downregulate either production or conversion of T4 to fT3, respectively.

In P2, our cohort were not chronic long-term hyperinsulinaemic. Hence, they had the earlier phase of greater demand of thyroid hormone (TH). If those 21 days turned into 21 years, it is arguable that, over that time frame, TH may become low, more specifically T3, in concordance with the research literature in T2DM hyperinsulinaemic populations. The aforementioned low T3 levels in our cohort when in ketosis, P1, and P3, could lead clinicians to mistakenly diagnose a metabolic phenotype 1 individual (see methods 2.2) with hypothyroidism. Therefore, it is worth highlighting that T3 levels were still within normative ranges; however, this information should assist clinicians and researchers by indicating a need for nuance and metabolic context when interpreting thyroid biomarkers.

3.4. OGTT

Our data indicate that the participants enrolled in this study were able to maintain normal glycaemic responses throughout the OGTT following each of the phases (Figure 5), highlighting that prolonged ketosis did not hinder metabolic flexibility. However, in the first 2 h of insulin response, there appears to be a shift to the right for P1 and P3. This is likely due to a reduced frequency and load from a large glucose bolus exposure, therefore increasing the time to peak for bolus insulin synthesis and secretion. Concurrently, plasma glucose appears to be greater during the first 2 h in these two phases, and higher peaks of glucose are also seen in P1 and P3 compared to the P2 phase (although these are not statistically significant). This pattern may be incorrectly labelled as a lack of sensitivity to insulin; on the contrary, this is the sum of the exogenous glucose from the OGTT, plus hepatic glucose output, which does not abate from the one-time glucose bolus.

The dosage of dietary glucose administered during the OGTT compounds with the hepatic glucose output, and therefore contributes to an elevated peak of glucose concentrations during P1 and P3 (Figure 4A). When the participants were fat fuelling (metabolic phenotype 1), their glucose needs were largely dependent on hepatic provision via gluconeogenesis and glycogenolysis. Sustained glycogenolysis during an OGTT is also seen in hyperinsulinaemic individuals (metabolic phenotype 3), where the liver is pathway-selective insulin-signalling-resistant, suppressing ketogenesis and inhibiting beta-oxidation whilst increasing de novo lipogenesis, and glycogenolysis is not inhibited [72]. The chronic hyperinsulinaemic state is also coupled with a higher glucagon state [73,74], adding to hepatic signalling that maintains hepatic glucose provision to the wider system.

Hypothetically, under an evolutionary context, selection pressure would have favoured the ability to adapt to and maintain NK due to seasonal food availability and intermittent CR/fasting, meaning the body's glucose needs would have been met by hepatic gluconeogenesis and glycogenolysis. If an (infrequent) opportunity to consume a high carbohydrate load would have presented itself, the subsequent increased insulin secretion would likely not have inhibited gluconeogenesis and glycogenolysis during the one-time exposure, given that the body is adapted and reliant on hepatic glucose as its main glucose source [8,28]. If insulin, in this one instance, were to inhibit gluconeogenesis and glycogenolysis, whilst

facilitating oral glucose load myocyte uptake, a potential case of hypoglycaemia with hypoketonaemia and inhibition of beta-oxidation may simultaneously occur, which would be potentially fatal. In this metabolic phenotype 1 context [8], an infrequent one-time bolus insulin secretion does not inhibit gluconeogenesis and glycogenolysis; this may incorrectly be interpreted as hepatic insulin resistance, as is the case for hyperinsulinaemic T2DM (stage-3 metabolic phenotype 3) individuals [8]. This has not been observed in our cohort, as ketogenesis declined during the first 2 h, indicating that the liver is being affected by the bolus insulin release and is selectively responding based on metabolic phenotype physiological state and adaptation. Like switches and gates, the metabolic phenotype signature changes hepatic responses to a bolus insulin signal.

Overall, our data indicates that long-term ketosis does not appear to negatively affect the insulin-dependent glucose uptake nor reduce carbohydrate tolerance. In fact, following SuK P2, participants demonstrated a significantly elevated peak level of insulin in response to a glucose challenge across the OGTT, compared to P1 and P3. Furthermore, in all phases, insulin levels were at their lowest and plateauing after 240 min; glucose was on a rise upwards to basal P2 fasting levels after 180 min in P2, whereas glucose was further declining back to basal fasting levels in P1 and P3. Together, these findings indicate a lower total insulin requirement to maintain lower glucose levels when in a state of NK, whereas SuK was associated with an increased insulin requirement. Maintaining lower–normal glucose levels with lower insulin and IGF-1 levels is associated with improved health outcomes, decreasing risk of insulin resistance and T2DM, reducing chronic diseases, and also improving longevity and healthy ageing [56,57]. This suggests that maintaining a long-term metabolic phenotype 1 profile may aid in maintaining a healthier healthspan and lifespan.

We previously hypothesised that the addition of the BHB sensitivity assay in an OGTT challenge would help to differentiate between different metabolic phenotypes with improved resolution [6,8]. Here we show that, indeed, the combination of insulin and BHB measurements throughout the OGTT helps to differentiate early stage hyperinsulinaemic individuals (metabolic phenotype 3, stage 1 or 2) or prior metabolically unwell individuals who have restricted carbohydrates and gone into ketosis (metabolic phenotype 4) from long-standing healthy-ketosis-living individuals (metabolic phenotype 1). The combination of glucose, BHB, and insulin response measurements help to provide greater resolution in understanding metabolic health and helps clinicians and researchers to better classify individuals when designing trials or analysing data.

During P2, the participants were exposed to an increased frequency (ad libitum, spread over three times a day SUK diet recommended, which prevents TRF-induced ketosis), dose (glycaemic load, SUK diet recommendation to consume at least 267 g of carbohydrate per day), and duration (21-day intervention) of dietary glucose, consequently repeatedly triggering bolus insulin release (equating to an equivalent carbohydrate exposure of approximately 63 OGTTs in 21 days). The increased bolus insulin secretion signals to the liver to temporarily reduce glycogenolysis; this is considered hepatic insulin sensitivity (response seen in metabolic phenotype 2 and not phenotype 3, stage 3), as hepatic glucose output is reduced in response to the insulin signal. We have come to consider this the normal and healthy response, which is likely correct for those consuming a ketosis-suppressive diet and who do not have any chronic ageing and hyperinsulinaemia disease. However, if we were to consider humans under an evolutionary context, with less frequency, dose, and duration of exogenous carbohydrate exposure, then it is arguable that what we see in our cohort's response curves in P1 and P3 would be the normal/healthy physiological responses. Hepatic glucose provision under the context of being in sustained NK would not be inhibited by bolus insulin secretion, and therefore this is not a case of pathological insulin resistance which logically only would be the case under a chronic hyperinsulinaemia and not an acute context (one-time OGTT for a metabolic phenotype 1 individual maintaining NK as a lifestyle). High levels of BHB would not be observed in hyperinsulinaemic/T2DM/CVD individuals, and therefore analysis of BHB response during an OGTT and/or for several

consecutive days before the evening meal is essential for resolving a T2D glycaemic response (metabolic phenotype 3 spectrum) and those on the hyperinsulinaemic spectrum from those in ketosis [8].

We found that during both ketosis phases (P1 and P3), BHB concentrations began to recover following the glucose challenge at 180 min, with an overall U-shaped curve. However, participants in P2 did not mirror this response pattern and sustained low levels of ketones before and after 180 min following the glucose challenge, with a flat line pattern. These data indicate that consumption of a carbohydrate diet that suppresses ketosis for 21 days results in limited de novo ketogenesis, even following a 12 h fasting period, indicating adaptive changes and likely downregulation of cellular ketogenesis enzymes and activities.

3.5. Liver Markers

3.5.1. GGT

There were significant findings regarding the effects of ketosis suppression and subsequent return to ketosis on liver markers. It is recognised that GGT is a diagnostic marker for many diseases in humans, including a fatty liver, T2DM, MetS, and AD, which are typified by hyperinsulinaemia [75–77]. In our study, SuK (P2) resulted in a significant increase in GGT levels, an enzyme associated with oxidative stress, low-grade inflammation, and insulin resistance [78,79]. Furthermore, GGT participates in the direct generation of ROS via a glutathione (GSH)/transferrin system, where, in the presence of molecular oxygen and iron/copper ions from transferrin and in the presence of cysteinylglycine (a product of GGT/GSH reaction) results in a paradoxical generation of ROS. This results in increased free radical and oxidative damage to nucleic acids and protein and lipid peroxidation [80]. Our findings suggest that suppressing ketosis may impose some degree of oxidative stress and inflammation on the liver, leading to increased GGT levels. GGT levels returned to near-baseline levels after our participants discontinued suppressing ketosis, indicating that carbohydrate restriction is an effective tool in correcting the significant increases in GGT.

High levels of GGT have been found to be associated with increased risk of MetS and impaired fasting glucose [78,81,82]. Our participants' GGT levels in ketosis (P1 and P3) and SuK (P2) were within standard reference ranges. However, a study in a large nonobese population of nondiabetics, n = 1309, showed that a moderate elevation in GGT within normal reference ranges is a strong risk marker predictor for T2DM, independent of visceral fat, obesity, and HOMA [83]. Unwin et al. later corroborated this finding in a primary healthcare setting. After restricting dietary carbohydrates in 67 individuals, with a minimum of 3 months adherence and an average follow-up of 13 months, they found the improvements in GGT (reduction) had no correlation to weight loss [77]. GGT has also been shown to be associated with cognitive decline prior to vascular dementia in longitudinal observations (n = 452, average 80 years of age) [84]. With an increasingly growing aged population, monitoring GGT may also provide an ability to detect and intervene earlier in dementia prevention.

3.5.2. PAI-1

The antifibrinolytic plasminogen activator inhibitor-1 (PAI-1) is the primary inhibitor of plasminogen activators (PAs) via inhibition of tissue-type PA (tPA) and urokinase-type PA (uPA) that proteolytically cleave zymogen plasminogen to active plasmin [85]. Elevated PAI-1 levels propagate a prothrombotic state [85]. We found a significant increase in PAI-1 levels during P2 compared to P1. Insulin has been shown to stimulate the secretion of PAI-1 by adipocytes, and there is a strong positive correlation between hyperinsulinaemia and elevated PAI-1 [86–88]. However, although fat mass increased after SuK (P2), it also returned to baseline levels in the return to ketosis (P3), whilst PAI-1 also significantly increased after P2, yet trended back, but not significantly, to baseline after P3. With the loss of gained fat mass after P3, with only a trend back for PAI-1, this indicates that other mechanisms outside of adiposity were involved.

PAI-1 circulates in the plasma at low levels (5–50 ng/mL), and its main pool is in platelets (approximately 300 ng/mL) [89]. Platelet activation is increased via increased PI3K, Akt, and PKC intracellular signalling, all of which are increased by hyperinsulinaemia and more so when glucose uptake insulin resistance develops [90–92]. In 2016, CVD mortality accounted for about 17.8 million deaths worldwide, where ischemic heart disease (IHD) and stroke contributed to 87% [4]. Disseminated intravascular coagulopathy (DIC) and thrombosis cause blockages of the blood flow to either the heart or brain, resulting in insufficient blood supply as well as increased atherosclerosis [93]. These processes are strongly associated with increased levels of PAI-1. NK may provide an effective strategy to reduce risk of DIC and thrombosis.

Hyperinsulinaemia increases gene expression of and stabilises PAI-1. Semad et al. demonstrated insulin increases in PAI-1 gene expression through a different signalling pathway to insulin-mediated glucose transport. Indicating that in the hyperinsulinaemia insulin-resistant state, where glucose tolerance declines, signalling by hyperinsulinaemia to upregulate PAI-1 gene expression is unimpeded and regulated by a pathway that does not become insulin-resistant [30,86].

PAI-1 contributes to an inflammatory response via infiltration of immune cells, specifically macrophages, in adipose tissue [85]. Adipocytes are a source of PAI-1 [85,94]. Our participants returned to their baseline mass with a concurrent loss of fat mass, indicating that the non-significant trend of PAI-1 returning to baseline from P2 to P3 was not associated with the significant loss of fat mass. As such, we may also conclude that, in our participants, the surge in PAI-1 during P2 was not due to enhanced adiposity.

A state of chronic inflammation, typical of conditions like obesity, T2DM, and MetS, is distinguished by the augmented expression of inflammatory adipokines, such as IL-6 and TNF-α [95]. These adipokines are known to upregulate PAI-1 expression within adipose tissue [96]. In our study's data, however, we did not identify any marked alterations in these inflammatory markers across the different phases. These observations may explain how the inflammatory cytokines, notably IL-6 and TNF-α, did not precipitate PAI-1 increase from P1 to P2, suggesting that this increase was not caused by a surge in these cytokines.

Elevated, dose-dependent levels of PAI-1 are pro-tumourigenic, pro-angiogenic, and anti-apoptotic [97,98]. PAI-1 is one of the most highly induced proteins in metastatic invasive tumours and the tumourigenesis process [85,99]. PAI-1 binds to the low-density lipoprotein receptor-related protein 1 (LRP1) receptor, activating intracellular signalling cascades and modulating cell migration, such as mast cells in gliomas [85,100]. PAI-1 is a highly reliable prognostic and biomarker in a host of cancers, including breast [101–105], bladder [106,107], colon [108], gliomas [100,109], ovarian [110–112], non-small cell lung cancer [113], and renal [114] cancers.

PAI-1 is seen to increase with age; furthermore, PAI-1 is a part of the senescence-associated secretory phenotype (SASP) paracrine signalling pathway, inducing the SASP profile in neighbouring cells, therefore acting as both a marker and maker of cellular ageing and ageing-related pathologies [85,115,116]. Our data suggests that reducing PAI-1 through adopting a ketogenic diet may have the potential to carry wide health benefits.

3.6. Cytokines

It is often posited that inflammation precedes hyperinsulinaemia; however, our data does not support this. Where we observed increases in insulin and glucose, at the end of P2, CRP, interleukins, and TNF-α remained unchanged with no increase in CRP nor interleukin cytokines or TNF-α, indicating that inflammation known to be caused by these molecules, was not mediating the increased insulin levels that suppress ketogenesis. There were, however, increases in growth factors VEGF, EGF, and MCP which are discussed below.

3.6.1. VEGF and EGF

Following SuK (P2), we observed significant increases in VEGF and EGF compared to baseline ketosis (P1) (Table 6; Figure 6A,B). This indicates that being in a state of ketosis

does not overstimulate the production of these growth factors and chemokines, whereas being in a state of carbohydrate metabolism promotes their production. The concentration of these growth factors then trended back towards the baseline values after 21 days of returning to ketosis (P3). However, the concentrations were not significantly different compared to P2. This indicates that the relatively short period of carbohydrate fuelling is a sufficient time to elevate the concentrations of these growth factors in a way that cannot be fully recovered in 21 days after returning to ketosis.

Pericytes are supportive cells which wrap around blood vessels, serving as multilineage progenitor cells, and are essential for the development of new blood vessels [117]. Insulin stimulates pericytes to increase their production of VEGF, which, in turn, stimulates endothelial cells to grow and proliferate, facilitating angiogenesis [118]. The lower levels of insulin in the NK phases likely account for the lower levels of VEGF. Insulin and IGF-1 have been shown to promote the upregulation of VEGF or EGF [119–122]. EGF signalling is one of the key pathways involved in tumour development [123,124]. Ketogenic metabolic therapy (KMT) may aid in reducing the expression of EGF and VEGF.

Considering that our cohort is female, it is imperative to recognise the links between hyperinsulinaemia, metabolic health, and breast cancer. Women with diabetes have been shown to exhibit poorer outcomes for breast cancer compared to their non-diabetic counterparts [66]. Consistent with these findings, in vitro research has shown that treating cancer cells, particularly breast and pancreatic, with high levels of glucose initiates molecular alterations such as phosphorylation of EGFR, which promotes their proliferation [125–127]. The implications of hyperglycaemia also extend to treatment outcomes, with heightened glucose levels during chemotherapy leading to increased chemoresistance in tumour cells [128]. Beyond direct cellular growth effects, the hyperglycaemic state appears to compromise the body's innate anti-tumour defences, notably by inhibiting neutrophil mobilisation, thereby granting tumour cells an immunological escape route and enhancing their metastatic capabilities [129].

SuK (P2) involved routine 3x a day feeding containing around 267 g of carbohydrate, resulting in the occurrence of hyperglycaemic and hyperinsulinaemic excursions equivalent to 3 OGGTs per day for 21 days, totalling 63 OGTTs. These periodic increases in glucose and consequent bolus insulin would not be captured in a fasting glucose/insulin test. This likely contributed to the upregulation of VEGF, EGF, and PAI-1.

3.6.2. MCP-1

Following SuK (P2), MCP-1 expression significantly increased, returning to baseline following P3 (Table 6; Figure 6C). MCP-1 is a chemokine (also termed CCL2) involved in the recruitment of monocytes and produced by a range of cell types including monocytes/macrophages, epithelial, adipocytes, endothelial, and smooth muscle cells [130], cells which express high levels of insulin receptors. Insulin has been shown to increase levels of MCP-1 in adipose tissue of both lean and obese individuals [131]. Thus, carbohydrate-rich diets that suppress ketosis, resulting in elevated insulin during P2, may help to explain the significantly increased levels of MCP-1.

There are multiple lines of evidence from both human and murine studies which suggest that MCP-1 appears to be a key player in insulin resistance. MCP-1-deficiency ameliorates insulin resistance in mice via downregulation of ERK and p38MAPK phosphorylation in the liver [132]. Moreover, MCP-1 has been shown to mediate skeletal muscle inflammation and localised insulin resistance in mouse muscle in T2DM models [133]. Thus, NK may not only assist with regulating glycaemic control in T2DM, reducing insulin demand and exposure [134], but may help to ameliorate further MCP-1-mediated insulin resistance, further reducing insulin demand.

The reduction in MCP-1 during the ketosis phases indicate one manner by which a ketogenic state may possess protective effects. Murine studies have shown that insulin can increase the expression of MCP-1 via adipocytes [135], and stimulation of adipose tissue with MCP-1 can also induce dedifferentiation, which may contribute to the pathologies

observed in obesity, such as cancer cell dedifferentiation, which occurs in their malignant transformation [135]. Elevated levels of MCP-1 have also been indicated in the pathophysiology of many other diseases, including age-related macular degeneration [136], allergic asthma [137,138], COVID-19, and CVD [139].

Our cohort showed no significant changes in the interleukins, except for a decrease in IL-1b from P2 to P3 (Table 6). IL-1b is a potent pro-inflammatory cytokine which becomes upregulated in response to pathogens and also in chronic disease [140]. IL-1b is a cytokine mainly produced by activated monocytes/macrophages [141]. Elevation of IL-1b in P2 compared to P3 correlates with the increased expression of MCP-1 we observed in this phase. However, we did not see an increase in IL-1b after SuK P2 from P1; this may be because our participants were in an anti-inflammatory state (P1) that persisted during the early days of SuK, which may have buffered/slowed down any change during that time.

Given that VEGF, EGF, and MCP-1 are often elevated in many cancers [142–145], KMT may be an effective way to support the action of certain cancer therapies, along with using the glucose-ketone index (GKI) calculator to measure therapeutic efficacy in metabolic management of brain cancers and likely other cancers [146]. Furthermore, KMT may be an effective stand-alone therapy for cancer. There have, indeed, been various human studies indicating that a ketogenic diet is tolerable for individuals with cancer [147], but also effective in reducing tumour burden and symptomatic disease [148]. Tumour cells are not well adapted to metabolising ketones, but instead predominantly depend on glucose for fuelling [149]. Limiting glucose availability for tumours by adapting into ketosis may therefore create a metabolically unfavourable environment for tumour growth, whilst also reducing insulin and IGF-1's growth and division stimulating signals [150]. The data presented here indicate that long-term ketosis is safe in healthy populations; well-designed clinical trials would elucidate the value of such an approach in cancer therapy.

4. Strengths and Limitations

Our study is the first investigating a non-athletic, healthy pre-menopausal female population living in a long-term (> 1 year, group average of 3.9 years) habitual ketosis lifestyle for more than 80% of their year. Our study was also culturally and ethnically diverse, whereby participants followed their own lifestyle and food preferences. However, they were controlled (via photos and daily dietary diary) for each meal throughout the day during the 9-week experimental period. Extensive nutritional instructions/guidance (e.g., reminders to follow the SUK dietary recommendations during the SuK phase) were also given to each participant.

Another strength of our study is participant adherence and compliance, as participants recorded and photographed daily capillary measurements (glucose and BHB), for a 6-month pre-trial (between 4–6 pm as a more rigorous threshold as opposed to morning overnight fasted measures) and throughout the experimental period (9 weeks) four times per day. The use of standardised procedures, including laboratory visit, blood sampling time, and testing measurements, is another strength of this study. The combination of anthropometric and metabolic indices as well as various biomarkers and OGTT assessments provides further knowledge of the underlying metabolic responses of hyperinsulinaemia, and subsequently on potential healthspan/lifespan. Finally, all our main results withstand a p-value correction, indicating this study was fully powered.

On the other hand, variations of findings between studies may occur due to the study population (e.g., females vs. males); female participants may respond differently to male cohorts due to hormonal changes. In addition to this, training status of the participants (trained vs. untrained) [151,152] or the duration of the study protocol may play a role [153]. Further work on metabolic health between different age groups (e.g., young vs. elderly population) and in healthy individuals in NK (as controls) vs. people with pathologies (i.e., cancer, T2D or elderly population with ageing-associated diseases, such as sarcopenia) in larger cohorts is also needed. Future investigations should also

conduct in-depth profiling analysis using RNA sequencing, proteomics, and metabolomics in response to hyperinsulinaemia.

Being a study on a wide range of physiological biomarkers (with different intrinsic magnitudes of variance), it is expected to have a large variability of p-values. We would like to highlight that while only insulin and IGF were used to calculate the sample size, most biomarkers showed statistical significance clearly below our multiple comparisons corrected p-value. Therefore, the borderline statistically significant findings in the measured cytokines and growth factors should be interpreted within the context of likely statistically underpowered tests on highly variable biomarkers and warrant further investigation as their trends are in resonance with the protective metabolic and inflammatory patterns we observed and that have been documented elsewhere.

5. Translational Importance

Recently, it has been shown in a large cohort study that a diet with a high proportion of carbohydrates significantly increases the risk of CVD [154]. This study, coupled with the studies discussed above, indicates that higher levels of insulin and IGF-1 are associated with increased morbidity and mortality risk. The current SUK Eatwell guideline recommendation to consume at least 267 g of carbohydrates a day effectively suppressed ketosis to a degree that by the end of this 21-day intervention, our participants were waking up with undetectable ketones on a capillary meter, indicating that insulin demand, secretion, and exposure had been enough to downregulate ketogenesis to even prevent a return to ketosis after an overnight fast. We therefore propose that the reduced concentration of BHB, accompanied by higher concentrations of insulin and IGF-1, may confer increased risk of morbidity and mortality over time, potentially increasing biological ageing rate.

