

Article

Ketosis Suppression and Ageing (KetoSAge) Trial: Effect of Hypoketonaemia on Extracellular Vesicle Profiles in Healthy Premenopausal Women

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Abstract

Extracellular vesicles (EVs) mediate endocrine and metabolic signalling. EV responses to shifts in substrate utilisation, from euketonaemia to hypoketonaemia, remain poorly understood. Chronic hyperinsulinaemia suppresses ketogenesis, causing hypoketonaemia; however, its impact on EV profiles in metabolically healthy individuals has not been characterised. We investigated the effects of hypoketonaemia on EV profiles in the KetoSAge cohort, examining associations between EV concentration, mean size, and modal size and metabolic–endocrine biomarkers. Ten lean (BMI, 20.52 ± 1.39 kg/m²), healthy premenopausal women (mean age, 32.30 ± 8.97 years) maintaining euketonaemia (mean 3.9 ± 2.3 years) completed a three-phase crossover trial, comprising 21 days each of: euketonaemia (baseline), induced-hypoketonaemia, and return-to-euketonaemia. No statistically significant phase-level differences in EV concentration or size parameters were detected. Linear mixed-effects analyses identified nominally significant associations between EV concentration and adiponectin, free T3, bilirubin, luteinising hormone, and epidermal growth factor, between EV mean size and HDL-cholesterol and between EV modal size and insulin, HOMA-IR, beta-hydroxybutyrate, GKI, thyroid hormones, ALT, AST, IL-6 and oestrogen (all unadjusted $p < 0.05$). However, none of these associations remained statistically significant after false discovery rate correction (FDR < 0.10). Hypoketonaemia via insulin-compensated euglycaemia revealed coordinated EV–biomarker relationships across metabolic–endocrine, hepatic and inflammatory pathways. Although exploratory, these findings suggest that EV profiles may reflect an insulin-responsive signalling network linked to the systemic bioenergetic state, highlighting their potential as early indicators of metabolic–endocrine adaptation.



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1. Introduction

The global ageing population has significant implications, particularly in relation to ageing-related diseases. These include hyperinsulinaemia and metabolic syndrome (MetS) which encompasses a range of risk factors, such as hyperglycaemia, dyslipidaemia, hypertension, obesity, and insulin resistance [1]. Hyperinsulinaemia significantly increases the risk of non-communicable chronic diseases, including cardiovascular diseases (CVD), Alzheimer's disease (AD), type 2 diabetes mellitus (T2DM), metabolic dysfunction-associated steatotic liver disease (MASLD), chronic inflammation, and cancer [1–4].

Hyperinsulinaemia is a central driver of metabolic dysfunction, influencing multiple cell types, tissues, and organs, as well as inflammatory signalling pathways, circulating factors, and nutrient processing. Central to MetS is hyperinsulinaemia, often termed insulin resistance, characterised by altered cellular responses to insulin, which for a long period of time maintains euglycaemia through compensatory mechanisms [4,5]. If sustained chronically, dysregulated glucose metabolism may occur and further compensatory hyperinsulinemia ensues [6,7]. Excess adiposity further exacerbates this process through the secretion of adipokines and inflammatory mediators. Whilst blood-based biomarkers such as insulin, HOMA-IR, glucose, and HbA1c are established biomarkers of metabolic–endocrine dysfunction, understanding interactions across metabolic phenotypes is essential for identifying early mechanisms of disease progression.

Extracellular vesicles (EVs) are small membrane-bound particles released by cells into the extracellular environment and play a key role in intercellular communication. They carry various biomolecules, such as proteins, lipids, nucleic acids (including DNA, mRNA, and microRNAs), and metabolites [8,9]. EVs can be profiled into different subtypes based on size, biogenesis pathways, and molecular cargo, making them valuable biomarkers for disease diagnosis and prognosis [9–14]. EVs have also been proposed as biomarkers of ageing, reflecting the complexity of cellular communication and systemic metabolic regulation. Previous work supports the hypothesis that interventions targeting cellular health and EV-mediated signalling may contribute to improved healthspan and lifespan [10,15–18]. Indeed, recent studies further highlight the anti-ageing potential of EVs in regulating age-related phenotypes and their possible therapeutic relevance in cancer, T2DM, CVD, and neurodegenerative disorders [19,20].

In addition, emerging evidence suggests a significant role of EVs in the development of diseases associated with hyperinsulinaemia, MetS, and T2DM [8,11,19,21–26]. It has also been proposed that adipocyte-derived EVs are important mediators of obesity-associated metabolic dysfunctions [12]. Moreover, key features of hyperinsulinaemia/MetS have been linked to alterations in circulating EV concentration and size distribution. Obesity has been characterised with increased EV secretion both locally within adipose tissue and systemically in circulation [24,27]. Additional mechanisms of adipose tissue communication have also been suggested, including the release of long non-coding RNAs and microRNAs, many of which are transported within EV cargo [24].