6. Materials and Methods

6.1. Ethical Approval

Ethical approval was obtained by the College of Liberal of Arts and Sciences Research Ethics Committee, University of Westminster, United Kingdom (ETH2122-0634). All procedures were conducted in accordance with the Declaration of Helsinki and UK legislation. Written informed consent was obtained from all participants prior to their participation.

6.2. Participants

Ten healthy, habitually keto-adapted (living ketosis lifestyle prior to starting trial, self-reported average of 3.85 years), pre-menopausal women [age, 32.30 years $\pm$ 8.97; body mass index (BMI), 20.52 $\pm$ 1.39] were recruited. Participants were not receiving hormonal birth control and were classified as "metabolic phenotype 1" as defined by capillary BHB (> 0.3 mmol/L) and low fasting insulin < 130 pmol/L, with normo-glycaemia [30]. Habitual ketosis was determined by once-daily capillary BHB measurements between 4–6 p.m., before the evening meal, for 6 months prior to commencement of the study. A summary of the participants' characteristics at baseline is given in Table 1.

Exclusion criteria included smoking, taking any medication, and evidence of metabolic, immunological, or CVD. Participants were required to complete a medical history questionnaire to confirm that they were free from any of the above diseases.

6.3. Study Design

The study was an open-labelled, non-randomised cross-over trial with three phases: baseline nutritional ketosis (NK) (Phase 1; P1), suppression of ketosis (SuK) (Phase 2; P2) and removal of intervention, returning to NK (Phase 3; P3) (Figure 7).

For the duration of the study, participants were required to monitor their capillary glucose and ketone BHB concentrations (mmol/L) at four time points throughout the day to ascertain compliance (Tables 2 and 3). These time points were between 7:30–9:30 a.m., 11:30–13:30 p.m., 15:30–17:30 p.m. and 21:30–23:30 p.m. Participants determined capillary glucose and BHB using a Keto-Mojo™ GKI multi-function meter (Keto-Mojo, Napa,

CA, United States). This equipment was selected for its reliability and good diagnostic performance [155].

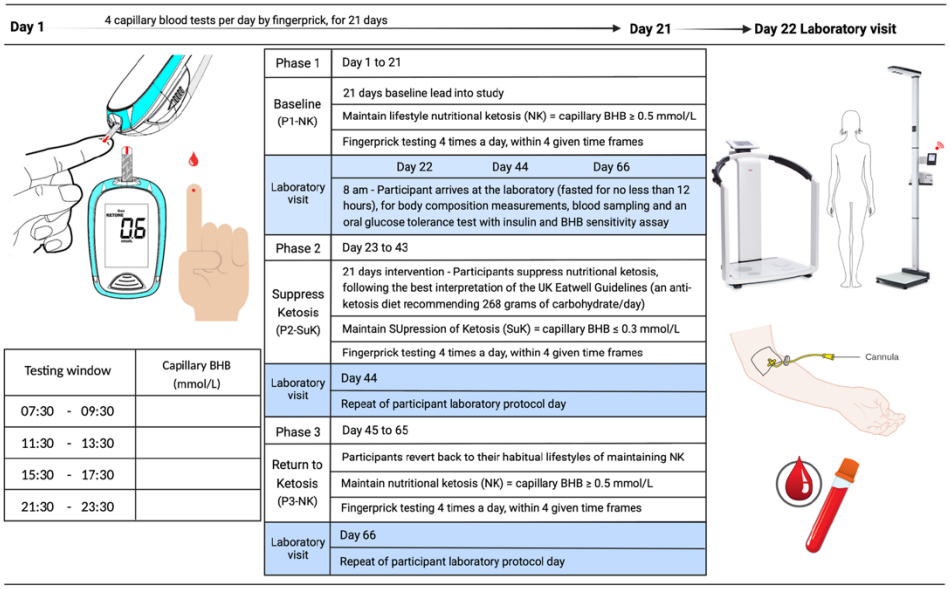

Figure 7. KetoSAge study design. Phase 1 and 3 covered the participants' habitual ketosis lifestyle. Phase 2 was the interventional phase to suppress ketosis (SuK). Each phase was monitored via finger prick testing of capillary beta-hydroxybutyrate (BHB) concentration (mmol/L). Testing was conducted four times per day, prior to mealtimes at evenly spaced intervals. At the end of each phase, participants underwent laboratory testing for body composition and blood sampling for biomarkers associated with chronic diseases and ageing, and were given an oral glucose tolerance test (75 g glucose in 250 mL water). Blood samples were taken at seven time points over 5 h. Whole blood glucose and BHB were measured sequentially in real time using the Keto-Mojo™ Meter, and plasma insulin sensitivity assay was conducted later using ELISA.

During P1, participants maintained lifestyle NK, as determined by maintenance of capillary blood concentration of BHB $\geq$ 0.5 mmol/L, through ad libitum consumption of a very-low-carbohydrate high-fat diet (VCHF), % carbohydrate:protein:fat = 8:17:75 [22,156] (this ratio is modulable according the metabolic health), ad libitum feeding within a time-restricted feeding (TRF) window, or a mixture of both (Tables 2 and 3).

On day 22 (visit 1), participants attended the Human Physiology Laboratory at the University of Westminster at the same time of day (8 a.m.) in an overnight fasted state (> 12 h) for baseline testing. The baseline visit included anthropometric measurements, metabolic measurements, including exchange analysis (VO_2, VCO_2), venous blood sample, and an oral glucose tolerance test (OGTT) with BHB sensitivity. On day 23, participants suppressed ketosis (P2) and capillary BHB was targeted to be sustained at < 0.3 mmol/L for 21 days. Participants adapted out of ketosis during days 23 to 43 by following their healthiest interpretation (ad libitum) of the UK Eatwell Guidelines (% carbohydrate:protein:fat = 55:20:25), which recommend consuming at least 267 g of carbohydrate per day, divided over at least three meals.

On day 44 (visit 2), participants reported to the laboratory at 8 a.m. having fasted overnight to complete the same measurements as during visit 1. On day 45, the trial intervention was removed, and participants returned to their habitual lifestyle patterns, resulting in a return to NK (P3), and during days 45 to 65, they continued to monitor their

capillary blood glucose and ketones, where BHB was maintained at ≥0.5 mmol/L, as in P1. On day 46 (visit 3), participants returned to the laboratory to repeat identical measurements as on previous visits. An overview of the study design is presented in Figure 7.

6.4. Anthropometric Measurements

Upon arrival at the laboratory, height (to nearest 0.1 cm) was measured using a stadiometer (Marsden HM-250P Leicester Height Measure), and body weight (to nearest 0.1 kg), BMI, fat mass, and total body water (TBW) were measured by bioelectrical impedance (BIA) using Seca® (mBCA 514 Medical Body Composition Analyzer, Gmbh&Co. KG, Hamburg, Germany) with participants being 12 h fasted, with an empty bladder, and with standardised clothing. In addition, waist and hip circumference measures were obtained with a non-stretch anthropometric circumference measuring tape (Seca® 201) while participants stood upright on both feet. The average value (cm) of three measurements was used for analysis.

6.5. Metabolic Measurements

Respiratory quotient (RQ) was measured by indirect calorimetry using a Quark RMR (COSMED srl, Rome, Italy) and was defined as the ratio of carbon dioxide (CO_2) production to oxygen (O_2) consumption. RQ was determined with the participants lying down at rest and with 15 min of lead time to allow respiration to equilibrate before measurements were taken. After RQ was determined, blood pressure was taken using an automatic upper arm blood pressure monitor (OMRON HEALTHCARE Co., Ltd., Kyoto, Japan).

6.6. Blood Collection

Following anthropometric measurements, a single-use sterile 22G Terumo (Japan, Tokyo) Versatus Winged and Ported IV Catheter (Cannula) was inserted into the participants' antecubital vein for blood sampling. Saline solution flushes (0.9% NaCl, 5 mL, BD PosiFlush SP Syringe) were delivered in order to keep the intravenous line patent. A total of 2 mL of blood was drawn and discarded prior to each blood draw to prevent blood sampling saline dilution.

Blood was drawn into tubes anti-coagulated with either ethylenediaminetetraacetic acid (EDTA) or lithium heparin (BD, Oxford, UK), ready for analysis by SYNLAB (see Section 6.7). Blood was also drawn into serum SST™ II Advance tubes with thrombin rapid clot activator and separation gel (BD, Oxford, UK) and left for 30 min at room temperature. Serum tubes were then centrifuged (Hettich Zentrifugen, Universal 320 R, Tuttlingen, Germany) at 3857× *g* for 10 min at room temperature. Serum samples were either sent to SYNLAB for analysis or aliquoted into cryovial tubes under sterile conditions and stored at −80 °C for later analysis by Randox (see Section 6.7).

6.7. Blood Profiling Analysis

Following blood draw, the blood samples were immediately sent to SYNLAB Belgium (Alexander Fleming, 3–6220 Heppignies–Company No: 0453.111.546) to determine the concentrations of the following markers: insulin, insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein 3 (IGFBP-3), C-reactive protein (CRP), gamma-glutamyl transferase (GGT), cortisol, plasminogen activator inhibitor-1 (PAI-1), total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, triglycerides, thyroid stimulating hormone (TSH), free triiodothyronine (T3), reverse T3, and thyroxine (T4).

At the end of the trial, frozen serum samples were sent to Randox Ireland (55 Diamond Road, Crumlin, Co. Antrim, BT29 4QY, company number: NI015738) to determine the concentrations of various cytokines and growth factors. These included: epithelial growth factor (EGF), vascular endothelial growth factor (VEGF), interferon-gamma (INF-γ), monocyte chemotactic protein (MCP-1), tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1a, IL-1b, IL-2, IL-4, IL-6, IL-8, and IL-10.

6.8. Oral Glucose Tolerance Test

Following anthropometric and metabolic measurements and blood sampling, participants were subjected to an OGTT. 75 g of glucose in 250 mL water (prepared fresh on each day) was consumed by participants within 5 min. Blood samples were then drawn into EDTA tubes via cannula at 7 time points: 0 min (before glucose bolus), 30, 60, 120, 180, 240, and 300 min. All samples were immediately spun at 3857× *g* for 10 min at 4 °C to obtain the plasma fraction. Plasma was aliquoted under sterile conditions and stored at −80 °C for later batch analysis. Plasma insulin concentrations were determined by Quantikine ELISA (R&D Systems), following the manufacturer's instructions. Samples were thawed once and analysed in triplicate. Throughout the OGTT, at each time point, venous whole blood was used to measure glucose and BHB concentrations, measured in triplicate, using a Keto-Mojo™ GKI multi-function meter.

6.9. Statistical Analysis

All data was found to be normally distributed by the Shapiro–Wilk test, and therefore parametric analyses were conducted. Repeated measures one-way analysis of variance (ANOVA) was used to evaluate differences in various parameters between the three phases (baseline ketosis P1, suppression of ketosis P2, and return to ketosis P3), or across time points (0, 30, 60, 120, 180, 240, and 300 min) for OGTT on glucose, insulin, and BHB. Tukey's HSD test was used for post hoc analysis to perform pairwise comparisons, and p values < 0.05 were considered statistically significant. Data are presented as mean ± standard deviation, unless otherwise stated. Statistical analysis was performed and all figures were generated in GraphPad Prism (v9; San Diego, CA, USA).

6.10. Sample Size Calculation

Sample size was calculated based on pilot feasibility data with 5 participants put through all 3 phases. We calculated sample size using changes in fasted insulin and IGF-1 concentrations. The sample size was estimated using G*Power (v3.1) with an alpha level of 0.05, a power (1-β) of 0.80, a medium effect size of f = 0.5, and a conservative intra-measurement correlation of 0.5. This analysis recommended a sample size of n = 9, that predicted to produce results with an effect size of 1.1 d_z for paired comparisons.

7. Conclusions

Evolutionary evidence suggests that ancestral populations were predominantly adapted to patterns of intermittent and time-restricted feeding, as opposed to continuous nutritional intake, rich in farinaceous and sucrose carbohydrates that stimulate bolus insulin secretion. The escalating prevalence of T2DM, obesity, CVD, AD, and cancer observed in populations adhering to multiple substantial carbohydrate-dominated meals in developed nations is a testament to this. Individuals maintaining long-standing habitual NK, when subjected to 21 days of consuming carbohydrate to suppress ketosis, followed with restricting carbohydrate, reverted to an evolutionary ketotic state within one day, indicate metabolic flexibility and health. The negative changes in biomarkers associated with chronic diseases and ageing, which occur from a one-time excursion in a 1-year period of 21 consecutive days of suppressing ketosis, are rapidly restored after restoring the baseline dietary lifestyle of carbohydrate restriction which does not overstimulate insulin demand and secretion. Our data show that long-standing NK appears to provide major health benefits in the maintenance of euglycaemia, with low insulin and IGF-1, the triad of markers most strongly associated with chronic diseases and biological ageing. NK serves as a reliable surrogate marker for these parameters to understand an individual's metabolic phenotype, and therefore risk. This study was conducted to establish a detailed metabolic phenotype biomarker profile in a long-standing healthy ketosis cohort, providing a NK control group for other studies to establish metabolic phenotypes in people with cancer, CVD, AD, T2DM, and ageing, and to assess treatment efficacy using KMT in gaining better health. Overall, sustained NK may mitigate hyperinsulinemia without impairing metabolic flexibility and carbohydrate

tolerance in metabolically healthy individuals. Maintaining low insulin requirement and IGF-1 levels through endogenous NK may offer lower chronic disease risk, resulting in benefits to both lifespan and healthspan.

Author Contributions: I.D.C. conceived the hypothesis and study design, wrote the original draft, designed the figures, and reviewed and edited the final manuscript. I.D.C. and Y.K. conducted the trial. I.D.C., Y.K., S.L.J., V.B., V.N. and R.A.-F. collected and compiled data. I.D.C., Y.K., K.E. and L.P. performed ex vivo bench top assays. I.D.C., Y.K., K.E. and L.P. analysed data, designed tables and graphs. I.D.C., Y.K., K.E., L.P., T.N.S., T.D., A.S.-M., A.S., K.B. and B.T.E. contributed to writing and reviewed and edited the final manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the College of Liberal of Arts and Sciences Research Ethics Committee, University of Westminster. Ethical approval was obtained by the College of Liberal of Arts and Sciences Research Ethics Committee, University of Westminster, United Kingdom (ETH2122-0634).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

Acknowledgments: The authors would like to acknowledge the time and dedication of all participants who participated in this study. The authors would like to acknowledge Camilla Holland for human trials laboratory technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. O'Connor, D.; Molloy, A.M.; Laird, E.; Kenny, R.A.; O'Halloran, A.M. Sustaining an ageing population: The role of micronutrients in frailty and cognitive impairment. *Proc. Nutr. Soc.* **2023**, *82*, 315–328. [CrossRef]
2. UN. World Population Ageing 2019: Highlights. 2019. Available online: https://www.un.org/en/development/desa/population/publications/pdf/ageing/WorldPopulationAgeing2019-Report.pdf (accessed on 13 September 2023).
3. ONS. Death Registration Summary Statistics, England and Wales—Office for National Statistics. 2023. Available online: https://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriages/deaths/articles/deathregistrationsummarystatisticsenglandandwales/2022 (accessed on 14 September 2023).
4. WHO. Global Health Estimates: Leading Causes of Death. 2019. Available online: https://www.who.int/data/gho/data/themes/mortality-and-global-health-estimates/ghe-leading-causes-of-death (accessed on 13 September 2023).
5. Araújo, J.; Cai, J.; Stevens, J. Prevalence of optimal metabolic ealth in american adults: National health and nutrition examination survey 2009–2016. *Metab. Syndr. Relat. Disord.* **2018**, *17*, 46–52. [CrossRef]
6. Cooper, I.D.; Kyriakidou, Y.; Petagine, L.; Edwards, K.; Elliott, B.T. Bio-hacking better health—Leveraging metabolic biochemistry to maximise healthspan. *Antioxidants* **2023**, *12*, 1749. [CrossRef]
7. Crofts, C.A.P.; Zinn, C.; Wheldon, M.; Schofield, M. Hyperinsulinemia: A unifying theory of chronic disease? *Diabesity* **2015**, *1*, 34. [CrossRef]
8. Cooper, I.D.; Brookler, K.H.; Kyriakidou, Y.; Elliott, B.T.; Crofts, C.A.P. Metabolic phenotypes and step by step evolution of type 2 diabetes: A new paradigm. *Biomedicines* **2021**, *9*, 800. [CrossRef] [PubMed]
9. Ambroselli, D.; Masciulli, F.; Romano, E.; Catanzaro, G.; Besharat, Z.M.; Massari, M.C.; Ferretti, E.; Migliaccio, S.; Izzo, L.; Ritieni, A.; et al. New advances in metabolic syndrome, from prevention to treatment: The role of diet and food. *Nutrients* **2023**, *15*, 640. [CrossRef]
10. Hayflick, L.; Moorhead, P.S. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **1961**, *25*, 585–621. [CrossRef] [PubMed]
11. Veech, R.L.; Bradshaw, P.C.; Clarke, K.; Curtis, W.; Pawlosky, R.; King, M.T. Ketone bodies mimic the life span extending properties of caloric restriction. *IUBMB Life* **2017**, *69*, 305–314. [CrossRef]
12. Saxton, R.A.; Sabatini, D.M. mTOR Signaling in Growth, Metabolism, and Disease. *Cell* **2017**, *168*, 960–976. [CrossRef] [PubMed]
13. Pignatti, C.; D'adamo, S.; Stefanelli, C.; Flamigni, F.; Cetrullo, S. Nutrients and pathways that regulate health span and life span. *Geriatrics* **2020**, *5*, 95. [CrossRef]
14. Ottens, F.; Franz, A.; Hoppe, T. Build-UPS and break-downs: Metabolism impacts on proteostasis and aging. *Cell Death Differ.* **2021**, *28*, 505–521. [CrossRef]

15. Roberts, M.N.; Wallace, M.A.; Tomilov, A.A.; Zhou, Z.; Marcotte, G.R.; Tran, D.; Perez, G.; Gutierrez-Casado, E.; Koike, S.; Knotts, T.A.; et al. A ketogenic diet extends longevity and healthspan in adult mice. *Cell Metab.* **2017**, *26*, 539–546.e5. [CrossRef]
16. Napoleão, A.; Fernandes, L.; Miranda, C.; Marum, A.P. Effects of calorie restriction on health span and insulin resistance: Classic calorie restriction diet vs. ketosis-inducing diet. *Nutrients* **2021**, *13*, 1302. [CrossRef]
17. Newman, J.C.; Verdin, E. Ketone bodies as signaling metabolites. *Trends Endocrinol. Metab.* **2014**, *25*, 42–52. [CrossRef]
18. Shimazu, T.; Hirschey, M.D.; Newman, J.; He, W.; Shirakawa, K.; Le Moan, N.; Grueter, C.A.; Lim, H.; Saunders, L.R.; Stevens, R.D.; et al. Suppression of oxidative stress by β-hydroxybutyrate, an endogenous histone deacetylase inhibitor. *Science* **2013**, *339*, 211–214. [CrossRef] [PubMed]
19. Grabacka, M.; Pierzchalska, M.; Dean, M.; Reiss, K. Regulation of ketone body metabolism and the role of PPARα. *Int. J. Mol. Sci.* **2016**, *17*, 2093. [CrossRef]
20. Wang, L.; Chen, P.; Xiao, W. β-hydroxybutyrate as an anti-aging metabolite. *Nutrients* **2021**, *13*, 3420. [CrossRef]
21. Puchalska, P.; Crawford, P.A. Multi-dimensional roles of ketone bodies in fuel metabolism, signaling, and therapeutics. *Cell Metab.* **2017**, *25*, 262–284. [CrossRef] [PubMed]
22. Miller, V.J.; Villamena, F.A.; Volek, J.S. Nutritional ketosis and mitohormesis: Potential implications for mitochondrial function and human health. *J. Nutr. Metab.* **2018**, *2018*, 5157645. [CrossRef]
23. Qu, C.; Keijer, J.; Adjobo-Hermans, M.J.W.; van de Wal, M.; Schirris, T.; van Karnebeek, C.; Pan, Y.; Koopman, W.J.H. The ketogenic diet as a therapeutic intervention strategy in mitochondrial disease. *Int. J. Biochem. Cell Biol.* **2021**, *138*, 106050. [CrossRef] [PubMed]
24. Benjamin, D.I.; Both, P.; Benjamin, J.S.; Nutter, C.W.; Tan, J.H.; Kang, J.; Machado, L.A.; Klein, J.D.D.; de Morree, A.; Kim, S.; et al. Fasting induces a highly resilient deep quiescent state in muscle stem cells via ketone body signaling. *Cell Metab.* **2022**, *34*, 902–918.e6. [CrossRef]
25. Zhu, H.; Bi, D.; Zhang, Y.; Kong, C.; Du, J.; Wu, X.; Wei, Q.; Qin, H. Ketogenic diet for human diseases: The underlying mechanisms and potential for clinical implementations. *Signal Transduct. Target. Ther.* **2022**, *7*, 11. [CrossRef]
26. Nadal, A.; Marrero, P.F.; Haro, D. Down-regulation of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by insulin: The role of the forkhead transcription factor FKHRL1. *Biochem. J.* **2002**, *366*, 289–297. [CrossRef]
27. DiNicolantonio, J.J.; Bhutani, J.; OKeefe, J.H.; Crofts, C. Postprandial insulin assay as the earliest biomarker for diagnosing pre-diabetes, type 2 diabetes and increased cardiovascular risk. *Open Heart* **2017**, *4*, e000656. [CrossRef]
28. Cooper, I.D.; Brookler, K.H.; Crofts, C.A.P. Rethinking fragility fractures in type 2 diabetes: The link between hyperinsulinaemia and osteofragilitas. *Biomedicines* **2021**, *9*, 1165. [CrossRef]
29. PHE. *Government Dietary Recommendations: Government Recommendations for Energy and Nutrients for Males and Females Aged 1–18 Years and 19+ Years*; PHE Publications Gateway Number: 2016202; Department of Health: London, UK, 2016.
30. Cooper, I.D.; Crofts, C.A.P.; DiNicolantonio, J.J.; Malhotra, A.; Elliott, B.; Kyriakidou, Y.; Brookler, K.H. Relationships between hyperinsulinaemia, magnesium, vitamin D, thrombosis and COVID-19: Rationale for clinical management. *Open Heart* **2020**, *7*, e001356. [CrossRef] [PubMed]
31. Dilliraj, L.N.; Schiuma, G.; Lara, D.; Strazzabosco, G.; Clement, J.; Giovannini, P.P.; Trapella, C.; Narducci, M.; Rizzo, R. The evolution of ketosis: Potential impact on clinical conditions. *Nutrients* **2022**, *14*, 3613. [CrossRef]
32. Bronisz, A.; Ozorowski, M.; Hagner-Derengowska, M. Pregnancy ketonemia and development of the fetal central nervous system. *Int. J. Endocrinol.* **2018**, *2018*, 1242901. [CrossRef] [PubMed]
33. Kinzig, K.P.; Honors, M.A.; Hargrave, S.L. Insulin sensitivity and glucose tolerance are altered by maintenance on a ketogenic diet. *Endocrinology* **2010**, *151*, 3105–3114. [CrossRef]
34. Skow, S.L.; Jha, R.K. A ketogenic diet is effective in improving insulin sensitivity in individuals with type 2 diabetes. *Curr. Diabetes Rev.* **2022**, *19*, e250422203985. [CrossRef] [PubMed]
35. Paoli, A.; Bianco, A.; Moro, T.; Mota, J.F.; Coelho-Ravagnani, C.F.; Paoli, A.; Bianco, A.; Moro, T.; Felipe Mota, J.; Coelho-Ravagnani, C.F. The effects of ketogenic diet on insulin sensitivity and weight loss, which came first: The chicken or the egg? *Nutrients* **2023**, *15*, 3120. [CrossRef]
36. Jiang, Z.; Yin, X.; Wang, M.; Chen, T.; Wang, Y.; Gao, Z.; Wang, Z. Effects of ketogenic diet on neuroinflammation in neurodegenerative diseases. *Aging Dis.* **2022**, *13*, 1146. [CrossRef]
37. Koh, S.; Dupuis, N.; Auvin, S. Ketogenic diet and neuroinflammation. *Epilepsy Res.* **2020**, *167*, 106454. [CrossRef] [PubMed]
38. Arima, Y.; Nakagawa, Y.; Takeo, T.; Ishida, T.; Yamada, T.; Hino, S.; Nakao, M.; Hanada, S.; Umemoto, T.; Suda, T.; et al. Murine neonatal ketogenesis preserves mitochondrial energetics by preventing protein hyperacetylation. *Nat. Metab.* **2021**, *3*, 196–210. [CrossRef] [PubMed]
39. Schutz, Y.; Ravussin, E. Respiratory quotients lower than 0.70 in ketogenic diets. *Am. J. Clin. Nutr.* **1980**, *33*, 1317–1319. [CrossRef] [PubMed]
40. Goldenshluger, A.; Constantini, K.; Goldstein, N.; Shelef, I.; Schwarzfuchs, D.; Zelicha, H.; Meir, A.Y.; Tsaban, G.; Chassidim, Y.; Gepner, Y. Effect of dietary strategies on respiratory quotient and its association with clinical parameters and organ fat loss: A randomized controlled trial. *Nutrients* **2021**, *13*, 2230. [CrossRef]
41. Ramos-Jiménez, A.; Hernández-Torres, R.P.; Torres-Durán, P.V.; Romero-Gonzalez, J.; Mascher, D.; Posadas-Romero, C.; Juárez-Oropeza, M.A. The Respiratory Exchange Ratio is Associated with Fitness Indicators Both in Trained and Untrained Men: A Possible Application for People with Reduced Exercise Tolerance. *Clin. Med. Circ. Respirat. Pulm. Med.* **2008**, *2*, 1–9. [CrossRef]

42. Bergman, B.C.; Brooks, G.A. Respiratory gas-exchange ratios during graded exercise in fed and fasted trained and untrained men. *J. Appl. Physiol.* **1999**, *86*, 479–487. [CrossRef]
43. Nishida, C.; Ko, G.T.; Kumanyika, S. Body fat distribution and noncommunicable diseases in populations: Overview of the 2008 WHO Expert Consultation on Waist Circumference and Waist–Hip Ratio. *Eur. J. Clin. Nutr.* **2009**, *64*, 2–5. [CrossRef]
44. WHO. Waist Circumference and Waist–Hip Ratio. WHO Expert. 2011. Available online: https://www.who.int/publications/i/item/9789241501491 (accessed on 14 September 2023).
45. Choi, S.M.; Tucker, D.F.; Gross, D.N.; Easton, R.M.; DiPilato, L.M.; Dean, A.S.; Monks, B.R.; Birnbaum, M.J. Insulin regulates adipocyte lipolysis via an Akt-independent signaling pathway. *Mol. Cell. Biol.* **2010**, *30*, 5009. [CrossRef]
46. Althaher, A.R. An overview of hormone-sensitive lipase (HSL). *Sci. World J.* **2022**, *2022*, 1964684. [CrossRef] [PubMed]
47. Lan, Y.L.; Lou, J.C.; Lyu, W.; Zhang, B. Update on the synergistic effect of HSL and insulin in the treatmentof metabolic disorders. *Ther. Adv. Endocrinol. Metab.* **2019**, *10*, 2042018819877300. [CrossRef]
48. Sandhu, M.S.; Dunger, D.B.; Giovannucci, E.L. Insulin, insulin-like growth factor-I (IGF-I), IGF binding proteins, their biologic interactions, and colorectal cancer. *JNCI J. Natl. Cancer Inst.* **2002**, *94*, 972–980. [CrossRef]
49. Goalstone, M.L.; Leitner, J.W.; Wall, K.; Dolgonos, L.; Rother, K.I.; Accili, D.; Draznin, B. Effect of insulin on farnesyltransferase. *J. Biol. Chem.* **1998**, *273*, 23892–23896. [CrossRef]
50. Draznin, B.; Miles, P.; Kruszynska, Y.; Olefsky, J.; Friedman, J.; Golovchenko, I.; Stjernholm, R.; Wall, K.; Reitman, M.; Accili, D.; et al. Effects of insulin on prenylation as a mechanism of potentially detrimental influence of hyperinsulinemia. *Endocrinology* **2000**, *141*, 1310–1316. [CrossRef]
51. Goalstone, M.L.; Draznin, B. What does insulin do to Ras? *Cell. Signal.* **1998**, *10*, 297–301. [CrossRef]
52. Cao, J.; Yee, D. Disrupting insulin and IGF receptor function in cancer. *Int. J. Mol. Sci.* **2021**, *22*, 555. [CrossRef]
53. Pollak, M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat. Rev. Cancer* **2008**, *8*, 915–928. [CrossRef]
54. Zhang, W.B.; Aleksic, S.; Gao, T.; Weiss, E.F.; Demetriou, E.; Verghese, J.; Holtzer, R.; Barzilai, N.; Milman, S. Insulin-like growth factor-1 and IGF binding proteins predict all-cause mortality and morbidity in older adults. *Cells* **2020**, *9*, 1368. [CrossRef] [PubMed]
55. Narasimhan, S.D.; Yen, K.; Tissenbaum, H.A. Converging pathways in lifespan regulation. *Curr. Biol.* **2009**, *19*, R657. [CrossRef] [PubMed]
56. Barbieri, M.; Bonafè, M.; Franceschi, C.; Paolisso, G. Insulin/IGF-I-signaling pathway: An evolutionarily conserved mechanism of longevity from yeast to humans. *Am. J. Physiol.-Endocrinol. Metab.* **2003**, *285*, E1064–E1071. [CrossRef] [PubMed]
57. Holzenberger, M.; Dupont, J.; Ducos, B.; Leneuve, P.; Géloën, A.; Even, P.C.; Cervera, P.; Le Bouc, Y. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* **2003**, *421*, 182–187. [CrossRef] [PubMed]
58. Rahmani, J.; Montesanto, A.; Giovannucci, E.; Zand, H.; Barati, M.; Kopchick, J.J.; Mirisola, M.G.; Lagani, V.; Bawadi, H.; Vardavas, R.; et al. Association between IGF-1 levels ranges and all-cause mortality: A meta-analysis. *Aging Cell* **2022**, *21*, e13540. [CrossRef]
59. Jag, U.R.; Zavadil, J.; Stanley, F.M. Insulin acts through FOXO3a to activate transcription of plasminogen activator inhibitor type 1. *Mol. Endocrinol.* **2009**, *23*, 1587–1602. [CrossRef] [PubMed]
60. Webb, A.E.; Brunet, A. FOXO transcription factors: Key regulators of cellular quality control. *Trends Biochem. Sci.* **2014**, *39*, 159. [CrossRef] [PubMed]
61. Klotz, L.O.; Sánchez-Ramos, C.; Prieto-Arroyo, I.; Urbánek, P.; Steinbrenner, H.; Monsalve, M. Redox regulation of FoxO transcription factors. *Redox Biol.* **2015**, *6*, 51–72. [CrossRef] [PubMed]
62. Newman, J.C.; Verdin, E. β-hydroxybutyrate: A signaling metabolite. *Annu. Rev. Nutr.* **2017**, *37*, 51–76. [CrossRef]
63. Miyauchi, T.; Uchida, Y.; Kadono, K.; Hirao, H.; Kawasoe, J.; Watanabe, T.; Ueda, S.; Okajima, H.; Terajima, H.; Uemoto, S. Up-regulation of FOXO1 and reduced inflammation by β-hydroxybutyric acid are essential diet restriction benefits against liver injury. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 13533–13542. [CrossRef]
64. Willcox, B.J.; Donlon, T.A.; He, Q.; Chen, R.; Grove, J.S.; Yano, K.; Masaki, K.H.; Willcox, D.C.; Rodriguez, B.; Curb, J.D. FOXO3A genotype is strongly associated with human longevity. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 13987–13992. [CrossRef]
65. Visscher, T.L.S.; Seidell, J.C. The public health impact of obesity. *Annu. Rev. Public Health* **2001**, *22*, 355–375. [CrossRef]
66. Ahmed, S.B.M.; Radwan, N.; Amer, S.; Saheb Sharif-Askari, N.; Mahdami, A.; Samara, K.A.; Halwani, R.; Jelinek, H.F. Assessing the link between diabetic metabolic dysregulation and breast cancer progression. *Int. J. Mol. Sci.* **2023**, *24*, 11816. [CrossRef]
67. WHO. Breast Cancer. World Health Organization. 2023. Available online: https://www.who.int/news-room/fact-sheets/detail/breast-cancer#:~:text=In2020%2C (accessed on 19 September 2023).
68. Kautzky-Willer, A.; Harreiter, J.; Pacini, G. Sex and gender differences in risk, pathophysiology and complications of type 2 diabetes mellitus. *Endocr. Rev.* **2016**, *37*, 278–316. [CrossRef] [PubMed]
69. Bolla, A.M.; Caretto, A.; Laurenzi, A.; Scavini, M.; Piemonti, L. Low-carb and ketogenic diets in type 1 and type 2 diabetes. *Nutrients* **2019**, *11*, 962. [CrossRef]
70. Paradies, G.; Paradies, V.; Ruggiero, F.M.; Petrosillo, G. Role of cardiolipin in mitochondrial function and dynamics in health and disease: Molecular and pharmacological aspects. *Cells* **2019**, *8*, 728. [CrossRef] [PubMed]
71. Martinez, B.; Ortiz, R.M. Thyroid hormone regulation and insulin resistance: Insights from animals naturally adapted to fasting. *Physiology* **2017**, *32*, 141–151. [CrossRef] [PubMed]
72. Petagine, L.; Zariwala, M.G.; Patel, V.B. Non-alcoholic fatty liver disease: Immunological mechanisms and current treatments. *World J. Gastroenterol.* **2023**, *29*, 4831–4850. [CrossRef]

73. Demant, M.; Bagger, J.I.; Suppli, M.P.; Lund, A.; Gyldenløve, M.; Hansen, K.B.; Hare, K.J.; Christensen, M.; Sonne, D.P.; Holst, J.J.; et al. Determinants of fasting hyperglucagonemia in patients with type 2 diabetes and nondiabetic control subjects. *Metab. Syndr. Relat. Disord.* **2018**, *16*, 530–536. [CrossRef]
74. Kumpatla, S.; Parveen, R.; Murugan, P.; Juttada, U.; Devarajan, A.; Viswanathan, V. Hyperglucagonemia and impaired insulin sensitivity are associated with development of prediabetes and type 2 diabetes—A study from South India. *Diabetes Metab. Syndr. Clin. Res. Rev.* **2021**, *15*, 102199. [CrossRef]
75. Luchsinger, J.A.; Tang, M.X.; Shea, S.; Mayeux, R. Hyperinsulinemia and risk of Alzheimer disease. *Neurology* **2004**, *63*, 1187–1192. [CrossRef] [PubMed]
76. Grundy, S.M. Gamma-glutamyl transferase: Another biomarker for metabolic syndrome and cardiovascular risk. *Arterioscler. Thromb. Vasc. Biol.* **2007**, *27*, 4–7. [CrossRef] [PubMed]
77. Unwin, D.J.; Cuthbertson, D.J.; Feinman, R.; Sprung, V.S. A pilot study to explore the role of a low-carbohydrate intervention to improve GGT levels and HbA 1c. *Diabesity Pract.* **2015**, *4*, 102–108.
78. Kwak, J.; Seo, I.H.; Lee, Y.J. Serum γ-glutamyltransferase level and incidence risk of metabolic syndrome in community dwelling adults: Longitudinal findings over 12 years. *Diabetol. Metab. Syndr.* **2023**, *15*, 29. [CrossRef] [PubMed]
79. Gohel, M.G.; Chacko, A.N. Serum GGT activity and hsCRP level in patients with type 2 diabetes mellitus with good and poor glycemic control: An evidence linking oxidative stress, inflammation and glycemic control. *J. Diabetes Metab. Disord.* **2013**, *12*, 56. [CrossRef] [PubMed]
80. Drozdz, R.; Parmentier, C.; Hachad, H.; Leroy, P.; Siest, Ǵ.; Wellman, M. γ-glutamyltransferase dependent generation of reactive oxygen species from a glutathione/transferrin system. *Free Radic. Biol. Med.* **1998**, *25*, 786–792. [CrossRef]
81. Wang, Y.; Wu, T.; Zang, X.; Liu, X.; Xu, W.; Lai, P.; Wang, Y.; Teng, F.; Qiu, Q.; Geng, H.; et al. Relationship between serum gamma-glutamyl transferase level and impaired fasting glucose among chinese community-dwelling adults: A follow-up observation of 6 years. *Metab. Syndr. Relat. Disord.* **2021**, *19*, 100–106. [CrossRef]
82. Xing, M.; Gao, M.; Li, J.; Han, P.; Mei, L.; Zhao, L. Characteristics of peripheral blood Gamma-glutamyl transferase in different liver diseases. *Medicine* **2022**, *101*, E28443. [CrossRef]
83. Bonnet, F.; Ducluzeau, P.H.; Gastaldelli, A.; Laville, M.; Anderwald, C.H.; Konrad, T.; Mari, A.; Balkau, B. Liver enzymes are associated with hepatic insulin resistance, insulin secretion, and glucagon concentration in healthy men and women. *Diabetes* **2011**, *60*, 1660. [CrossRef]
84. Björk, M.P.; Johansson, B. Gamma-Glutamyltransferase (GGT) as a biomarker of cognitive decline at the end of life: Contrasting age and time to death trajectories. *Int. Psychogeriatr.* **2018**, *30*, 981–990. [CrossRef]
85. Sillen, M.; Declerck, P.J. A narrative review on plasminogen activator inhibitor-1 and Its (patho)physiological role: To target or not to target? *Int. J. Mol. Sci.* **2021**, *22*, 2721. [CrossRef]
86. Samad, F.; Pandey, M.; Bell, P.A.; Loskutoff, D.J. Insulin continues to induce plasminogen activator inhibitor 1 gene expression in insulin-resistant mice and adipocytes. *Mol. Med.* **2000**, *6*, 680–692. [CrossRef]
87. Altalhi, R.; Pechlivani, N.; Ajjan, R.A. PAI-1 in diabetes: Pathophysiology and role as a therapeutic target. *Int. J. Mol. Sci.* **2021**, *22*, 3170. [CrossRef]
88. Yarmolinsky, J.; Bordin Barbieri, N.; Weinmann, T.; Ziegelmann, P.K.; Duncan, B.B.; Inês Schmidt, M. Plasminogen activator inhibitor-1 and type 2 diabetes: A systematic review and meta-analysis of observational studies. *Sci. Rep.* **2016**, *6*, 17714. [CrossRef] [PubMed]
89. Booth, N.A.; Simpson, A.J.; Croll, A.; Bennett, B.; MacGregor, I.R. Plasminogen activator inhibitor (PAI-1) in plasma and platelets. *Br. J. Haematol.* **1988**, *70*, 327–333. [CrossRef] [PubMed]
90. Leitner, J.W.; Kline, T.; Carel, K.; Goalstone, M.; Draznin, B. Hyperinsulinemia potentiates activation of p21Ras by growth factors. *Endocrinology* **1997**, *138*, 2211–2214. [CrossRef] [PubMed]
91. Hopkins, B.D.; Pauli, C.; Xing, D.; Wang, D.G.; Li, X.; Wu, D.; Amadiume, S.C.; Goncalves, M.D.; Hodakoski, C.; Lundquist, M.R.; et al. Suppression of insulin feedback enhances the efficacy of PI3K inhibitors. *Nature* **2018**, *560*, 499–503. [CrossRef]
92. Bye, A.P.; Unsworth, A.J.; Gibbins, J.M. Platelet signaling: A complex interplay between inhibitory and activatory networks. *J. Thromb. Haemost.* **2016**, *14*, 918. [CrossRef]
93. Juhan-Vague, I.; Alessi, M.C.; Mavri, A.; Morange, P.E. Plasminogen activator inhibitor-1, inflammation, obesity, insulin resistance and vascular risk. *J. Thromb. Haemost.* **2003**, *1*, 1575–1579. [CrossRef]
94. Alessi, M.C.; Peiretti, F.; Morange, P.; Henry, M.; Nalbone, G.; Juhan-Vague, I. Production of plasminogen activator inhibitor 1 by human adipose tissue: Possible link between visceral fat accumulation and vascular disease. *Diabetes* **1997**, *46*, 860–867. [CrossRef]
95. Ellulu, M.S.; Patimah, I.; Khaza'ai, H.; Rahmat, A.; Abed, Y. Obesity and inflammation: The linking mechanism and the complications. *Arch. Med. Sci.* **2017**, *13*, 851. [CrossRef]
96. Pandey, M.; Loskutoff, D.J.; Samad, F. Molecular mechanisms of tumor necrosis factor-alpha-mediated plasminogen activator inhibitor-1 expression in adipocytes. *FASEB J.* **2005**, *19*, 1317–1319. [CrossRef]
97. Devy, L.; Blacher, S.; Grignet-Debrus, C.; Bajou, K.; Masson, V.; Gerard, R.D.; Gils, A.; Carmeliet, G.; Carmeliet, P.; Declerck, P.J.; et al. The pro- or antiangiogenic effect of plasminogen activator inhibitor 1 is dose dependent. *FASEB J.* **2002**, *16*, 147–154. [CrossRef]