Carbohydrate restriction (CR), fasting mimicking diets (FMD), and euketonaemia (formerly referred to as nutritional ketosis [1,4], characterised by euglycaemia with healthy low insulin levels ($<9 \mu\text{IU/mL}$) alongside elevated beta-hydroxybutyrate (BHB) levels ($>0.5 \text{ mmol/L}$)) have been associated with improved metabolic health, increased healthspan, and lifespan [1,2,4,28–33]. These effects are mediated through mechanisms

including increased autophagy, mitophagy and mitochondrial biogenesis [1,34–36]. Ketogenic metabolic–endocrine therapy (KMT) has therefore emerged as promising strategy for the prevention and management of hyperinsulinaemia and ageing-related diseases, including T2DM, CVD, cancer, and neurodegenerative disorders [37].

Chronic hyperinsulinaemia suppresses hepatic ketogenesis, resulting in hypoketonaemia, which precedes overt metabolic dysfunction despite apparently normal glucose homeostasis [4]. While euketonaemia has been associated with metabolic resilience and improved systemic health, the effects of suppressing ketosis on circulating EV release characteristics remain poorly understood. To our knowledge, no studies to date have examined the effect of long-standing euketonaemia (nutritional ketosis) and subsequent ketosis suppression (hyperinsulinaemia-mediated hypoketonaemia) in metabolically healthy, premenopausal women on circulating EV profiles. Therefore, this study aimed to isolate, quantify, and characterise plasma EVs in participants who maintain euketonaemia as their normal metabolic phenotype 1 (MP1) lifestyle [1,7,33], and to investigate the interplay between habitual diet lifestyle changes (i.e., from euketonaemia to hypoketonaemia), metabolic–endocrine biomarkers, and changes in EV release profiles.

2. Materials and Methods

2.1. Participant Characteristics and Study Design

Ten lean (weight, 52.99 ± 4.24 kg; height, 160.95 ± 7.28 cm; BMI, 20.52 ± 1.39 kg/m²), healthy premenopausal women (age, 32.30 ± 8.97 years) who habitually followed a ketogenic diet participated in the KetoSAge study. This was an open-labelled, non-randomised crossover trial comprising three phases: baseline nutritional ketosis (NK; Phase 1, P1), suppression of ketosis (SuK; Phase 2, P2) and return to NK following removal of the intervention (Phase 3, P3) as previously described [1,2,4,31,32]. Participants self-reported adherence to a lifestyle maintaining NK for ≥ 6 months (mean 3.9 ± 2.3 years), providing sufficient time for metabolic adaptations. Baseline characteristics, adherence measures during experimental period, details on ketosis adaptation, and macronutrient composition with statistical analysis between phases have been reported previously [1,2,4,31,32]. At the end of each study phase, participants attended the laboratory at 8:00 a.m. after a 12 h overnight fast for anthropometric measurements and blood sampling (Figure 1).

2.2. Anthropometric Measurements

Upon arrival at the laboratory, height was measured (to the nearest 0.1 cm) using a stadiometer (Marsden HM-250P Leicester Height Measure, Rotherham, UK). Body weight was measured (to the nearest 0.1 kg), and waist and hip circumference were obtained using a non-stretch anthropometric measuring tape seca[®] 201 (seca GmbH & Co. KG, Hamburg, Germany) while participants stood upright with feet together. The average of three measurements (cm) was used for analysis. Body mass index (BMI), fat mass and fat-free mass (FFM) were measured by bioelectrical impedance (BIA) using seca[®] mBCA 514 Medical Body Composition Analyser (seca GmbH & Co. KG, Hamburg, Germany). All measurements were taken following a 12 h overnight fast, with participants being with an empty bladder and wearing standardised light clothing [1,2,4,31,32].

2.3. Blood Collection and Measurement

Blood was drawn into tubes containing ethylenediaminetetraacetic acid (EDTA) or lithium heparin (BD, Oxford, UK) before being centrifuged at $3857 \times g$ for 10 min at 4 °C (Hettich Universal 320 R, Andreas Hettich GmbH, Tuttlingen, Germany), according to previously described methods [1]. 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 at $3857 \times g$ for 10 min at room temperature. Samples were either sent to SYNLAB Belgium SRL (Heppignies, Belgium) for analysis or aliquoted into cryovial tubes under sterile conditions and stored at -80°C for later analysis by Randox Laboratories Ltd. (Crumlin, Northern Ireland, UK), Clinilabs Limited (London, UK), or in-house testing [1,2,4,31,32].