98. Balsara, R.D.; Ploplis, V.A. Plasminogen activator inhibitor-1: The double-edged sword in apoptosis. *Thromb. Haemost.* **2008**, *100*, 1029–1036. [CrossRef]
99. Bajou, K.; Noël, A.; Gerard, R.D.; Masson, V.; Brunner, N.; Holst-Hansen, C.; Skobe, M.; Fusenig, N.E.; Carmeliet, P.; Collen, D.; et al. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat. Med.* **1998**, *4*, 923–928. [CrossRef]
100. Roy, A.; Coum, A.; Marinescu, V.D.; Põlajeva, J.; Smits, A.; Nelander, S.; Uhrbom, L.; Westermark, B.; Forsberg-Nilsson, K.; Pontén, F.; et al. Glioma-derived plasminogen activator inhibitor-1 (PAI-1) regulates the recruitment of LRP1 positive mast cells. *Oncotarget* **2015**, *6*, 23647–23661. [CrossRef]
101. Harbeck, N.; Kates, R.E.; Gauger, K.; Willems, A.; Kiechle, M.; Magdolen, V.; Schmitt, M. Urokinase-type plasminogen activator (uPA) and its inhibitor PAI-I: Novel tumor-derived factors with a high prognostic and predictive impact in breast cancer. *Thromb. Haemost.* **2004**, *92*, 47–53. [CrossRef]
102. Leissner, P.; Verjat, T.; Bachelot, T.; Paye, M.; Krause, A.; Puisieux, A.; Mougin, B. Prognostic significance of urokinase plasminogen activator and plasminogen activator inhibitor-1 mRNA expression in lymph node- and hormone receptor-positive breast cancer. *BMC Cancer* **2006**, *6*, 216. [CrossRef] [PubMed]
103. Duffy, M.J.; O'Donovan, N.; McDermott, E.; Crown, J. Validated biomarkers: The key to precision treatment in patients with breast cancer. *Breast* **2016**, *29*, 192–201. [CrossRef]
104. Jevrić, M.; Matić, I.Z.; Krivokuća, A.; Crnogorac, M.D.; Besu, I.; Damjanović, A.; Branković-Magić, M.; Milovanović, Z.; Gavrilović, D.; Susnjar, S.; et al. Association of uPA and PAI-1 tumor levels and 4G/5G variants of PAI-1 gene with disease outcome in luminal HER2-negative node-negative breast cancer patients treated with adjuvant endocrine therapy. *BMC Cancer* **2019**, *19*, 71. [CrossRef]
105. Sobocan, M.; Ledinek, Z.; Crnobrnja, B.; Sikošek, N.Č.; Dovnik, N.F.; Kavalar, R.; Knez, J.; Takač, I. The value of uPA and PAI-1 levels in triple negative breast cancer. *Eur. J. Cancer* **2020**, *138*, S73–S74. [CrossRef]
106. Becker, M.; Szarvas, T.; Wittschier, M.; Vom Dorp, F.; Tötsch, M.; Schmid, K.W.; Rübben, H.; Ergün, S. Prognostic impact of plasminogen activator inhibitor type 1 expression in bladder cancer. *Cancer* **2010**, *116*, 4502–4512. [CrossRef]
107. Chan, O.T.M.; Furuya, H.; Pagano, I.; Shimizu, Y.; Hokutan, K.; Dyrskjøt, L.; Jensen, J.B.; Malmstrom, P.U.; Segersten, U.; Janku, F.; et al. Association of MMP-2, RB and PAI-1 with decreased recurrence-free survival and overall survival in bladder cancer patients. *Oncotarget* **2017**, *8*, 99707–99721. [CrossRef] [PubMed]
108. Herszényi, L.; Farinati, F.; Cardin, R.; István, G.; Molnár, L.D.; Hritz, I.; De Paoli, M.; Plebani, M.; Tulassay, Z. Tumor marker utility and prognostic relevance of cathepsin B, cathepsin L, urokinase-type plasminogen activator, plasminogen activator inhibitor type-1, CEA and CA 19-9 in colorectal cancer. *BMC Cancer* **2008**, *8*, 194. [CrossRef]
109. Iwadate, Y.; Hayama, M.; Adachi, A.; Matsutani, T.; Nagai, Y.; Hiwasa, T.; Saeki, N. High serum level of plasminogen activator inhibitor-1 predicts histological grade of intracerebral gliomas. *Anticancer Res.* **2008**, *28*, 415–418. [PubMed]
110. Kuhn, W.; Schmalfeldt, B.; Reuning, U.; Pache, L.; Berger, U.; Ulm, K.; Harbeck, N.; Späthe, K.; Dettmar, P.; Höfler, H.; et al. Prognostic significance of urokinase (uPA) and its inhibitor PAI-1 for survival in advanced ovarian carcinoma stage FIGO IIIc. *Br. J. Cancer* **1999**, *79*, 1746–1751. [CrossRef]
111. Nakatsuka, E.; Sawada, K.; Nakamura, K.; Yoshimura, A.; Kinose, Y.; Kodama, M.; Hashimoto, K.; Mabuchi, S.; Makino, H.; Morii, E.; et al. Plasminogen activator inhibitor-1 is an independent prognostic factor of ovarian cancer and IMD-4482, a novel plasminogen activator inhibitor-1 inhibitor, inhibits ovarian cancer peritoneal dissemination. *Oncotarget* **2017**, *8*, 89887. [CrossRef]
112. Koensgen, D.; Stope, M.B.; Tuerbachova, I.; Bruennert, D.; Kohlmann, T.; Braicu, I.; Sehouli, J.; Denkert, C.; Darb-Esfahani, S.; Stickeler, E.; et al. Expression, intracellular localization, and prognostic value of plasminogen activator inhibitor 1 and PAI-1 RNA-binding Protein 1 in primary and recurrent ovarian cancer: A study of the tumor bank ovarian cancer network. *Gynecol. Obstet. Investig.* **2018**, *83*, 508–514. [CrossRef] [PubMed]
113. Ostheimer, C.; Evers, C.; Bache, M.; Reese, T.; Vordermark, D. Prognostic implications of the co-detection of the urokinase plasminogen activator system and osteopontin in patients with non-small-cell lung cancer undergoing radiotherapy and correlation with gross tumor volume. *Strahlenther. Onkol.* **2018**, *194*, 539–551. [CrossRef]
114. Zubac, D.P.; Wentzel-Larsen, T.; Seidal, T.; Bostad, L. Type 1 plasminogen activator inhibitor (PAI-1) in clear cell renal cell carcinoma (CCRCC) and its impact on angiogenesis, progression and patient survival after radical nephrectomy. *BMC Urol.* **2010**, *10*, 20. [CrossRef]
115. Vaughan, D.E.; Rai, R.; Khan, S.S.; Eren, M.; Ghosh, A.K. PAI-1 is a marker and a mediator of senescence. *Arterioscler. Thromb. Vasc. Biol.* **2017**, *37*, 1446. [CrossRef] [PubMed]
116. Jiang, C.S.; Rana, T.; Jin, L.W.; Farr, S.A.; Morley, J.E.; Qin, H.; Liu, G.; Liu, R.M. Aging, plasminogen activator inhibitor 1, brain cell senescence, and Alzheimer's Disease. *Aging Dis.* **2023**, *14*, 515–528. [CrossRef]
117. Ahmed, T.A.; El-Badri, N. Pericytes: The role of multipotent stem cells in vascular maintenance and regenerative medicine. *Adv. Exp. Med. Biol.* **2018**, *1079*, 69–86. [CrossRef]
118. Escudero, C.A.; Herlitz, K.; Troncoso, F.; Guevara, K.; Acurio, J.; Aguayo, C.; Godoy, A.S.; González, M. Pro-angiogenic role of insulin: From physiology to pathology. *Front. Physiol.* **2017**, *8*, 224224. [CrossRef] [PubMed]
119. Miele, C.; Rochford, J.J.; Filippa, N.; Giorgetti-Peraldi, S.; Van Obberghen, E. Insulin and Insulin-like Growth Factor-I Induce Vascular Endothelial Growth Factor mRNA Expression via Different Signaling Pathways. *J. Biol. Chem.* **2000**, *275*, 21695–21702. [CrossRef] [PubMed]

120. Hale, L.J.; Hurcombe, J.; Lay, A.; Santamaría, B.; Valverde, A.M.; Saleem, M.A.; Mathieson, P.W.; Welsh, G.I.; Coward, R.J. Insulin directly stimulates VEGF-A production in the glomerular podocyte. *J Physiol Ren. Physiol* **2013**, *305*, 182–188. [CrossRef] [PubMed]
121. Hill, D.J.; Milner, R.D.G. Insulin as a growth factor. *Pediatr. Res.* **1985**, *19*, 879–886. [CrossRef]
122. Steller, M.A.; Delgado, C.H.; Zou, Z. Insulin-like growth factor II mediates epidermal growth factor-induced mitogenesis in cervical cancer cells. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 11970–11974. [CrossRef]
123. Ayati, A.; Moghimi, S.; Salarinejad, S.; Safavi, M.; Pouramiri, B.; Foroumadi, A. A review on progression of epidermal growth factor receptor (EGFR) inhibitors as an efficient approach in cancer targeted therapy. *Bioorg. Chem.* **2020**, *99*, 103811. [CrossRef]
124. Mabeta, P.; Steenkamp, V. The VEGF/VEGFR Axis Revisited: Implications for Cancer Therapy. *Int. J. Mol. Sci.* **2022**, *23*, 15585. [CrossRef]
125. Flores-López, L.A.; Martínez-Hernández, M.G.; Viedma-Rodríguez, R.; Díaz-Flores, M.; Baiza-Gutman, L.A. High glucose and insulin enhance uPA expression, ROS formation and invasiveness in breast cancer-derived cells. *Cell. Oncol.* **2016**, *39*, 365–378. [CrossRef]
126. Hou, Y.; Zhou, M.; Xie, J.; Chao, P.; Feng, Q.; Wu, J. High glucose levels promote the proliferation of breast cancer cells through GTPases Breast Cancer-Targets and Therapy Dovepress High glucose levels promote the proliferation of breast cancer cells through GTPases. *Breast Cancer Targets Ther.* **2017**, *9*, 429–436. [CrossRef]
127. Han, L.; Ma, Q.; Li, J.; Liu, H.; Li, W.; Ma, G.; Xu, Q.; Zhou, S.; Wu, E. High glucose promotes pancreatic cancer cell proliferation via the induction of EGF expression and transactivation of EGFR. *PLoS ONE* **2011**, *6*, e27074. [CrossRef] [PubMed]
128. Zhao, W.; Chen, R.; Zhao, M.; Li, L.; Fan, L.; Che, X.M. High glucose promotes gastric cancer chemoresistance in vivo and in vitro. *Mol. Med. Rep.* **2015**, *12*, 843–850. [CrossRef] [PubMed]
129. Fainsod-Levi, T.; Gershkovitz, M.; Vö, S.; Grunewald, M.; Keshet, E.; Correspondence, Z.G.; Vö Ls, S.; Kumar, S.; Khawaled, S.; Sagiv, J.Y.; et al. Hyperglycemia impairs neutrophil mobilization leading to enhanced metastatic seeding. *Cell Rep.* **2017**, *21*, 2384–2392. [CrossRef] [PubMed]
130. Deshmane, S.L.; Kremlev, S.; Amini, S.; Sawaya, B.E. Monocyte chemoattractant protein-1 (MCP-1): An overview. *J. Interferon Cytokine Res.* **2009**, *29*, 313. [CrossRef] [PubMed]
131. Westerbacka, J.; Cornér, A.; Kolak, M.; Makkonen, J.; Turpeinen, U.; Hamsten, A.; Fisher, R.M.; Yki-Järvinen, H. Insulin regulation of MCP-1 in human adipose tissue of obese and lean women. *Am. J. Physiol.-Endocrinol. Metab.* **2008**, *294*, 841–845. [CrossRef]
132. Nio, Y.; Yamauchi, T.; Iwabu, M.; Okada-Iwabu, M.; Funata, M.; Yamaguchi, M.; Ueki, K.; Kadowaki, T. Monocyte chemoattractant protein-1 (MCP-1) deficiency enhances alternatively activated M2 macrophages and ameliorates insulin resistance and fatty liver in lipoatrophic diabetic A-ZIP transgenic mice. *Diabetologia* **2012**, *55*, 3350–3358. [CrossRef]
133. Patsouris, D.; Cao, J.J.; Vial, G.; Bravard, A.; Lefai, E.; Durand, A.; Durand, C.; Chauvin, M.A.; Laugerette, F.; Debard, C.; et al. Insulin resistance is associated with MCP1-mediated macrophage accumulation in skeletal muscle in mice and humans. *PLoS ONE* **2014**, *9*, e110653. [CrossRef]
134. Yuan, X.; Wang, J.; Yang, S.; Gao, M.; Cao, L.; Li, X.; Hong, D.; Tian, S.; Sun, C. Effect of the ketogenic diet on glycemic control, insulin resistance, and lipid metabolism in patients with T2DM: A systematic review and meta-analysis. *Nutr. Diabetes* **2020**, *10*, 38. [CrossRef]
135. Sartipy, P.; Loskutoff, D.J. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 7265. [CrossRef]
136. Du, Z.; Wu, X.; Song, M.; Li, P.; Wang, L. Oxidative damage induces MCP-1 secretion and macrophage aggregation in age-related macular degeneration (AMD). *Graefes Arch. Clin. Exp. Ophthalmol.* **2016**, *254*, 2469–2476. [CrossRef]
137. Quoc, Q.L.; Cao, T.B.T.; Moon, J.Y.; Jang, J.H.; Shin, Y.S.; Choi, Y.; Ryu, M.S.; Park, H.S. Contribution of monocyte and macrophage extracellular traps to neutrophilic airway inflammation in severe asthma. *Allergol. Int.* **2023**. [CrossRef] [PubMed]
138. Gonzalo, J.A.; Lloyd, C.M.; Wen, D.; Albar, J.P.; Wells, T.N.C.; Proudfoot, A.; Martinez-a, C.; Dorf, M.; Bjerke, T.; Coyle, A.J.; et al. The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J. Exp. Med.* **1998**, *188*, 157. [CrossRef] [PubMed]
139. Singh, S.; Anshita, D.; Ravichandiran, V. MCP-1: Function, regulation, and involvement in disease. *Int. Immunopharmacol.* **2021**, *101*, 107598. [CrossRef]
140. Kaneko, N.; Kurata, M.; Yamamoto, T.; Morikawa, S.; Masumoto, J. The role of interleukin-1 in general pathology. *Inflamm. Regen.* **2019**, *39*, 12. [CrossRef]
141. Lopez-Castejon, G.; Brough, D. Understanding the mechanism of IL-1β secretion. *Cytokine Growth Factor Rev.* **2011**, *22*, 189. [CrossRef]
142. Abdulla, M.H.; Sultana, S.A.; Vaali-Mohammed, M.A.; Al Khayal, K.A.; Bin Traiki, T.; Zubaidi, A.M.; Al-Johani, T.; Shakoor, Z.; Obeed, O.A. Al Expression of VEGF, EGF and HGF in early- and late-stage colorectal cancer. *Mol. Clin. Oncol.* **2021**, *15*, 251. [CrossRef]
143. Yang, Y.; Cao, Y. The impact of VEGF on cancer metastasis and systemic disease. *Semin. Cancer Biol.* **2022**, *86*, 251–261. [CrossRef] [PubMed]
144. Mulholland, B.S.; Hofstee, P.; Millar, E.K.A.; Bliuc, D.; O'Toole, S.; Forwood, M.R.; McDonald, M.M. MCP-1 expression in breast cancer and its association with distant relapse. *Cancer Med.* **2023**, *12*, 16221–16230. [CrossRef] [PubMed]
145. Wang, L.; Lan, J.; Tang, J.; Luo, N. MCP-1 targeting: Shutting off an engine for tumor development. *Oncol. Lett.* **2022**, *23*, 26. [CrossRef]

146. Meidenbauer, J.J.; Mukherjee, P.; Seyfried, T.N. The glucose ketone index calculator: A simple tool to monitor therapeutic efficacy for metabolic management of brain cancer. *Nutr. Metab.* **2015**, *12*, 12. [CrossRef]
147. Tan-Shalaby, J. Ketogenic diets and cancer: Emerging evidence. *Fed. Pract.* **2017**, *34*, 37S.
148. Weber, D.D.; Aminzadeh-Gohari, S.; Tulipan, J.; Catalano, L.; Feichtinger, R.G.; Kofler, B. Ketogenic diet in the treatment of cancer—Where do we stand? *Mol. Metab.* **2020**, *33*, 102. [CrossRef] [PubMed]
149. Liberti, M.V.; Locasale, J.W. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem. Sci.* **2016**, *41*, 211–218. [CrossRef] [PubMed]
150. Draznin, B. Mitogenic action of insulin: Friend, foe or "frenemy"? *Diabetologia* **2010**, *53*, 229–233. [CrossRef]
151. Volek, J.S.; Freidenreich, D.J.; Saenz, C.; Kunces, L.J.; Creighton, B.C.; Bartley, J.M.; Davitt, P.M.; Munoz, C.X.; Anderson, J.M.; Maresh, C.M.; et al. Metabolic characteristics of keto-adapted ultra-endurance runners. *Metabolism.* **2015**, *65*, 100–110. [CrossRef] [PubMed]
152. Kyriakidou, Y.; Cooper, I.; Kraev, I.; Lange, S.; Elliott, B.T. Preliminary investigations into the effect of exercise-induced muscle damage on systemic extracellular vesicle release in trained younger and older men. *Front. Physiol.* **2021**, *12*, 723931. [CrossRef]
153. Jansen, L.T.; Yang, N.; Wong, J.M.W.; Mehta, T.; Allison, D.B.; Ludwig, D.S.; Ebbeling, C.B. Prolonged glycemic adaptation following transition from a low- to high-carbohydrate diet: A randomized controlled feeding trial. *Diabetes Care* **2022**, *45*, 576–584. [CrossRef]
154. Jo, U.; Park, K. Carbohydrate-based diet may increase the risk of cardiovascular disease: A pooled analysis of two prospective cohort studies. *Clin. Nutr.* **2023**, *42*, 1301–1307. [CrossRef]
155. Moore, A.R.; Holland-Winkler, A.M.; Ansley, J.K.; Boone, E.D.H.; Schulte, M.K.O. Reliability and diagnostic performance of a new blood ketone and glucose meter in humans. *J. Int. Soc. Sports Nutr.* **2021**, *18*, 6. [CrossRef]
156. Veyrat-Durebex, C.; Reynier, P.; Procaccio, V.; Hergesheimer, R.; Corcia, P.; Andres, C.R.; Blasco, H. How can a ketogenic diet improve motor function? *Front. Mol. Neurosci.* **2018**, *11*, 15. [CrossRef]

APPENDIX C: Published version of Cooper, Kyriakidou, Petagine et al., (2024).

https://doi.org/10.3390/ biomedicines12071553

biomedicines

MDPI

Article

Ketosis Suppression and Ageing (KetoSAge) Part 2: The Effect of Suppressing Ketosis on Biomarkers Associated with Ageing, HOMA-IR, Leptin, Osteocalcin, and GLP-1, in Healthy Females

Isabella D. Cooper [1,*], Yvoni Kyriakidou [1], Lucy Petagine [1], Kurtis Edwards [2], Adrian Soto-Mota [3,4], Kenneth Brookler [5] and Bradley T. Elliott [1]

[1] Ageing Biology and Age-Related Diseases, School of Life Sciences, University of Westminster, 115 New Cavendish Street, London W1W 6UW, UK; y.kyriakidou@westminster.ac.uk (Y.K.); l.petagine@westminster.ac.uk (L.P.); b.elliott@westminster.ac.uk (B.T.E.)
[2] Cancer Biomarkers and Mechanisms Group, School of Life Sciences, University of Westminster, 115 New Cavendish Street, London W1W 6UW, UK; k.edwards3@westminster.ac.uk
[3] Metabolic Diseases Research Unit, National Institute of Medical Sciences and Nutrition Salvador Zubiran, Mexico City 14080, Mexico; adrian.sotom@incmnsz.mx
[4] School of Medicine, Tecnologico de Monterrey, Mexico City 14380, Mexico
[5] Retired former Research Collaborator, Aerospace Medicine and Vestibular Research Laboratory, Mayo Clinic, Scottsdale, AZ 85259, USA; kennethhbrookler@mac.com
* Correspondence: isabella@bellamitochondria.com or i.cooper@westminster.ac.uk

Abstract: Metabolic dysfunctions are among the best documented hallmarks of ageing. Cardiovascular disease, Alzheimer's disease, cancer, type 2 diabetes mellitus, metabolic-dysfunction-associated steatosis liver disease, and fragility fractures are diseases of hyperinsulinaemia that reduce life and healthspan. We studied the effect of suppressing ketosis in 10 lean (BMI 20.5 kg/m^2 ± 1.4), metabolically healthy, pre-menopausal women (age 32.3 ± 8.9 years) maintaining nutritional ketosis (NK) for an average of 3.9 years (± 2.3) who underwent three 21-day phases: nutritional ketosis (NK; P1), suppressed ketosis (SuK; P2), and returned to NK (P3). Ketosis suppression significantly increased insulin, 1.83-fold (p = 0.0006); glucose, 1.17-fold (p = 0.0088); homeostasis model assessment for insulin resistance (HOMA-IR), 2.13-fold (p = 0.0008); leptin, 3.35-fold (p = 0.0010); total osteocalcin, 1.63-fold (p = 0.0138); and uncarboxylated osteocalcin, 1.98-fold (p = 0.0417) and significantly decreased beta-hydroxybutyrate, 13.50-fold (p = 0.0012) and glucagon-like peptide-1 (GLP-1), 2.40-fold (p = 0.0209). Sustained NK showed no adverse health effects and may mitigate hyperinsulinemia. All biomarkers returned to basal P1 levels after removing the intervention for SuK, indicating that metabolic flexibility was maintained with long-term euketonaemia.

Keywords: ageing; cortisol; GLP-1; HOMA-IR; insulin resistance; hyperinsulinaemia; ketosis; leptin; metabolic syndrome; osteocalcin

Citation: Cooper, I.D.; Kyriakidou, Y.; Petagine, L.; Edwards, K.; Soto-Mota, A.; Brookler, K.; Elliott, B.T. Ketosis Suppression and Ageing (KetoSAge) Part 2: The Effect of Suppressing Ketosis on Biomarkers Associated with Ageing, HOMA-IR, Leptin, Osteocalcin, and GLP-1, in Healthy Females. *Biomedicines* **2024**, *12*, 1553. https://doi.org/10.3390/biomedicines12071553

Academic Editor: Alfredo Caturano

Received: 5 June 2024
Revised: 8 July 2024
Accepted: 9 July 2024
Published: 12 July 2024

1. Introduction

Ageing is typically understood in its chronological context, as the length of time that has passed since a person's birth, whereas biological age (BA) is the measure of functional age, often measured in terms of physical and mental performance, as well as morbidities that decrease quality of life and youth-span [1,2]. One-sixth of Europeans are expected to be over 60 years of age by 2030, and 25% of older adults will be above 85 [3], reflecting a chronologically aged population increase. Concurrently, chronic non-communicable diseases promote an earlier decline in BA. Additionally, cardiovascular disease (CVD), Alzheimer's disease (AD), and cancer are the leading causes of morbidity and mortality in the USA [4], with AD and other dementias being the leading causes for females, followed by CVD, cerebral vascular disease, and cancer in England and Wales [5]. This decline in BA was disturbingly evidenced in an analysis of 8,721 participants from 2009 to 2016

from the National Health and Nutrition Examination Survey (NHANES), showing that the proportion of metabolically unhealthy Americans increased from an already very high level of 80.1% to 87.8% over the span of 7 years, putting the vast majority of the population on the hyperinsulinaemia spectrum [2,6–8]. This increase in a younger BA onset and population proportion (living with morbidity earlier and for longer) is arguably the global pandemic of our time.

The chronic non-communicable diseases AD, CVD, cancer, type 2 diabetes mellitus (T2DM), metabolic syndrome (MetS), metabolic-dysfunction-associated steatotic liver disease (MASLD), and chronic inflammation are the consequence of lifestyle factors that stimulate chronic excess insulin demand and secretion, termed hyperinsulinaemia [2,7–11]. Hyperinsulinaemia is understood to be an ageing metabolo-endocrine state, and can be staged as metabolic phenotypes (MPs) [7,8]. Hyperinsulinaemia, when assessed as fasting insulin above the reference range only, likely does not capture the sub-clinical occult phase of hyperinsulinaemia [1]. We argue that hyperinsulinaemia can be detected before exceeding the population-derived broad reference range, which does not reflect an individual's optimal level of insulin.

A symphony of biomarkers taken together are more sensitive to aid in catching hyperinsulinaemia as early as possible, especially in those with a body mass index (BMI) less than 25 kg/m^2 [1,12–14], thus enabling earlier intervention, preventive care, and minimising the incorrect grouping of research participants, which may lead to increased false negatives in study results. A prospective cohort study from the NHANES, n = 12,563 with median age 45 years (20–85 years), found hyperinsulinaemia to be a greater risk marker for increased mortality compared to BMI [15]. We previously published a report on the effect of suppressing ketosis on ageing- and chronic-disease-associated biomarkers, which included fasting insulin, insulin-like growth factor 1 (IGF-1), glucose, beta-hydroxybutyrate (BHB), gamma-glutaryl transferase (GGT), plasminogen activator inhibitor-1 (PAI-1), monocyte chemotactic protein 1 (MCP-1), and more [7]. Part 2 investigates additional biomarkers strongly associated with morbidity, including homeostasis model assessment for insulin resistance (HOMA-IR), osteocalcin (OCN), leptin, and glucagon-like peptide-1 (GLP-1), which are biomarkers positively and negatively associated with chronic diseases and ageing. We aimed to investigate the effect of long-term sustained nutritional ketosis (NK), also known as euketonaemia, on these biomarkers, and if suppressing ketosis for 21 days would result in any measurable changes, allowing us to understand one lifestyle factor that may meaningfully impact these biomarkers, which could reduce the earlier onset of BA, meaning the potential to enhance youth-span as well as lifespan.

2. Materials and Methods

2.1. Ethics

Ethical approval was obtained by the College of Liberal of Arts and Sciences Research Ethics Committee, University of Westminster, United Kingdom (ETH2122-0634). All procedures were conducted in accordance with the Declaration of Helsinki and UK legislation. Written informed consent was obtained from all participants prior to their participation.

2.2. Participants

Ten healthy pre-menopausal habitually keto-adapted women, classified as metabolic phenotype 1 (MP1) [8], were recruited to take part in this three-phase study named KetoSAge [7]. The participants self-reported their adherence to a lifestyle that sustained NK for $\geq$ 6 months (mean 3.9 $\pm$ 2.3 years), ensuring sufficient time for metabolic adaptations. Ketosis adaptation was proven during a 6-month lead-in period, where the participants were required to take a once-daily capillary BHB reading between 4–6 p.m., before their evening meal, prior to the commencement of the study. This standardised evening measurement was chosen due to it being a more rigorous threshold to pass in order to be judged as sustaining NK over the majority of the 24 h day, in comparison to morning fasted measurements, thus increasing confidence in the participants maintaining NK most of

the time. Readings were taken with a Keto-Mojo™ GKI multi-function meter (Keto-Mojo, Napa, CA, USA; [16]). The baseline characteristics of these participants were described in our earlier publication [7].

For the duration of the study, the participants were required to monitor their capillary glucose and ketone BHB concentrations (mmol/L) at four time points throughout the day to ascertain compliance (previously published in Table 3 [7]; herein expressed in rank by concentration in Table 1 in the results section). These time points were between 7:30–9:30 a.m., 11:30 a.m.–13:30 p.m., 15:30–17:30 p.m., and 21:30–23:30 p.m. The participants determined their capillary glucose and BHB using a Keto-Mojo™ GKI multi-function meter. This equipment was selected for its reliability and good diagnostic performance [16].

The exclusion criteria for all participants included smoking, taking any medication, and evidence or history of metabolic syndrome, immunological issues, or CVD. The participants were required to complete a medical history questionnaire to confirm that they were free from all of the above diseases.

2.3. Study Design

The 10 KetoSAge participants took part in an open-labelled, non-randomised crossover trial with three 21-day phases: baseline NK was defined as BHB $\geq$ 0.5 mmol/L (Phase 1; P1) and the suppression of ketosis (SuK) as BHB < 0.3 mmol/L, with dietary carbohydrate reintroduction following the Standard U.K. diet (SUK) Eatwell Guidelines (Phase 2; P2), which recommend consuming a predominance of calories from carbohydrates (e.g., 55% kcal from carbohydrates on a 2000 kcal diet is 275 g/day net carbohydrates). The final phase was the removal of the intervention, returning to NK (Phase 3; P3) (Figure **??**). At the end of each of the 21-day study phases, on days 22, 44, and 66, the participants attended the laboratory for one day at 8 a.m. after a 12 h overnight fast to undertake anthropometric measurements and blood sampling. A detailed description of the study design was provided in our previous publication [7]. No participants withdrew from this study.

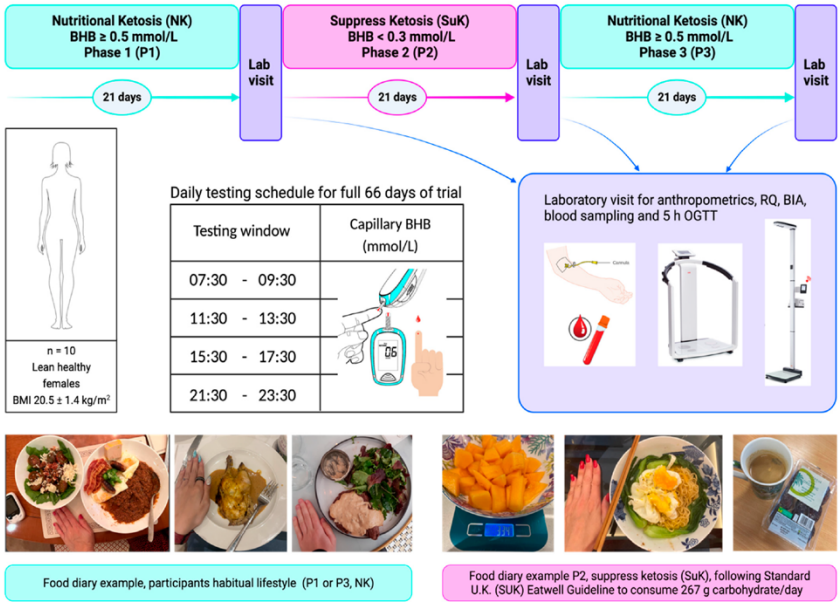

Figure 1. KetoSAge study design. Phase 1 and 3 covered the participants' habitual nutritional ketosis lifestyle. Phase 2 was the interventional phase to suppress ketosis (SuK). Each phase was monitored via finger prick testing of capillary beta-hydroxybutyrate (BHB) concentration (mmol/L). Testing was conducted four times per day, prior to mealtimes, at evenly spaced intervals. At the end of each phase, participants underwent a laboratory testing day for body composition and biochemical tests. Participants were given an oral glucose tolerance test (75 g glucose in 250 mL water) described in our earlier publication [7]. Blood samples were taken at seven time points over 5 hours. Whole blood glucose and BHB were measured sequentially in real time using the Keto-Mojo^{TM} meter, and plasma insulin sensitivity assay was conducted later using ELISA. Body mass index (BMI); oral glucose tolerance test (OGGT); respiratory quotient (RQ).

2.4. Anthropometric Measurements

Upon arrival at the laboratory, height (to the nearest 0.1 cm) was measured using a stadiometer (Marsden HM-250P Leicester Height Measure, Rotherham, UK), and body weight (to the nearest 0.1 kg), waist, and hip circumference measures were obtained with a non-stretch anthropometric circumference measuring tape (Seca® 201, Birmingham, UK) while participants stood upright on both feet. The average value (cm) of three measurements was used for analysis. All measurements were taken from following a 12 h fast wearing standardised clothing.

2.5. Blood Collection and Measurement

Blood was drawn from the antecubital vein into ethylenediaminetetraacetic acid (EDTA) tubes (BD, Oxford, UK) before being centrifuged at 3,857 × *g* for 10 min at 4 °C, as described previously [7]. Plasma was then aliquoted into airtight vials and frozen at −80 °C for batch analysis later.

Samples of blood were immediately sent to SYNLAB Belgium (Alexander Fleming, 3–6220 Heppignies–Company No: 0453.111.546) for the measurement of various markers (see below). Blood was additionally drawn into serum SST^{TM} II Advance tubes with thrombin rapid clot activator and separation gel (BD, Oxford, UK), then left to stand for 30 min at room temperature. Serum tubes were centrifuged (Hettich Zentrifugen, Universal 320 R, Tuttlingen, Germany) at 3,857 × *g* for 10 min at room temperature. Serum samples were aliquoted into cryovial tubes under sterile conditions and stored at −80 °C for later analysis.

2.6. Blood Marker Analysis

Serum insulin was measured via Simple Plex Assay (Ella™, Bio-Techne, Minneapolis, USA). Total osteocalcin (tOCN; DuoSet, R&D Systems, Minneapolis, MN, USA), uncarboxylated osteocalcin (unOCN; BioLegend, San Diego, CA, USA), melatonin, serotonin and serum GLP-1 (Abcam, Cambridge, UK), and leptin (DuoSet, R&D Systems, Minneapolis, MN, USA) were measured by enzyme-linked immunosorbent assay (ELISA) from frozen EDTA plasma or serum samples, according to the manufacturer's instructions. Cortisol was measured externally by SYNALB in the blood samples taken from the KetoSAge participants, as described previously [7]. Glucose concentrations were measured using a Biosen C-Line Clinic Glucose and Lactate analyser (EKF-Diagnostic, GmbH, Barleben, Germany).

2.7. Statistical Analysis

Data were checked for normality using the Shapiro–Wilk test. Different markers measured in the plasma between study phases for the KetoSAge participants were compared using the Friedman test with Dunn's correction for multiple comparisons or repeated measures ANOVA with Tukey's correction for multiple comparisons, depending on the results of the normality tests. Data are presented as mean ± SD. Data were analysed and graphed using GraphPad Prism (Version 9.1.2).

3. Results

3.1. Frequency of Rank-Ordered Capillary BHB Level

Assuming that each test was independent of the other, then the mean of a 21-day testing window had a 25% chance of being the highest. We saw in Phase 1 that 60% of the highest 21-day test window mean BHB concentrations were found in the third test of the day (pre-dinner), with the remaining 40% in the pre-lunch test (Table 1). All biomarkers assessed in this study are presented in below in Table 2.

Table 1. Frequency distribution of beta-hydroxybutyrate (BHB) (the mean of 21 days, BHB concentrations, per test window) expressed in rank, from 1 as the lowest to 4 as the highest concentration. Each participant took 4 tests per day, totalling 840 capillary BHB tests in 10 participants sustaining nutritional ketosis (NK) over 21 consecutive days, in two NK phases (P1 and P3), totalling 1,680 capillary BHB tests. The rank is given to the mean BHB concentration for that test window of 21 days, per phase, per participant. If a participant's test 4 has a rank score of 1, this indicates that the mean BHB concentration for 21 days of tests at bedtime is the lowest of the mean values of all the other test windows.

Phase 1 NK											
Participant	1011	1021	1031	1041	1051	1061	1071	1081	1091	1101	Rank 4 frequency
Test 1 (Wake up)	2	1	2	1	2	1	1	1	1	1	0
Test 2 (Pre-lunch)	3	3	4	3	3	4	4	2	4	2	4
Test 3 (Pre-dinner)	4	4	3	4	4	2	3	4	3	4	6
Test 4 (Bedtime)	1	2	1	2	1	3	2	3	2	3	0
Phase 3 NK											
Participant	1011	1021	1031	1041	1051	1061	1071	1081	1091	1101	Rank 4 frequency
Test 1 (Wake up)	2	1	2	1	2	1	1	1	1	2	0
Test 2 (Pre-lunch)	4	3	4	3	2	3	4	3	3	4	4
Test 3 (Pre-dinner)	3	4	3	4	4	4	3	4	4	3	6
Test 4 (Bedtime)	1	2	1	2	3	2	2	2	2	1	0

Table 2. BMI, fat mass, fasted insulin, glucose, BHB, HOMA-IR, total, carboxylated and uncarboxylated osteocalcin, leptin, cortisol, serotonin, and GLP-1 across all phases in KetoSAge participants. Measurements were taken following each of the study phases: baseline nutritional ketosis (NK), P1; intervention suppress ketosis (SuK), P2; and removal of SuK returning to NK, P3. Measurements were taken at 8 a.m. after a 12 h overnight fast; (n = 10). Values are presented as mean ± SD.

	P1	P2	P3	ANOVA *p* Value	P1 vs. P2	P2 vs. P3	P1 vs. P3
BMI (kg/m^2)	20.52 (±1.39)	21.54 (±1.30)	20.82 (±1.46)	<0.0001	<0.0001	0.0025	0.0197
Fat mass (kg)	14.21 (±2.55)	15.88 (±2.23)	14.78 (±2.20)	<0.0001	0.0008	0.0057	0.1016
Insulin (µIU/mL)	4.95 (±1.24)	9.06 (±2.14)	5.62 (±1.83)	<0.0001	0.0006	0.0027	0.3995
Glucose (mmol/L)	4.36 (±0.53)	5.12 (±0.59)	4.41 (±0.30)	0.0015	0.0088	0.0177	0.9469
BHB (mmol/L)	2.43 (±1.28)	0.18 (±0.13)	2.31 (±0.71)	0.0001	0.0012	<0.0001	0.9854
HOMA-IR	0.97 (±0.32)	2.07 (±0.61)	1.11 (±0.41)	<0.0001	0.0008	0.0013	0.4950
tOCN (ng/mL)	33.84 (±13.66)	55.31 (±29.71)	34.02 (±12.05)	0.0049	0.0138	0.0253	0.9978
cOCN (ng/mL)	31.54 (±12.59)	50.78 (±26.22)	32.02 (±11.13)	0.0040	0.0120	0.0246	0.9840
unOCN (ng/mL)	2.29 (±1.25)	4.54 (±3.79)	2.00 (±1.16)	0.0004	0.0417	0.0010	0.7907
tOCN % (% relative to P1)	100.00	162.36 (±46.53)	109.70 (±40.36)	0.0005	0.0065	0.0094	>0.9999
cOCN % (% relative to P1)	100.00	161.56 (±46.73)	111.52 (±42.90)	0.0007	0.0062	0.0080	0.6836
unOCN % (% relative to P1)	100.00	176.67 (±66.66)	87.11 (±21.43)	0.0028	0.0135	0.0076	0.2476
Leptin (ng/mL)	4.50 (±3.67)	15.08 (±8.00)	4.57 (±3.48)	<0.0001	0.0010	0.0052	>0.9999
Cortisol (ng/mL)	126.20 (±52.67)	112.70 (±58.46)	131.90 (±52.18)	0.4362	>0.9999	0.5391	>0.9999
Serotonin (ng/mL)	21.05 (±22.83)	18.38 (±16.03)	21.77 (±16.80)	0.6013	0.7907	>0.9999	>0.9999
GLP-1 (pg/mL)	1383.18 (±911.36)	576.72 (±452.43)	1471.85 (±1066.75)	0.0075	0.0209	0.0219	>0.9999

Beta-hydroxybutyrate (BHB); body mass index (BMI); carboxylated osteocalcin (cOCN); glucagon like peptide 1 (GLP-1); homeostasis model assessment for insulin resistance (HOMA-IR); total osteocalcin (tOCN); and uncarboxylated osteocalcin (unOCN).

3.2. Suppression of Ketosis is Associated with Increases in HOMA-IR

Following P2, HOMA-IR significantly increased from 0.97 (± 0.32, P1) to 2.07 (± 0.61, P2; $p = 0.0008$; Figure 2). This trend reversed following P3, where HOMA-IR significantly changed and returned to the participants' baseline levels of 1.12 (± 0.41, P3; $p = 0.0013$) compared to P2 (Figure 2).

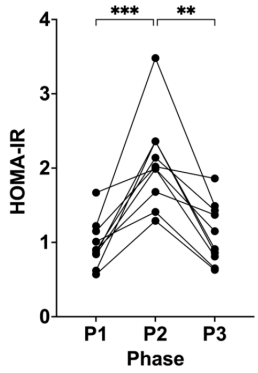

Figure 2. Homeostatic model assessment for insulin resistance (HOMA-IR) across all phases in KetoSAge participants. Fasting serum concentrations of insulin and plasma glucose were measured following each of the study phases: baseline nutritional ketosis (NK), P1; intervention to suppress ketosis (SuK), P2; and removal of SuK returning to NK, P3. Insulin was determined by via Simple Plex Assay (Ella™, Bio-Techne, Minneapolis, USA) and glucose was measured by Biosen C-Line Clinic Glucose and Lactate analyser. HOMA-IR adopts the following formula to index insulin resistance: fasting plasma insulin (uIU/mL) × fasting plasma glucose (mmol/L)/22.5 [1,17,18]. Homeostasis model assessment for insulin resistance (HOMA-IR). Samples were taken at 8 a.m. after a 12 h overnight fast (n = 10). Data were analysed by repeated measures one-way ANOVA. ** $p < 0.01$; and *** $p < 0.001$.

3.3. Suppressing Ketosis Increases all Forms of Osteocalcin

tOCN significantly increased after P1 from 33.84 ng/mL (± 13.66) to 55.31 ng/mL (± 29.71, P2, $p = 0.0138$; Figure 3), and then significantly decreased following the removal of SuK (P3) to 34.02 ng/mL (± 12.05, $p = 0.0253$) compared to P2, returning to similar baseline P1 values. cOCN significantly increased after P1 from 31.54 ng/mL (± 12.59) to 50.8 ng/mL (± 26.2, P2, $p = 0.0120$), then significantly decreased following the removal of SuK in P3 to 32.0 ng/mL (± 11.1, P3, $p = 0.0246$) compared to P2, returning to similar baseline P1 values. unOCN increased after P1 from 2.29 ng/mL (± 1.25) to 4.54 ng/mL (± 3.79, P2, $p = 0.0417$), then decreased following the removal of SuK in P3 to 2.00 ng/mL (± 1.16, $p = 0.0010$) compared to P2, returning to similar baseline P1 values.

We analysed tOCN, cOCN, and unOCN as percentages relative to each participant's own baseline values in P1. tOCN significantly increased after P1 from 100% as the standardised baseline (± 0.00%) to 162.36% (± 46.53%, $p = 0.0065$; Figure 4) relative to P1, then decreased following the removal of SuK in P3 to 109.70% (± 40.36%, P3, $p = 0.0094$), relative to baseline and compared to P2, returning to similar baseline P1 values. cOCN significantly increased after P1 from 100% as the standardised baseline (± 0.00%) to 161.56% (± 46.73%, P2, $p = 0.0062$) relative to P1, then decreased following the removal of SuK in P3 to 111.52% (± 42.90%, P3, $p = 0.0080$), relative to baseline and compared to P2, returning to similar baseline P1 values. unOCN significantly increased after P1 from 100% as the standardised baseline (± 0.00%) to 176.67% (± 66.66%, P2, $p = 0.0135$) relative to P1, then decreased following the removal of SuK in P3 to 87.11% (± 21.43%, P3, $p = 0.0076$), relative to baseline and compared to P2, returning to similar baseline P1 values.

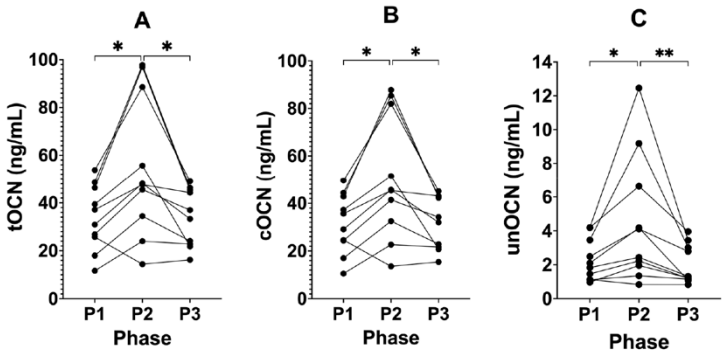

Figure 3. tOCN, cOCN, and unOCN across all phases in KetoSAge participants. Fasting plasma concentrations of (**A**) tOCN, (**B**) cOCN, and (**C**) unOCN were measured following each of the study phases: baseline nutritional ketosis (NK), P1; intervention to suppress ketosis (SuK), P2; and removal of SuK returning to NK, P3; total OCN (tOCN) and uncarboxylated OCN (unOCN) were determined by ELISA and carboxylated OCN (cOCN) was calculated by subtracting unOCN from tOCN. Samples were taken at 8 a.m. after a 12 h overnight fast; (n = 10); tOCN and cOCN data were analysed by repeated measures one-way ANOVA with Tukey's correction for multiple comparisons, unOCN data were analysed using the Friedman test with Dunn's correction for multiple comparisons. * $p < 0.05$ and ** $p < 0.01$.

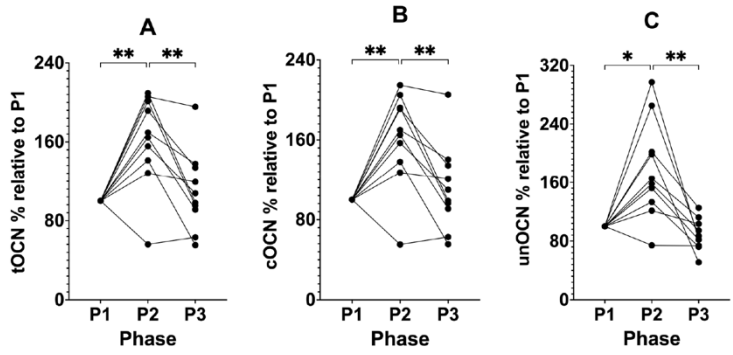

Figure 4. Percentage change from baseline P1 at 100% for tOCN, cOCN, and unOCN across all phases in KetoSAge participants. Fasting plasma concentrations of (**A**) tOCN, (**B**) cOCN, and (**C**) unOCN were measured following each of the study phases: baseline nutritional ketosis (NK), P1; intervention to suppress ketosis (SuK), P2; and removal of SuK returning to NK, P3; total OCN (tOCN) and uncarboxylated OCN (unOCN) were determined by ELISA, carboxylated OCN (cOCN) was calculated by subtracting unOCN from tOCN. Samples were taken at 8 a.m. after a 12 h overnight fast; (n = 10); data were analysed by repeated measures one-way ANOVA with Tukey's correction for multiple comparisons. * $p < 0.05$ and ** $p < 0.01$.

3.4. Suppression of Ketosis is Associated with Increased Leptin

Following P2, leptin significantly increased from 4.50 ng/mL (± 3.67, P1) to 15.08 ng/mL (± 8.00, P2; $p = 0.0010$; Figure 5). This trend reversed following P3, where leptin significantly decreased and returned to the participants' baseline levels of 4.57 ng/mL (± 3.48, P3; $p = 0.0005$) compared to P2.

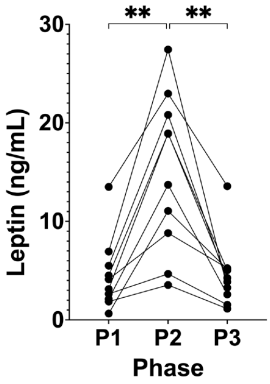

Figure 5. Serum leptin across all phases in KetoSAge participants. Fasting serum concentrations of leptin were measured following each of the study phases: baseline nutritional ketosis (NK), P1; intervention to suppress ketosis (SuK), P2; and removal of SuK returning to NK, P3. Leptin was determined by via ELISA (DuoSet, R&D Systems, Minneapolis, MN, USA). Samples were taken at 8 a.m. after a 12 h overnight fast; (n = 10). Data were analysed by repeated measures one-way ANOVA. ** $p < 0.01$.

3.5. Cortisol Levels Remain at Healthy Low Levels in Long-Term NK, and Serotonin Does Not Significantly Change

Following P2, cortisol did not significantly change after P1, changing from 126.20 ng/mL (± 52.67, P1) to 112.70 ng/mL (± 58.46, P2; $p \geq 0.9999$; Figure 6). Similarly, following P3, there was no significant change compared to P2 at 131.90 ng/mL (± 52.18, P3, $p = 0.5391$). Following P2, plasma serotonin did not significantly change from P1 at 21.05 pmol/L (± 22.83, $p = 0.7907$; Figure 6). With the removal of the intervention for SuK with a return to NK for 21 days at the end of P3, there was no significant change in serotonin compared to P2 at 21.77 pmol/L (± 16.80, P3, $p \geq 0.9999$).

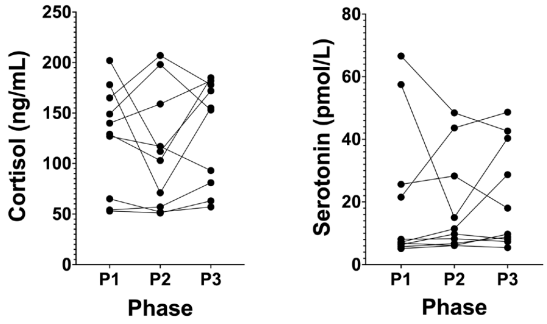

Figure 6. Serum cortisol and serotonin across all phases in KetoSAge participants. Fasting serum concentrations of cortisol and plasma serotonin were measured following each of the study phases: baseline nutritional ketosis (NK), P1; intervention to suppress ketosis (SuK), P2; and removal of SuK returning to NK, P3; cortisol was measured externally by SYNLAB Belgium (Alexander Fleming, 3–6220 Heppignies–Company No: 0453.111.546), serotonin was measured by ELISA (Abcam, Cambridge, UK). Samples were taken at 8 a.m. after a 12 h overnight fast; (n = 10). Data were analysed by Friedman test with Dunn's correction for multiple comparisons.

3.6. Suppression of Ketosis is Associated with Decreased GLP-1

Following P2, active GLP-1 significantly decreased from 1,064.98 pg/mL (± 500.15, P1) to 504.98 pg/mL (± 246.43, P2; $p = 0.0209$; Figure 7). This trend reversed following P3,

where GLP-1 significantly increased and returned to the participants' baseline levels of 1,032.26 pg/mL (± 453.88, P3; $p = 0.0210$) compared to P2.

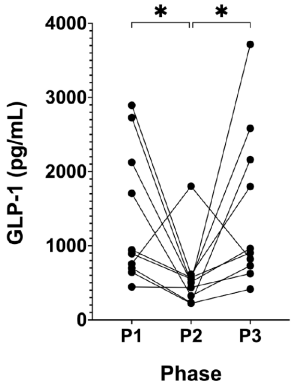

Figure 7. Serum concentrations of active GLP-1 across all phases in KetoSAge participants. Serum concentrations of active GLP-1 were measured following each of the study phases: baseline nutritional ketosis (NK), P1; intervention to suppress ketosis (SuK), P2; and removal of SuK returning to NK, P3. GLP-1 was determined by ELISA (Abcam, Cambridge, UK). Glucagon-like peptide-1 (GLP-1). Samples were taken at 8 a.m. after a 12 h overnight fast; (n = 10). Data were analysed by repeated measures one-way ANOVA. * $p < 0.05$.

4. Discussion

4.1. When to Measure Capillary BHB

The 6-month 4–6 p.m. capillary ketone testing standardised evening measurement as a criterion for eligibility for the trial was chosen due to it being a more rigorous threshold to pass in order to be judged as sustaining NK over the majority of the 24 h day, in comparison to morning fasted measurements, thus increasing the confidence in the participants maintaining NK most of the time. Most people embarking on a ketogenic lifestyle, typically via carbohydrate restriction, time-restricted feeding, or other forms of fasting-mimicking diets, will measure their capillary ketones in the morning, much the same way that morning fasting blood glucose is taken. However, morning ketone readings for hyperinsulinaemic and insulin-resistant individuals will likely produce some measurable levels (potentially up to 0.5 mmol/L) due to an overnight fast. If this was performed on individuals only daily, they could mistakenly then assume that they are successfully maintaining a metabolic state of ketosis. However, if they were to measure their capillary ketones at least 3 to 4 hours after lunch, then this test would capture their breakfast and/or lunch if they had consumed anything, allowing them to truly know if their lifestyle was supportive of maintaining euketonaemia via minimising lifestyle practices that stimulate excess insulin demand, which would inhibit (lower) ketone production.

In our cohort, our participants noted that their pre-lunch and dinner readings were often their highest levels of ketones out of the four times a day tests, with their pre-dinner (4–6 p.m.) reading showing a slightly higher frequency of being greater than their second test of the day (pre-lunch). This informed our participants of how their lifestyle was supporting them in maintaining nutritional levels of ketones ($\geq$ 0.5 mmol/L) throughout the day [7].

4.2. Hypoketonaemia, HOMA-IR, and Fasting Insulin

Throughout the KetoSAge study, the participants maintained healthy fasting glucose levels in all phases, 4.36 mmol/L (± 0.53, P1), 5.12 mmol/L (± 0.59, P2), and 4.41 mmol/L (± 0.30, P3), which are considered to be extremely healthy [17]. HbA1c was not measured in this study, due to the three-phase blood sampling timing that would have resulted in

prior blood sampling affecting the next blood sampling results (P2 affected by P1, and P3 affected by P2). HbA1c is used to assess cumulative glucose exposure, which glycates the haemoglobin (HbA1c) in blood. This provides evidence of average blood glucose levels over the previous two to three months, which is the average half-life of red blood cells [18]. A percentage of glycation damage occurs as a result of hyperglycaemia, which is associated with an increased risk of atherosclerosis, CVD, cerebrovascular disease, all-cause dementia (ACD), and AD. For example, in the AgeCoDe cohort study of 1,342 elderly individuals, a higher HbA1c level was found to be associated with increased ACD and AD. HbA1c $\geq$ 6.5% was associated with an increased risk of ACD by 2.8-fold (p = 0.027) and for AD (p = 0.047). There was an associated 5-fold increased risk of incident ACD (p = 0.001) and a 4.7-fold increased risk of AD (p = 0.004) with HbA1c levels $\geq$ 7% [19]. However, hyperinsulinaemia has been shown to precede hyperglycaemia by up to 24 years [20]. Therefore, there is a weakness when relying on HbA1c to detect earlier disease risk, in that there is a sub-clinical phase (occult stage), during which, a healthy HbA1c value is seen. This is due to an increased insulin concentration that reduces the plasma glucose, whilst the elevated basal insulin still has non-glucose-related signalling effects, such as increasing PI3K and MAPK signalling for growth and division [21–24] and increasing ceramide production, which increases mitochondrial reaction oxygen species generation, therefore increasing oxidative stress in the system [25–27].

An example of how a reduced HbA1c due to more insulin exposure can be less positive for health is found in the ACCORD group randomised controlled trial (RCT) study of 10,251 patients, with a mean age 63 years, who had an 8.1% median HbA1c. All participants were already on exogenous insulin. Half of the group was randomised to a more tailored intensive insulin therapy regimen to achieve a decrease in their HbA1c to 6%, whilst the other group was to follow their standard insulin therapy to target a value of 7.0–7.9%. The three primary outcomes measured in this RCT were non-fatal stroke, non-fatal myocardial infarction, and CVD death. This trial was stopped after 3.5 years due to ethical reasons, as the intervention group receiving the intensive insulin therapy (more insulin) in order to lower their HbA1c by a greater amount had a significantly higher mortality rate [28]. Whilst successfully lowering their HbA1c with more insulin, there was an increase in deaths, indicating that relying on a lower HbA1c may lure physicians and patients into a false sense of security whilst patients are actually exposed to greater insulin levels and, therefore, their subsequent potential harms [2,8,24,27,29–31].

HOMA-IR is recognised as a diagnostic index for insulin resistance, where a higher concentration of fasting insulin to maintain a low fasting glucose results in a higher HOMA-IR value [1]. Insulin resistance is generally accepted as having an HOMA-IR > 2 [32–34], whilst hyperinsulinaemia is considered where the fasting plasma insulin is > 28.8 uIU/mL [8,9,22,35]. Our participants' group mean of fasting insulin levels was 9.06 uIU/mL ($\pm$ 2.13) after 21 days of suppressing ketosis, with the highest individual fasting insulin measuring a value of 11.92 uIU/mL. However, HOMA-IR was able to detect a significant change, where, in both NK phases, our participants' HOMA-IR was at the very healthy levels of 0.97 ($\pm$ 0.32, P1) and 1.11 ($\pm$ 0.41, P3). Conversely, suppressing ketosis managed to significantly double their mean HOMA-IR to 2.07 ($\pm$ 0.61, P2), bringing it into the beginning range of insulin resistance, with one participant having an HOMA-IR of > 3 after suppressing ketosis for 21 days. All participants' HOMA-IR values returned to their healthy baseline values within only 21 days of returning to NK by removing the intervention for SuK via the ingestion of carbohydrates. Given HOMA-IR's strong association and even predictive value in chronic disease risk, it would be of high value to understand how lifestyle may modulate this marker index measured alongside other biomarkers strongly associated with disease risk, such as GGT, IGF-1, MCP-1, and ageing, to provide further confidence and resolution in diagnostics and the more accurate phenotyping/categorisation of patients in studies [7].

In a cross-sectional analysis study of 12,266 participants from the NHANES database, a positive correlation was found between participants' HOMA-IR levels and their BA in US adults [1]. A prospective cohort study of 3,741 asymptomatic employees of Santander Bank

in Madrid, aged between 40–55 years upon recruitment, with no known CVD, investigated the relationship between early insulin resistance, as measured using the HOMA-IR index in normoglycaemic individuals assumed to be at a low risk of atherosclerosis, in addition to the effect of cardiovascular risk factors in individuals with normal HbA1c. The presence and progression of sub-clinical atherosclerosis (SA) were assessed using non-invasive vascular imaging modalities, multiterritorial vascular ultrasound, and coronary arterial calcification (CAC) scans [36]. The study found that HOMA-IR had a direct association with the multiterritorial extent of SA and CACs ($p < 0.001$). In summary, 85.1% of the reference group (HOMA-IR < 2) were free of coronary artery calcium (14% had a CACS of > 0). This proportion of participants with CAC increased as insulin resistance increased, with a HOMA-IR between 2 to 3, the proportion increased to 25.6%, and with a HOMA-IR > 3, the proportion increased further to 39.3%. A bibliometric study of 1,500 publications found HOMA-IR insulin resistance to be strongly associated with cerebral small vessel disease, ischemic stroke incidence, post-stroke depression, and early neurological deterioration in patients who suffered stroke [29].

The 21 days of suppressing ketosis resulted in hypoketonaemia due to insulin's suppressive control of ketogenesis [28]. Whereas P1 and P3 were typified by euketonaemia, along with healthy low insulin and HOMA-IR values. As our cohort was exclusively female, it is imperative to recognise the links between hyperinsulinaemia, metabolic health, and breast cancer [4,30]. In a retrospective case–control study, 80 non-diabetic patients with pre-menopausal and post-menopausal breast tumours were compared to 60 women with normal mammograms as a control. Hyperinsulinaemia and insulin resistance HOMA-IR have been found to be associated with an increased risk of breast cancer in non-diabetic (normoglycaemic) women. Whilst hyperinsulinaemia determined by fasting insulin levels of > 28 uIU/mL may proceed hyperglycaemia for up to 24 years [2,7–9,31], early sub-clinical (occult) hyperinsulinaemia may be considered when there is chronic hypoketonaemia measured between 4–6 p.m. for at least three consecutive days (a longer duration would increase confidence). Measurement at this time of day would reduce the false-positive acceptance of NK compliance from a test result resulting from waking up with ketosis levels (> 0.3 mmol/L) due to an overnight fast [7,32].

In our KetoSAge trial, the chronic suppression of ketosis (P2) intervention resulted in significantly increased fasting insulin levels, which would still be considered as healthy values, yet significantly increased HOMA-IR values into the insulin resistant range. Our participant data show that long-term NK maintains and reduces HOMA-IR to very healthy levels, adding to existing evidence that sustaining a lifestyle that promotes ketosis (minimising insulin demand and secretion) is an effective modality in preventing insulin resistance and its associated diseases, including cancer, CVD, dementia, T2DM, and earlier biological ageing [1,7,9,33–35]. It was very reassuring to see that our participants' HOMA-IR levels returned to their baseline mean value of 1.11 (± 0.41, P3) after the removal of carbohydrates, returning to sustained NK, indicating that restricting carbohydrates is a viable and practical method to reduce the risk of insulin-resistance-related morbidities known to affect healthspan and lifespan. Additionally, our data indicate that the effect of 21 days of suppressing ketosis on HOMA-IR, can be reversed by a subsequent period of sustained NK, in individuals who habitually maintain a ketogenic lifestyle.

4.3. Osteocalcin

Human OCN has a molecular weight of 5 kDa, is 49 amino acids long, and is the tenth most abundant protein in the body [37]. It has been shown to have an endocrine function in glucose homeostasis, insulin sensitivity, neurogenesis, cognitive health, and mitochondrial biogenesis [38–43]. The majority of OCN is synthesised by osteoblasts and osteocytes in the bone. Its carboxylated form (cOCN) is both deposited into the bone and released into the system by osteoblasts and osteocytes. unOCN is synthesised and released into the circulation by osteoblasts and osteocytes, as well as resorbed from the bone by osteoclasts [39]. cOCN is required for the correct alignment of hydroxyapatite (HA), which

confers bone its torsion and tensile ductility, giving it resistance to fragility fractures, which is not captured via bone mineral density (BMD) assessment [39,44].

In the KetoSAge trial, all forms of osteocalcin (tOCN, cOCN, and unOCN) significantly increased after 21 days of suppressing ketosis and then decreased back to baseline with the return to ketosis. Interestingly, OCN is synthesised and secreted from adipocytes only during adipogenesis [45]. Weight gain as fat mass occurred during the 21-day intervention phase (P2) to suppress ketosis via the re-introduction of dietary carbohydrates following the SUK Eatwell Guideline recommendations (data previously published in [7]). This may explain the increases in all forms of OCN.

tOCN, cOCN, and unOCN increased from P1 to P2 in all but one participant (ID: 1091). From P2 to P3, tOCN and cOCN decreased in all but one participant, which was the same individual (ID: 1091). Upon further investigation, participant ID: 1091 regularly reported consuming beef bone broth during P1 and P3. Since OCN is the second most abundant protein in bone, it is plausible that this broth may have served as an exogenous source of OCN, thus explaining the increase [38]. Removal of their data in unOCN analysis results in a significant decrease from P2 at 4,691.87 pg/mL (± 3,751.66 pg/mL) to P3 at 1,873.10 pg/mL (± 1,173.00 pg/mL; $p = 0.0489$). Further investigation into the exposure of exogenous OCN from foodstuffs is, therefore, warranted.

An increased HOMA-IR increases coronary atherosclerosis risk; furthermore, insulin resistance has a sub-clinical occult phase potentially detectable via repeated consecutive days blood ketosis and glucose testing (between 4–6 p.m.), where a capillary BHB value of < 0.3 would indicate hypoketonaemia due to occult hyperinsulinaemia. Endothelial progenitor cells (EPCs), which aid in the repair of the vasculature, may also come with vascular calcification, as they have been shown to express OCN genes in insulin-resistant chronic disease conditions such as atherosclerosis [36]. EPCs from 72 patients with coronary atherosclerosis undergoing invasive coronary assessment have been shown to express OCN genes, indicating epigenetic osteogenic transformation [46]. Other studies of patients with hyperinsulinaemia conditions, such as in CVD and chronic kidney disease, have detected vascular smooth muscle cell osteogenic differentiation and aortic valve tissue having an increased expression of sclerostin [39,47–50], corroborating the relationship between chronic increased insulin exposure altering gene expression in the osteogenic profile.

In a study of 2,493 individuals with MetS, plasma OCN levels were found to be inversely correlated with HOMA-IR, fasting insulin and glucose, leptin, and BMI ($p < 0.0010$ for each biomarker) [51]. Interestingly, OCN has been found to be lower in obese people [52,53] and those with T2DM, such as seen in a correlation analysis of 204 patients with T2DM, where an inverse relationship between OCN and HOMA-IR was found [52,54]. With this information, it would be easy to then make the assumption that a higher level of OCN would, therefore, be better. However, our KetoSAge trial showed an opposite pattern, where P1 and P3 had the healthiest low HOMA-IR values and significantly lower OCN (all forms) relative to P2, indicating OCN was positively associated with HOMA-IR in this cohort. Furthermore, as BMI and fat mass increased in P2 [7], so too did all forms of OCN in the present study. In corroboration with Ferron's work [55], OCN increased in P2, where insulin also increased, indicating a potential effect of OCN increasing insulin secretion. However, our trial did not corroborate an increase in sensitising glucose uptake, given that P2 came with a significant increase in fasting blood glucose, although the mean value was still in a healthy range. It is possible that OCN was rescuing the situation, meaning that, if OCN did not increase, perhaps there would have been a greater increase in glycaemia and a trend towards IR. The OCN patterns in MP1 individuals may behave much like free triiodothyronine (T3), where T3 demand increases as mitochondrial reactive oxygen species increase, causing oxidative damage to mitochondrial (mt) oxidative phosphorylation (OXPHOS) proteins and resulting in an increased demand of T3 to upregulate the synthesis of mtOXPHOS electron transport chain proteins. Furthermore, like the pattern seen in our prior work [7,32], we saw a lower T3 when in long-term ketosis and an increase in T3 after 21 days of suppressing ketosis, likely indicating an increased T3 sensitivity and

decreased demand, which may be a similar case to OCN. If we assume that, in a healthy setting, OCN would be synthesised and deposited into the bone and that only a low dose of OCN is steadily released into the circulation, therefore, bone OCN deposition would confer a healthier bone tensile strength, and there may be a potential greater sensitivity to the OCN in the circulation [7,32].

OCN has been shown to stimulate GLP-1 synthesis [56], a pattern that was not corroborated in our trial. However, there may be a difference between endogenous versus oral exposure to OCN. It is interesting that, as OCN significantly increased in P2, GLP-1 conversely decreased, which is an opposite trend to other experiments showing OCN activating GLP-1 synthesis [56,57]. Research into human physiology often investigates pathology and tries to reverse engineer relationships, or very often uses a control group labelled as the "healthy" group, where, in actual fact, they are the "common" group and not necessarily a group reflecting human evolutionary phenotypic living, which is likely to be in a state of ketosis for the majority of each 24 h day [2,8,58]. This would result in a metabolic signature that is different to non-pathology-presenting long-term suppressed ketosis metabolic phenotype 2 (MP2) people [8]. A low OCN concentration in healthy long-term ketosis should not be confused with a low OCN level as seen in obesity, T2DM, CVD, and AD, where these cohorts are also seen to have higher rates of fragility fractures with a normal to high bone mineral density, which is hyperinsulinaemia osteofragilitas and not osteoporosis (Cooper, Brookler and Crofts, 2021 [39]). This indicates poor bone health, with hyper mineralisation as a form of osteocyte fossilisation, rendering them unable to thrive nor survive, nor produce the necessary OCN, not only for the alignment of HA to confer bone tensile strength, but to also to be released into the circulation to play its part in healthy metabolic homeodynamic regulation [39]. In this context, a low plasma OCN concentration is indicative of pathology. It is, therefore, clear that clinicians and researchers need to be aware of understanding biomarkers within the metabolic phenotype profile of each individual, so as not to misdiagnose/categorise according to a single marker out of the patient's metabolic context.

4.4. Leptin Increases with Chronic Suppression of Ketosis a Risk of Hyperleptinaemia

Leptin is an adipokine, synthesised and secreted by white adipocytes, which has a 24 h circadian rhythm [59] and may be a more sensitive marker for hyperinsulinaemia than BMI. In a study of 119 normal-weight 18- to 24-year-old participants with a BMI of < 25, it was found that fasting leptin levels were significantly associated with fasting insulin ($\beta \pm SE = 0.30 \pm 0.06$, $p < 0.001$) and HOMA-IR ($\beta \pm SE = 0.41 \pm 0.20$, $p < 0.001$) [14]. These relationships were independent of age, gender, and total body fat. This indicates that, although leptin increases with obesity, it may be, that which causes obesity or excess body fat, which can exist in normal-BMI individuals, often termed TOFI (thin on the outside, fat on the inside) [60,61], also causes hyperleptinaemia and hyperinsulinaemia. These share a common root cause, and, therefore, explain why we see leptin and insulin rising where obesity may not, or may rise later on, meaning that obesity was not the first mover in causing the rises in these biomarkers associated with chronic metabolic diseases. Chronic excess insulin is the likely root cause, since insulin is so intrinsically linked to the regulation of leptin, and that excess may not be captured by a fasting insulin nor an oral glucose tolerance test (OGTT). Insulin tightly regulates ketogenesis, therefore, many consecutive days of BHB testing between 4–6 p.m. acts as a proxy to understanding individual hyperinsulinaemia, if we accept hyperinsulinaemia to mean "more than is ideal for optimal health" for an individual [28,62]. Our KetoSAge participants' leptin levels were healthily low in their natural habitual ketosis states, at levels classified/categorised as the healthiest in terms of CVD risk [63]. With the 21-day suppression of ketosis, which resulted in a significant increase in fasting insulin, there was a significant increase in fasting leptin too, to levels that re-classify into less healthy ranges associated with poorer health outcome [63]. Returning to ketosis showed a complete return to baseline levels, indicating

how responsive leptin is to dietary carbohydrate-stimulated insulin secretion in long-term habitually keto-adapted females.

The KetoSAge trial participants' weight and fat mass did increase significantly after 21 days of SuK, however, their BMI stayed well within the healthy range. This corroborates the understanding that, although BMI is a good tool for investigating disease risk, there are subsets of people with sub-clinical (occult) conditions that are being pooled into research, which decreases accurate reflections of physiology and pathophysiology in trial findings and data analysis. A BMI of < 25 kg/m^2 is considered as healthy, however, there are subsets of people with a normal BMI who have metabolic diseases and even some with a BMI of > 25 kg/m^2 that may have occult hyperinsulinaemia for many years when their BMI was < 25 kg/m^2 [60,61,64–66]. Boden et al. showed, using a 72 h euglycaemia-hyperinsulinaemia and hyperglycaemic clamp, that prolonged hyperinsulinaemia and not hyperglycaemia increased serum leptin in 28 healthy normal-weight males. Furthermore, high levels of free fatty acids also did not affect leptin release [59]. Whilst this trial intervention for SuK for 21 days resulted in these significant changes, it is thought-provoking to consider if it had lasted months, if not years.

Higher levels of leptin have been shown to be associated with increased frailty in older adults. Fragility fracture rate increases with hyperinsulinaemia [39], which is also associated with ageing, given that the "chronic" in chronic-elevated insulin indicates a long duration of time exposure, which chronological age reflects. In an investigation between leptin levels and incident frailty in 1,573 individuals from the Seniors-ENRICA cohort, aged $\geq$ 60 years and without T2DM [67], those with leptin levels in the highest tertile had a significantly increased risk of frailty (odds ratio [OR]: 2.12; 95% confidence interval [CI]: 1.47–3.06; p-trend < 0.001). In a multivariate linear regression analysis, leptin levels were shown to be positively associated with insulin resistance in 398 middle-aged and elderly Taiwanese individuals ($\beta = 0.226$, $p < 0.01$) [68]. Leptin has also been shown to be positively associated with atherosclerosis assessed by CAC in a cross-sectional study on 200 participants, aged between 35 and 75 years with T2DM (which is hyperinsulinaemia with hyperglycaemia, with insulin resistance) [63]. Given that the HOMA-IR and progression of early SA-CNIC-Santander study (NCT01410318) showed a significantly increased risk of SA with an increase in HOMA-IR, the KetoSAge trial provides us with valuable information on increasing our knowledge about how to maintain or modulate these biomarkers associated with one of the global leading causes of chronic disease mortality, CVD [36]. Our KetoSAge trial showed lower leptin levels during ketosis, coupled with very low HOMA-IR (no insulin resistance), and the suppression of ketosis increased leptin in lockstep with increasing insulin resistance. The removal of dietary farinaceous carbohydrates to no longer stimulate an increased insulin demand repeatedly showed how responsive these chronic disease biomarkers are to ketosis, the metabolic state that, throughout history, humans likely spent most of their time in.

4.5. Cortisol and Serotonin

The steroid glucocorticoid hormone cortisol is mainly synthesised in the adrenal cortex by the zona fasciculata and, to a much lesser degree, by the thymus, brain, intestine, and skin [69]. Further, cortisol is involved in the regulation of gluconeogenesis and is often considered to be the stress response or related hormone [70]. In 919 participants aged 60–75 years from the Edinburgh Type 2 Diabetes prospective study, elevated fasting plasma cortisol was positively associated with increased ischaemic heart disease [71]. The univariate analysis found an associated increased odds of ischaemic heart disease with cortisol concentrations of > 800 nmol/L (290 ng/mL). The standard reference range for cortisol in plasma is 33–246 ng/mL [72]. Our KetoSAge trial mean fasting serum cortisol levels were within healthy ranges in all phases of the trial, with ketosis phases' mean concentrations of 126.20 ng/mL (± 52.67, P1) (348.13 nmol/L) and 131.90 ng/mL (± 52.18, P3) (363.90 nmol/L), and after 21 days of SuK (P2), 112.70 ng/mL (± 58.46, P2) (310.90 nmol/L). It has been suggested that chronic gluconeogenesis can induce a "fight

or flight" emergency survival process, resulting in chronic elevated cortisol secretion [73]. Interestingly, although there were no significant changes in cortisol in the KetoSAge trial, the overall healthy low levels of cortisol should be noted, which indicated that ketosis via carbohydrate restriction with subsequent reliance on gluconeogenesis for a long period of time did not appear to result in hypercortisolaemia. Furthermore, as the KetoSAge participants were, on average, in long-term ketosis for 3.9 years, their plasma glucose source was predominantly from gluconeogenesis, despite having perfectly healthy cortisol levels. Together, these data strongly negate claims that long-term ketosis gluconeogenesis is a fight or flight emergency survival process.

OCN has been shown to modulate insulin sensitivity and secretion via stimulating β-cell serotonin synthesis and secretion to modulate pancreatic islet α cells [8,39], however, this modulation of serotonin may be intracellular, and its secretory actions act in a paracrine manner within the pancreas and, thus, are undetectable in the blood stream. We did not see significant changes in plasma serotonin in the KetoSAge trial.

4.6. GLP-1

The current "blockbuster" drugs on the market for diabetes and weight loss are classed as GLP-1 agonists. Our participants in long-term NK showed significantly higher levels of GLP-1, whilst suppressing ketosis significantly decreased GLP-1. Lifestyle practices that stimulate chronic excess insulin demand and secretion result in lower GLP-1 levels and, therefore, less GLP-1 receptor (GLP-1R) activation, which is associated with increased insulin resistance, obesity, and T2DM [74]. Interestingly, in a competing risk regression analysis of 462 incident first cancer cases with 2,417 controls, it was found that a higher fasting GLP-1 level was significantly associated with a lower risk of incident first cancer (sub-hazard ratio 0.90; 95% CI 0.82–0.99; $p = 0.022$) [75].

GLP-1 is excreted primarily by ileo-colonic (L) enteroendocrine cells in the distal small bowel and colon [76]. Interestingly, GLP-1 stimulates proinsulin gene expression, resulting in activating the replenishment of insulin stores [77]. GLP-1 activates GLP-1R on pancreatic islet α cells, inhibiting glucagon secretion, resulting in a lowering of plasma glucose independent of insulin [78,79]. We were unable to measure glucagon in our participants' samples due to the low sensitivity of the assays trialled, and potentially as a result of glucagon's rapid degradation ex vivo [80]. However, fasting glucose and insulin were both significantly lower in both NK phases, along with a significantly higher concentration of GLP-1, whilst fasting glucose was significantly higher during the P2 suppression of ketosis with significantly lower GLP-1 levels and higher fasting insulin. This suggests that the lower fasting glucose levels during both NK phases were likely due to the GLP-1 lowering of glucagon, thus resulting in a lower fasting glucose, independent of insulin, and, subsequently, the NK phases having lower fasting insulin values. GLP-1 is an incretin, however, and it enhances insulin secretion in a glucose-dependent manor [81]. Participants' low HOMA-IR values in both NK phases were half those of when suppressing ketosis (P2), meaning they had a significantly greater insulin sensitivity when in ketosis, which would mean that less basal insulin was required to "do the job" of regulating plasma glucose, if one were to ascribe this as the primary role of insulin. However, in the long-term NK state, basal insulin's primary role is not likely to be the regulation of plasma glucose and may instead be the regulation of fat oxidation and ketogenesis, as well as modulating hormones and growth factors [2,7], whilst GLP-1 may regulate basal glucose levels via glucagon.

OCN, more specifically, the uncarboxylated form unOCN, stimulates GLP-1 synthesis via the promiscuous GPRC6A receptor [82]. However, the KetoSAge participants had lower OCN (all forms) in both NK phases with higher GLP-1, indicating that the elevated GLP-1 was not likely to be due to endogenous OCN action. As the incretin effect of GLP-1 is glucose dependent, therefore, elevated basal GLP-1 in long-standing euketonaemia is likely acting on its none-incretin actions, such as the slowing of gastric emptying [83]. This may aid in increasing nutrient uptake during food scarcity periods, or when only consuming one meal a day, to facilitate maximal nutrient absorption which would confer a survival

benefit. The slowing of gastric emptying may assist in extending the time provided for intestinal processing and maximising the absorption of nutrients from the acidic chyme. This may explain the evolutionary purpose of elevated basal GLP-1 levels in humans who likely spent a great amount of time in ketosis and, thus, not exposed to dietary farinaceous nor sucrose-rich foods that would trigger bolus GLP-1 secretion.

Euketonaemia is achievable without fasting through carbohydrate restriction, which results in a fasting-mimicking state. Palmitic-acid-9-hydroxy-stearic-acid (9-PAHSA), a branched fatty acid ester of hydroxy fatty acids (FAHFAs), stimulates GLP-1 secretion. 9-PAHSA is endogenously synthesised and regulated by a fasting state (euketonaemia/NK) and dietary saturated fatty acid consumption [84]. Insulin-resistant people have lower levels of 9-PAHSA in their serum and adipose tissue, and serum 9-PAHSA levels are significantly correlated with insulin sensitivity shown in humans assessed via euglycaemic clamp [84]. FAHFAs are found in the fat of ruminant meat, milk, eggs, fish [85,86], and breast milk, although they are lower in the breast milk of obese lactating mothers [87]. Interestingly, omnivores have significantly higher serum levels of FAHFA than vegetarians/vegans, and in a 1-week over-feeding study in 15 lean males with a BMI < 27, the consumption of saturated fatty acids from whipping cream was shown to increase serum FAFHA [88,89]. All of these foods are common in many ketosis-supportive lifestyles.

The stimulation of basal GLP-1 secretion has been mainly studied in people in suppressed ketosis (hypoketonaemia) states, who are in the lean and overweight categories, or in pathology. It is arguable that stimulators of basal GLP-1 secretion should be studied in healthy long-term sustained NK (euketonaemia) individuals, who may better reflect the metabolic state that humans likely evolved in, where they would have had less frequent meals, with only seasonally available low farinaceous carbohydrate availability, and, thus, would have been naturally in ketosis for a greater number of hours in their 24 h day and overall year [2,7,39,90,91].

Trials administering oral glucose to hypoketonaemic participants have shown dietary glucose-causing bolus-associated GLP-1 secretion [79]. During the NK phases of the KetoSAge trial, the participants did not consume enough carbohydrates to cause the suppression of ketosis, whilst they consumed 267 g of carbohydrate spread over three meals a day during phase 2 to suppress ketosis. As can be seen, basal GLP-1 was significantly higher in the two phases with the least dietary glucose exposure, whilst after the participants had followed the SUK dietary guidelines to habitually consume the equivalent of three OGTTs worth of glucose for 21 days straight, which would be equivalent to 63 OGTTs, the KetoSAge participants' mean basal fasting GLP-1 levels were significantly lower, with higher fasting glucose and insulin. This might be explained by increased GLP-1 clearance in a hypoketonaemia state, via increased DDP-4 enzymatic breakdown or increased renal clearance. Alternatively, euketonaemia may decrease DDP-4 activity, thus reducing GLP-1 clearance [83,92]. This warrants further investigation.

Given that the "blockbuster" drugs for the current treatment for T2DM, and now used for the treatment of obesity to induce weight loss, are GLP-1R agonists [93], it would be beneficial to understand the physiological role of basal GLP-1 in healthy humans in a metabolic state reflective of how we evolved, namely, euketonaemia MP1. The fasting-mimicking-state euketonaemia effectuates weight normalisation, shown to bring back weight homeostasis, meaning that the overweight will lose weight and the underweight will return to a healthier weight, whilst resolving many metabolic and neurological health conditions in the process [94–99].

5. Strengths and Limitations

Our study is a novel investigation of the biomarkers in chronic diseases of ageing in a healthy pre-menopausal female population living in long-term ketosis. OCN and GLP-1 have not been measured in this cohort before. Further work assessing these biomarkers in larger cohorts across different ages and in pathologies should be considered. Due to the low sensitivity of several trialled glucagon assays, we were unable to measure glucagon in

this cohort of participant samples. Further details on strengths and limitations have been previously discussed in detail in our earlier publication [7].

6. Conclusions

Long-term ketosis does not result in chronic elevated cortisol levels, indicating that ketosis is not a chronic stress-inducing nor fight or flight state. Long-term NK, euketonaemia, results in elevated GLP-1 levels that would presumably stimulate GLP-1R, which is associated with maintaining a healthier weight or weight loss and satiety, a method employed by T2DM and weight loss GLP-1R agonists. Long-term sustained euketonaemia results in lower levels of leptin, which is associated with increased satiety sensitivity and healthier metabolic profiles. Whilst OCN is considered to be necessary for insulin secretion, given that we understand that a low level of insulin that does not suppress ketogenesis is a healthy level of insulin, MP1, euketonaemia, presents with lower plasma OCN levels. This likely indicates that the OCN remains in the bone, and would confer greater bone health. This is understood to be corroborated in those with low HOMA-IR values also having a lower risk of fragility fractures, indicating insulin sensitivity and, thus, not being hyperinsulinaemic. Furthermore, the increased plasma OCN seen after suppressing ketosis may be from osteoclast bone resorption, which may, over time, result in the hyper-mineralisation of the bones (increased BMD seen in T2DM and obesity), but also be coupled with an increased brittleness, resulting in increased fragility fractures rate, as seen in obesity and T2DM individuals. Lower OCN levels are found in the healthy euketonaemic state, and are accompanied with lower fasting glucose, insulin, insulin sensitivity HOMA-IR, and leptin and higher GLP- 1. Sustained NK showed no adverse health effects and may mitigate hyperinsulinemia. All biomarkers returned to basal P1 levels after removing the intervention for SuK, indicating that metabolic flexibility is maintained with long-term euketonaemia.

Author Contributions: I.D.C. conceived the hypothesis and study design, wrote the original draft, designed the figures, and reviewed and edited the final manuscript. I.D.C. and Y.K. conducted the trial. I.D.C., Y.K. and L.P. collected and compiled data. I.D.C., Y.K., L.P. and K.E. performed ex vivo bench top assays. I.D.C., Y.K., L.P. and K.E. analysed data. I.D.C. designed tables and graphs. I.D.C., Y.K., L.P., K.E., A.S.-M., K.B. and B.T.E. contributed to writing and reviewed and edited the final manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the College of Liberal of Arts and Sciences Research Ethics Committee, University of Westminster. Ethical approval was obtained by the College of Liberal of Arts and Sciences Research Ethics Committee, University of Westminster, United Kingdom (ETH2122-0634).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

Acknowledgments: The authors would like to acknowledge the time and dedication of all participants who participated in this study.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

9-PAHSA	palmitic-acid-9-hydroxy-stearic-acid
ACD	all-cause dementia
AD	Alzheimer's disease
BA	biological ageing
BHB	beta-hydroxybutyrate
BMI	body mass index

CAC	coronary artery calcification
CI	confidence interval
cOCN	carboxylated osteocalcin
CVD	cardiovascular disease
EPC	endothelial progenitor cells
FAHFA	fatty acid ester of hydroxy fatty acids
GGT	gamma-glutamyl transferase
GLP-1	glucagon like peptide-1
GLP-1R	glucagon like peptide-1 receptor
HA	hydroxyapatite
HOMA-IR	homeostatic model assessment for insulin resistance
IGF-1	insulin like growth factor-1
L	ileo-colonic
MASLD	metabolic-dysfunction-associated steatosis liver disease
MCP-1	monocyte chemotactic protein-1
MetS	metabolic syndrome
Mt	mitochondrial
MP	metabolic phenotype
NK	nutritional ketosis
OGTT	oral glucose tolerance test
OR	odds ratio
OXPHOS	oxidative phosphorylation
P1	Phase 1
P2	Phase 2
P3	Phase 3
RCT	randomised control trial
SA	sub-clinical atherosclerosis
SFA	saturated fatty acid
SUK	standard U.K. diet
SuK	suppression of ketosis
TOFI	thin on the outside fat on the inside
T2DM	type 2 diabetes mellitus
T3	triiodothyronine
tOCN	total osteocalcin
unOCN	uncarboxylated osteocalcin

References

1. Yang, H.; Gong, R.; Liu, M.; Deng, Y.; Zheng, X.; Hu, T. HOMA-IR is positively correlated with biological age and advanced aging in the US adult population. *Eur. J. Med. Res.* **2023**, *28*, 470. [CrossRef] [PubMed]
2. Cooper, I.D.; Kyriakidou, Y.; Petagine, L.; Edwards, K.; Elliott, B.T. Bio-hacking better health—Leveraging metabolic biochemistry to maximise healthspan. *Antioxidants* **2023**, *12*, 1749. [CrossRef] [PubMed]
3. UN World Population Ageing 2019: Highlights. Available online: https://www.un.org/en/development/desa/population/publications/pdf/ageing/WorldPopulationAgeing2019-Report.pdf (accessed on 13 September 2023).
4. WHO. Global Health Estimates: Leading Causes of Death. 2019. Available online: https://www.who.int/data/gho/data/themes/mortality-and-global-health-estimates/ghe-leading-causes-of-death (accessed on 13 September 2023).
5. ONS. Death Registration Summary Statistics, England and Wales—Office for National Statistics. ONS Website. 2023. Available online: https://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriages/deaths/articles/deathregistrationsummarystatisticsenglandandwales/2022 (accessed on 14 September 2023).
6. Araújo, J.; Cai, J.; Stevens, J. Prevalence of optimal metabolic ealth in american adults: National health and nutrition examination survey 2009–2016. *Metab. Syndr. Relat. Disord.* **2018**, *17*, 46–52. [CrossRef] [PubMed]
7. Cooper, I.D.; Kyriakidou, Y.; Edwards, K.; Petagine, L.; Seyfried, T.N.; Duraj, T.; Soto-Mota, A.; Scarborough, A.; Jacome, S.L.; Brookler, K.; et al. Ketosis Suppression and Ageing (KetoSAge): The effects of suppressing ketosis in long term keto-adapted non-athletic females. *Int. J. Mol. Sci.* **2023**, *24*, 15621. [CrossRef] [PubMed]
8. Cooper, I.D.; Brookler, K.H.; Kyriakidou, Y.; Elliott, B.T.; Crofts, C.A.P. Metabolic phenotypes and step by step evolution of type 2 diabetes: A new paradigm. *Biomedicines* **2021**, *9*, 800. [CrossRef] [PubMed]

9. Crofts, C.A.P.; Zinn, C.; Wheldon, M.; Schofield, M. Hyperinsulinemia: A unifying theory of chronic disease? *Diabesity* **2015**, *1*, 34. [CrossRef]
10. Petagine, L.; Zariwala, M.G.; Patel, V.B. Non-alcoholic fatty liver disease: Immunological mechanisms and current treatments. *World J. Gastroenterol.* **2023**, *29*, 4831–4850. [CrossRef] [PubMed]
11. Younossi, Z.M.; Rinella, M.E.; Sanyal, A.J.; Harrison, S.A.; Brunt, E.M.; Goodman, Z.; Cohen, D.E.; Loomba, R. From NAFLD to MAFLD: Implications of a Premature Change in Terminology. *Hepatology* **2021**, *73*, 1194–1198. [CrossRef] [PubMed]
12. Salvatore, T.; Galiero, R.; Caturano, A.; Rinaldi, L.; Criscuolo, L.; Di Martino, A.; Albanese, G.; Vetrano, E.; Catalini, C.; Sardu, C.; et al. Current knowledge on the pathophysiology of lean/normal-weight type 2 diabetes. *Int. J. Mol. Sci.* **2022**, *24*, 658. [CrossRef]
13. Nabulsi, A.A.; Folsom, A.R.; Heiss, G.; Weir, S.S.; Chambless, L.E.; Watson, R.L.; Eckfeldt, J.H. Atherosclerosis Risk in Communities Study Investigators Fasting hyperinsulinemia and cardiovascular disease risk factors in nondiabetic adults: Stronger associations in lean versus obese subjects. *Metabolism* **1995**, *44*, 914–922. [CrossRef]
14. Kempf, A.M.; Strother, M.L.; Li, C.; Kaur, H.; Huang, T.T.K. Leptin as a marker of body fat and hyperinsulinemia in college students. *J. Am. Coll. Health* **2006**, *55*, 175–180. [CrossRef]
15. Wiebe, N.; Muntner, P.; Tonelli, M. Associations of body mass index, fasting insulin, and inflammation with mortality: A prospective cohort study. *Int. J. Obes.* **2022**, *46*, 2107–2113. [CrossRef]
16. Moore, A.R.; Holland-Winkler, A.M.; Ansley, J.K.; Boone, E.D.H.; Schulte, M.K.O. Reliability and diagnostic performance of a new blood ketone and glucose meter in humans. *J. Int. Soc. Sports Nutr.* **2021**, *18*, 1–9. [CrossRef]
17. The Emerging Risk Factors Collaboration Diabetes mellitus, fasting glucose, and risk of cause-specific death. *N. Engl. J. Med.* **2011**, *364*, 829–841. [CrossRef]
18. Sherwani, S.I.; Khan, H.A.; Ekhzaimy, A.; Masood, A.; Sakharkar, M.K. Significance of HbA1c test in diagnosis and prognosis of diabetic patients. *Biomark. Insights* **2016**, *11*, 95. [CrossRef]
19. Ramirez, A.; Lange, C.; Kaduszkiewicz, H.; Weyerer, S.; Werle, J.; Pentzek, M.; Fuchs, A.; Riedel-Heller, S.G.; Luck, T.; Mösch, E.; et al. Elevated HbA1c is associated with increased risk of incident dementia in primary care patients. *J. Alzheimers Dis.* **2015**, *44*, 1203–1212. [CrossRef]
20. Crofts, C.; Schofield, G.; Zinn, C.; Wheldon, M.; Kraft, J. Identifying hyperinsulinaemia in the absence of impaired glucose tolerance: An examination of the Kraft database. *Diabetes Res. Clin. Pract.* **2016**, *118*, 50–57. [CrossRef]
21. Goalstone, M.L.; Leitner, J.W.; Wall, K.; Dolgonos, L.; Rother, K.I.; Accili, D.; Draznin, B. Effect of insulin on farnesyltransferase. *J. Biol. Chem.* **1998**, *273*, 23892–23896. [CrossRef]
22. Draznin, B. Mitogenic action of insulin: Friend, foe or "frenemy"? *Diabetologia* **2010**, *53*, 229–233. [CrossRef]
23. Goalstone, M.L.; Draznin, B. What does insulin do to Ras? *Cell. Signal.* **1998**, *10*, 297–301. [CrossRef] [PubMed]
24. Draznin, B. Mechanism of the mitogenic influence of hyperinsulinemia. *Diabetol. Metab. Syndr.* **2011**, *3*, 10. [CrossRef] [PubMed]
25. Hodson, A.E.; Tippetts, T.S.; Bikman, B.T. Insulin treatment increases myocardial ceramide accumulation and disrupts cardiometabolic function. *Cardiovasc. Diabetol.* **2015**, *14*, 153. [CrossRef] [PubMed]
26. Smith, M.E.; Tippetts, T.S.; Brassfield, E.S.; Tucker, B.J.; Ockey, A.; Swensen, A.C.; Anthonymuthu, T.S.; Washburn, T.D.; Kane, D.A.; Prince, J.T.; et al. Mitochondrial fission mediates ceramide-induced metabolic disruption in skeletal muscle. *Biochem. J.* **2013**, *456*, 427–439. [CrossRef] [PubMed]
27. Hansen, M.E.; Tippetts, T.S.; Anderson, M.C.; Holub, Z.E.; Moulton, E.R.; Swensen, A.C.; Prince, J.T.; Bikman, B.T. Insulin increases ceramide synthesis in skeletal muscle. *J. Diabetes Res.* **2014**, *2014*, 765784. [CrossRef] [PubMed]
28. Action to Control Cardiovascular Risk in Diabetes Study Group; Gerstein, H.C.; Miller, M.E.; Byington, R.P.; Goff, D.C., Jr.; Bigger, J.T.; Buse, J.B.; Cushman, W.C.; Genuth, S.; Ismail-Beigi, F.; et al. Effects of intensive glucose lowering in type 2 diabetes. *N. Engl. J. Med.* **2008**, *358*, 2545–2559. [CrossRef]
29. Zhou, X.; Kang, C.; Hu, Y.H.; Wang, X.C. Study on insulin resistance and ischemic cerebrovascular disease: A bibliometric analysis via CiteSpace. *Front. Public Health* **2023**, *11*, 1021378. [CrossRef] [PubMed]
30. Ahmed, S.B.M.; Radwan, N.; Amer, S.; Saheb Sharif-Askari, N.; Mahdami, A.; Samara, K.A.; Halwani, R.; Jelinek, H.F. Assessing the link between diabetic metabolic dysregulation and breast cancer progression. *Int. J. Mol. Sci.* **2023**, *24*, 11816. [CrossRef]
31. Cooper, I.D.; Crofts, C.A.P.; DiNicolantonio, J.J.; Malhotra, A.; Elliott, B.; Kyriakidou, Y.; Brookler, K.H. Relationships between hyperinsulinaemia, magnesium, vitamin D, thrombosis and COVID-19: Rationale for clinical management. *Open Hear.* **2020**, *7*, e001356. [CrossRef]
32. Cooper, I.D.; Sanchez-Pizarro, C.; Norwitz, N.G.; Feldman, D.; Kyriakidou, Y.; Edwards, K.; Petagine, L.; Elliot, B.T.; Soto-Mota, A. Thyroid markers and body composition predict LDL-cholesterol change in lean healthy women on a ketogenic diet: Experimental support for the lipid energy model. *Front. Endocrinol.* **2023**, *14*, 1326768. [CrossRef]
33. Paoli, A.; Rubini, A.; Volek, J.S.; Grimaldi, K.A. Beyond weight loss: A review of the therapeutic uses of very-low-carbohydrate (ketogenic) diets. *Eur. J. Clin. Nutr.* **2013**, *67*, 789–796. [CrossRef]
34. Gershuni, V.M.; Yan, S.L.; Medici, V. Nutritional ketosis for weight management and reversal of metabolic syndrome. *Curr. Nutr. Rep.* **2018**, *7*, 97–106. [CrossRef]
35. Janssen, J.A.M.J.L.; Street, E. Hyperinsulinemia and its pivotal role in aging, obesity, type 2 diabetes, cardiovascular disease and cancer. *Int. J. Mol. Sci.* **2021**, *22*, 7797. [CrossRef] [PubMed]

36. Iglesies-Grau, J.; Garcia-Alvarez, A.; Oliva, B.; Mendieta, G.; García-Lunar, I.; Fuster, J.J.; Devesa, A.; Pérez-Herreras, C.; Fernández-Ortiz, A.; Brugada, R.; et al. Early insulin resistance in normoglycemic low-risk individuals is associated with subclinical atherosclerosis. *Cardiovasc. Diabetol.* **2023**, *22*, 350. [CrossRef] [PubMed]
37. Hauschka, P.V.; Lian, J.B.; Cole, D.E.; Gundberg, C.M. Osteocalcin and matrix Gla protein: Vitamin K-dependent proteins in bone. *Phys. Rev.* **1989**, *69*, 990–1047. [CrossRef] [PubMed]
38. Mizokami, A.; Wang, D.G.; Tanaka, M.; Gao, J.; Takeuchi, H.; Matsui, T.; Hirata, M. An extract from pork bones containing osteocalcin improves glucose metabolism in mice by oral administration. *Biosci. Biotechnol. Biochem.* **2016**, *80*, 2176–2183. [CrossRef] [PubMed]
39. Cooper, I.D.; Brookler, K.H.; Crofts, C.A.P. Rethinking fragility fractures in type 2 diabetes: The link between hyperinsulinaemia and osteofragilitas. *Biomedicines* **2021**, *9*, 1165. [CrossRef] [PubMed]
40. Nakamura, M.; Imaoka, M.; Takeda, M. Interaction of bone and brain: Osteocalcin and cognition. *Int. J. Neurosci.* **2020**, *131*, 1115–1123. [CrossRef] [PubMed]
41. Wang, J.S.; Mazur, C.M.; Wein, M.N. Sclerostin and osteocalcin: Candidate bone-produced hormones. *Front. Endocrinol.* **2021**, *12*, 584147. [CrossRef] [PubMed]
42. Puig, J.; Blasco, G.; Daunis-I-Estadella, J.; Moreno, M.; Molina, X.; Alberich-Bayarri, A.; Xifra, G.; Pedraza, S.; Ricart, W.; Fernández-Aranda, F.; et al. Lower serum osteocalcin concentrations are associated with brain microstructural changes and worse cognitive performance. *Clin. Endocrinol.* **2016**, *84*, 756–763. [CrossRef]
43. Ferron, M.; Lacombe, J. Regulation of energy metabolism by the skeleton: Osteocalcin and beyond. *Arch. Biochem. Biophys.* **2014**, *561*, 137–146. [CrossRef]
44. Dede, A.D.; Tournis, S.; Dontas, I.; Trovas, G. Type 2 diabetes mellitus and fracture risk. *Metabolism* **2014**, *63*, 1480–1490. [CrossRef]
45. Foresta, C.; Strapazzon, G.; De Toni, L.; Gianesello, L.; Calcagno, A.; Pilon, C.; Plebani, M.; Vettor, R. Evidence for osteocalcin production by adipose tissue and its role in human metabolism. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 3502–3506. [CrossRef] [PubMed]
46. Gössl, M.; Mödder, U.I.; Atkinson, E.J.; Lerman, A.; Khosla, S. Osteocalcin expression by circulating endothelial progenitor cells in patients with coronary atherosclerosis. *J. Am. Coll. Cardiol.* **2008**, *52*, 1314. [CrossRef] [PubMed]
47. Zhu, D.; Mackenzie, N.C.W.; Millán, J.L.; Farquharson, C.; MacRae, V.E. The appearance and modulation of osteocyte marker expression during calcification of vascular smooth muscle cells. *PLoS ONE* **2011**, *6*, e19595. [CrossRef] [PubMed]
48. Brandenburg, V.M.; Kramann, R.; Koos, R.; Krüger, T.; Schurgers, L.; Mühlenbruch, G.; Hübner, S.; Gladziwa, U.; Drechsler, C.; Ketteler, M. Relationship between sclerostin and cardiovascular calcification in hemodialysis patients: A cross-sectional study. *BMC Nephrol.* **2013**, *14*, 219. [CrossRef] [PubMed]
49. De Maré, A.; D'haese, P.C.; Verhulst, A. The role of sclerostin in bone and ectopic calcification. *Int. J. Mol. Sci.* **2020**, *21*, 3199. [CrossRef] [PubMed]
50. Tyson, J.; Bundy, K.; Roach, C.; Douglas, H.; Ventura, V.; Segars, M.F.; Schwartz, O.; Simpson, C.L. Mechanisms of the osteogenic switch of smooth muscle cells in vascular calcification: WNT signaling, BMPs, mechanotransduction, and EndMT. *Bioengineering* **2020**, *7*, 88. [CrossRef] [PubMed]
51. Saleem, U.; Mosley, T.H.; Kullo, I.J. Serum osteocalcin is associated with measures of insulin resistance, adipokine levels, and the presence of metabolic syndrome. *Arterioscler. Thromb. Vasc. Biol.* **2010**, *30*, 1474–1478. [CrossRef] [PubMed]
52. Yaylali, Y.T.; Fidan-Yaylali, G.; Dedeoglu, O.; Senol, H. Osteocalcin and epicardial adipose tissue in obesity: New hints for epicardial adipose tissue–bone crosstalk. *Scand. Cardiovasc. J.* **2019**, *53*, 296–298. [CrossRef] [PubMed]
53. Bao, Y.; Xiaojing, M.; Yang, R.; Wang, F.; Hao, Y.; Dou, J.; He, H.; Jia, W. Inverse relationship between serum osteocalcin levels and visceral fat area in chinese men. *J. Clin. Endocrinol. Metab.* **2013**, *98*, 345–351. [CrossRef]
54. Lei, H.; Liu, J.; Wang, W.; Yang, X.; Feng, Z.; Zang, P.; Lu, B.; Shao, J. Association between osteocalcin, a pivotal marker of bone metabolism, and secretory function of islet beta cells and alpha cells in Chinese patients with type 2 diabetes mellitus: An observational study. *Diabetol. Metab. Syndr.* **2022**, *14*, 160. [CrossRef]
55. Ferron, M. Endocrine Functions of Bone. In *Principles of Endocrinology and Hormone Action*; Belfiore, A., LeRoith, D., Eds.; Springer: Cham, Switzerland, 2018; ISBN 978-3-319-44675-2.
56. Mizokami, A.; Yasutake, Y.; Gao, J.; Matsuda, M.; Takahashi, I.; Takeuchi, H.; Hirata, M. Osteocalcin induces release of glucagon-like peptide-1 and thereby stimulates insulin secretion in mice. *PLoS ONE* **2013**, *8*, 57375. [CrossRef]
57. Mizokami, A.; Mukai, S.; Gao, J.; Kawakubo-Yasukochi, T.; Otani, T.; Takeuchi, H.; Jimi, E.; Hirata, M. GLP-1 signaling is required for improvement of glucose tolerance by osteocalcin. *J. Endocrinol.* **2020**, *244*, 285–296. [CrossRef] [PubMed]
58. Ben-Dor, M.; Gopher, A.; Hershkovitz, I.; Barkai, R. Man the fat hunter: The demise of Homo erectus and the emergence of a new Hominin lineage in the middle pleistocene (ca. 400 kyr) Levant. *PLoS ONE* **2011**, *6*, e28689. [CrossRef] [PubMed]
59. Boden, G.; Chen, X.; Kolaczynski, J.W.; Polansky, M. Effects of prolonged hyperinsulinemia on serum leptin in normal human subjects. *J. Clin. Investig.* **1997**, *100*, 1107. [CrossRef] [PubMed]
60. Pluta, W.; Dudzińska, W.; Lubkowska, A. Metabolic obesity in people with normal body weight (MONW)—review of diagnostic criteria. *Int. J. Environ. Res. Public Health* **2022**, *19*, 624. [CrossRef]
61. Ruderman, N.B.; Schneider, S.H.; Berchtold, P. The "metabolically-obese," normal-weight individual. *Am. J. Clin. Nutr.* **1981**, *34*, 1617–1621. [CrossRef] [PubMed]

62. Veech, R.L. The therapeutic implications of ketone bodies: The effects of ketone bodies in pathological conditions: Ketosis, ketogenic diet, redox states, insulin resistance, and mitochondrial metabolism. *Prostaglandins Leukot. Essent. Fat. Acids* **2004**, *70*, 309–319. [CrossRef] [PubMed]
63. Reilly, M.P.; Iqbal, N.; Schutta, M.; Wolfe, M.L.; Scally, M.; Localio, A.R.; Rader, D.J.; Kimmel, S.E. Plasma leptin levels are associated with coronary atherosclerosis in type 2 diabetes. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 3872–3878. [CrossRef]
64. Zdrojewicz, Z.; Popowicz, E.; Szyca, M.; Michalik, T.; Śmieszniak, B. TOFI phenotype-its effect on the occurrence of diabetes. *Pediatr. Endocrinol. Diabetes. Metab.* **2017**, *23*, 96–100. [CrossRef] [PubMed]
65. Ghosh, A.; Abdo, S.; Zhao, S.; Wu, C.-H.; Shi, Y.; Lo, C.-S.; Chenier, I.; Alquier, T.; Filep, J.G.; Ingelfinger, J.R.; et al. Insulin Inhibits Nrf2 Gene Expression via Heterogeneous Nuclear Ribonucleoprotein F/K in Diabetic Mice. *Endocrinology* **2019**, *158*, 903–919. [CrossRef] [PubMed]
66. Ruderman, N.; Chisholm, D.; Pi-Sunyer, X.; Schneider, S. The metabolically obese, normal-weight individual revisited. *Diabetes* **1998**, *47*, 699–713. [CrossRef] [PubMed]
67. Lana, A.; Valdés-Bécares, A.; Buño, A.; Rodríguez-Artalejo, F.; Lopez-Garcia, E. Serum leptin concentration is associated with incident frailty in older adults. *Aging Dis.* **2017**, *8*, 240–249. [CrossRef] [PubMed]
68. Shih, Y.L.; Huang, T.C.; Shih, C.C.; Chen, J.Y. Relationship between leptin and insulin resistance among community-dwelling middle-aged and elderly populations in Taiwan. *J. Clin. Med.* **2022**, *11*, 5357. [CrossRef] [PubMed]
69. Stalder, T.; Kirschbaum, C. Cortisol. In *Encyclopedia of Behavioral Medicine*; Gellman, M.D., Ed.; Springer: Cham, Switzerland, 2020; pp. 561–567. ISBN 978-3-030-39903-0.
70. Yan, Y.X.; Xiao, H.B.; Wang, S.S.; Zhao, J.; He, Y.; Wang, W.; Dong, J. Investigation of the relationship between chronic stress and Insulin resistance in a chinese population. *J. Epidemiol.* **2016**, *26*, 355–360. [CrossRef] [PubMed]
71. Reynolds, R.M.; Labad, J.; Strachan, M.W.J.; Braun, A.; Fowkes, F.G.R.; Lee, A.J.; Frier, B.M.; Seckl, J.R.; Walker, B.R.; Price, J.F. Elevated fasting plasma cortisol is associated with ischaemic heart disease and its risk factors in people with type 2 diabetes: The Edinburgh Type 2 Diabetes Study. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 1602. [CrossRef] [PubMed]
72. Kushnir, M.M.; Neilson, R.; Roberts, W.L.; Rockwood, A.L. Cortisol and cortisone analysis in serum and plasma by atmospheric pressure photoionization tandem mass spectrometry. *Clin. Biochem.* **2004**, *37*, 357–362. [CrossRef] [PubMed]
73. Seal, S.V.; Turner, J.D. The 'Jekyll and Hyde' of gluconeogenesis: Early life adversity, later life stress, and metabolic disturbances. *Int. J. Mol. Sci.* **2021**, *22*, 3344. [CrossRef] [PubMed]
74. Popoviciu, M.S.; Păduraru, L.; Yahya, G.; Metwally, K.; Cavalu, S. Emerging role of GLP-1 agonists in obesity: A comprehensive review of randomised controlled trials. *Int. J. Mol. Sci.* **2023**, *24*, 10449. [CrossRef] [PubMed]
75. Jujić, A.; Godina, C.; Belting, M.; Melander, O.; Juul Holst, J.; Ahlqvist, E.; Gomez, M.F.; Nilsson, P.M.; Jernström, H.; Magnusson, M. Endogenous incretin levels and risk of first incident cancer: A prospective cohort study. *Sci. Rep.* **2023**, *13*, 382. [CrossRef]
76. Drucker, D.J. The biology of incretin hormones. *Cell Metab.* **2006**, *3*, 153–165. [CrossRef]
77. Drucker, D.J.; Philippe, J.; Mojsov, S.; Chick, W.L.; Habener, J.F. Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 3434. [CrossRef] [PubMed]
78. Ramracheya, R.; Chapman, C.; Chibalina, M.; Dou, H.; Miranda, C.; González, A.; Moritoh, Y.; Shigeto, M.; Zhang, Q.; Braun, M.; et al. GLP-1 suppresses glucagon secretion in human pancreatic alpha-cells by inhibition of P/Q-type Ca^{2+} channels. *Physiol. Rep.* **2018**, *6*, e13852. [CrossRef] [PubMed]
79. Kreymann, B.; Ghatei, M.A.; Williams, G.; Bloom, S.R. Glucagon-like peptide-1 7-36: A physiological incretin in man. *Lancet* **1987**, *330*, 1300–1304. [CrossRef] [PubMed]
80. Albrechtsen, N.J.W.; Bak, M.J.; Hartmann, B.; Christensen, L.W.; Kuhre, R.E.; Deacon, C.F.; Holst, J.J. Stability of glucagon-like peptide 1 and glucagon in human plasma. *Endocr. Connect.* **2015**, *4*, 50. [CrossRef] [PubMed]
81. Ebou, M.H.; Singh-Estivalet, A.; Launay, J.M.; Callebert, J.; Tronche, F.; Ferré, P.; Gautier, J.F.; Guillemain, G.; Bréant, B.; Blondeau, B.; et al. Glucocorticoids inhibit basal and hormone-induced serotonin synthesis in pancreatic beta cells. *PLoS ONE* **2016**, *11*, 149343. [CrossRef] [PubMed]
82. Pi, M.; Nishimoto, S.K.; Quarles, L.D. GPRC6A: Jack of all metabolism (or master of none). *Mol. Metab.* **2017**, *6*, 185–193. [CrossRef]
83. Müller, T.D.; Finan, B.; Bloom, S.R.; D'Alessio, D.; Drucker, D.J.; Flatt, P.R.; Fritsche, A.; Gribble, F.; Grill, H.J.; Habener, J.F.; et al. Glucagon-like peptide 1 (GLP-1). *Mol. Metab.* **2019**, *30*, 72–130. [CrossRef] [PubMed]
84. Yore, M.M.; Syed, I.; Moraes-Vieira, P.M.; Zhang, T.; Herman, M.A.; Homan, E.A.; Patel, R.T.; Lee, J.; Chen, S.; Peroni, O.D.; et al. Discovery of a class of endogenous mammalian lipids with anti-diabetic and anti-inflammatory effects. *Cell* **2014**, *159*, 318–332. [CrossRef] [PubMed]
85. Manful, C.F.; Pham, T.H.; Wheeler, E.; Nadeem, M.; Adigun, O.A.; Walsh, N.; Thomas, R.H. Assessing the fate of fatty acid esters of hydroxy fatty acids, diglycerides and monoacetyldiacylglycerides in grilled ruminant meats marinated with unfiltered beer-based marinades. *J. Food Drug Anal.* **2022**, *30*, 234. [CrossRef]
86. An, N.; Wang, Y.; He, D.X.; Mei, P.C.; Zhu, Q.F.; Feng, Y.Q. A dataset of branched fatty acid esters of hydroxy fatty acids diversity in foods. *Sci. Data* **2023**, *10*, 790. [CrossRef]
87. Brezinova, M.; Kuda, O.; Hansikova, J.; Rombaldova, M.; Balas, L.; Bardova, K.; Durand, T.; Rossmeisl, M.; Cerna, M.; Stranak, Z.; et al. Levels of palmitic acid ester of hydroxystearic acid (PAHSA) are reduced in the breast milk of obese mothers. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2018**, *1863*, 126–131. [CrossRef] [PubMed]

88. Ott, B.; Skurk, T.; Lagkouvardos, L.; Fischer, S.; Büttner, J.; Lichtenegger, M.; Clavel, T.; Lechner, A.; Rychlik, M.; Haller, D.; et al. Short-term overfeeding with dairy cream does not modify gut permeability, the fecal microbiota, or glucose metabolism in young healthy men. *J. Nutr.* **2018**, *148*, 77–85. [CrossRef] [PubMed]
89. Kellerer, T.; Kleigrewe, K.; Brandl, B.; Hofmann, T.; Hauner, H.; Skurk, T. Fatty acid esters of hydroxy fatty acids (FAHFAs) are associated with diet, BMI, and age. *Front. Nutr.* **2021**, *8*, 691401. [CrossRef] [PubMed]
90. Rollo, F.; Ubaldi, M.; Ermini, L.; Marota, I. Ötzi's last meals: DNA analysis of the intestinal content of the neolithic glacier mummy from the Alps. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 12594–12599. [CrossRef] [PubMed]
91. Ben-Dor, M.; Sirtoli, R.; Barkai, R. The evolution of the human trophic level during the Pleistocene. *Am. J. Phys. Anthropol.* **2021**, *175*, 27–56. [CrossRef] [PubMed]
92. Meier, J.J.; Nauck, M.A.; Kranz, D.; Holst, J.J.; Deacon, C.F.; Gaeckler, D.; Schmidt, W.E.; Gallwitz, B. Secretion, Degradation, and Elimination of Glucagon-Like Peptide 1 and Gastric Inhibitory Polypeptide in Patients with Chronic Renal Insufficiency and Healthy Control Subjects. *Diabetes* **2004**, *53*, 654–662. [CrossRef] [PubMed]
93. Fareed, A.; Hussain, A. The expanding role of GLP-1: From diabetes management to cancer treatment. *Clin. Med. Insights Endocrinol. Diabetes* **2023**, *16*. [CrossRef] [PubMed]
94. Hallberg, S.J.; McKenzie, A.L.; Williams, P.T.; Bhanpuri, N.H.; Peters, A.L.; Campbell, W.W.; Hazbun, T.L.; Volk, B.M.; McCarter, J.P.; Phinney, S.D.; et al. Effectiveness and safety of a novel care model for the management of type 2 diabetes at 1 Year: An open-label, non-randomized, controlled study. *Diabetes Ther.* **2018**, *9*, 583–612. [CrossRef] [PubMed]
95. Danan, A.; Westman, E.C.; Saslow, L.R.; Ede, G. The ketogenic diet for refractory mental illness: A retrospective analysis of 31 inpatients. *Front. Psychiatry* **2022**, *13*, 951376. [CrossRef]
96. Norwitz, N.G.; Hu, M.T.; Clarke, K. The mechanisms by which the ketone body D-β-hydroxybutyrate may improve the multiple cellular pathologies of Parkinson's disease. *Front. Nutr.* **2019**, *6*, 63. [CrossRef]
97. Kelly, T.; Unwin, D.; Finucane, F. Low-carbohydrate diets in the management of obesity and type 2 diabetes: A review from clinicians using the approach in practice. *Int. J. Environ. Res. Public Health* **2020**, *17*, 2557. [CrossRef] [PubMed]
98. Athinarayanan, S.J.; Adams, R.N.; Hallberg, S.J.; McKenzie, A.L.; Bhanpuri, N.H.; Campbell, W.W.; Volek, J.S.; Phinney, S.D.; McCarter, J.P. Long-term effects of a novel continuous remote care intervention including nutritional ketosis for the management of type 2 diabetes: A 2-year nonrandomized clinical trial. *Front. Endocrinol.* **2019**, *10*, 348. [CrossRef] [PubMed]
99. Norwitz, N.G.; Hurn, M.; Forcen, F.E.; Norwitz, N. Animal-based ketogenic diet puts severe anorexia nervosa into multi-year remission: A case series. *J. Metab. Health* **2023**, *6*, 8. [CrossRef]