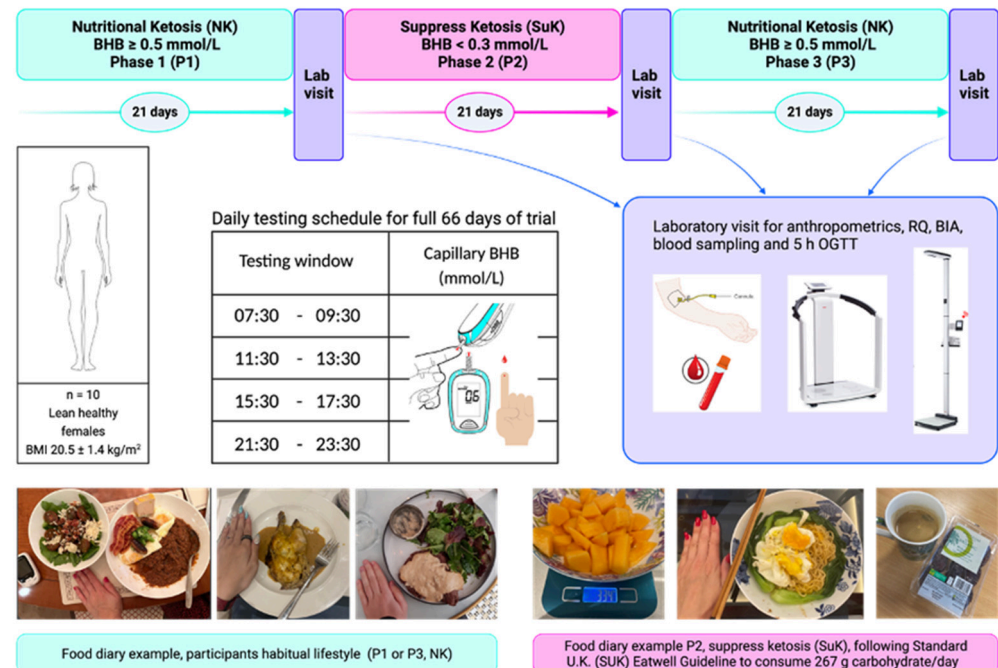


Figure 1. KetoSAGE study design. Phases 1 and 3 covered the participants' habitual nutritional ketosis lifestyle. Phase 2 was the interventional phase aimed at suppressing ketosis (SuK). Each phase was monitored via finger-prick testing of capillary beta-hydroxybutyrate (BHB) concentration (mmol/L). Testing was conducted four times per day, before consuming any food, 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) as described in our earlier publication [1]. Blood samples were taken at seven time points over 5 h. Whole-blood glucose and BHB were measured in real time using the Keto-Mojo™ meter, and a plasma insulin sensitivity assay was conducted later using an enzyme-linked immunosorbent assay (ELISA). Abbreviations: BIA, bioelectrical impedance; BMI, body mass index; NK, nutritional ketosis; OGTT, oral glucose tolerance test; P1, phase 1; P2, phase 2; P3, phase 3; RQ, respiratory quotient. Figure previously published in [1,2,4,31,32].

2.4. Blood Marker Analysis

Serum fasting insulin was measured using a Simple Plex Assay (Ella™, Bio-Techne, Minneapolis, MN, USA). Fasted venous whole blood glucose and BHB concentrations were measured using the Keto-Mojo™ GKI multi-function meter (Keto-Mojo, Napa, CA, USA) [1,2,31,38]. The glucose ketone index (GKI) was calculated from whole blood readings using a Keto-Mojo™ meter as follows: $\text{GKI} = \text{glucose (mmol/L)} \div \text{ketones (mmol/L)}$. Insulin resistance was estimated using the homeostatic model assessment (HOMA-IR): $\text{HOMA-IR} = (\text{Insulin } [\mu\text{IU/mL}] \times \text{glucose [mmol/L]}) / 22.5$.

Leptin (DuoSet, R&D Systems, Minneapolis, MN, USA), glucagon-like peptide (GLP-1; Abcam, Cambridge, UK), total osteocalcin (tOCN; DuoSet, R&D Systems, Minneapolis, MN, USA) and uncarboxylated osteocalcin (unOCN; BioLegend, San Diego, CA, USA) were quantified by ELISA from frozen serum or EDTA samples, according to the manufacturer's instructions. Monocyte chemoattractant protein-1 (MCP-1), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) were measured using Quantikine ELISA

kits (R&D Systems, Minneapolis, MN, USA). ELISA assays met predefined quality control criteria. Standard curves demonstrated excellent goodness of fit (mean $R^2 \geq 0.99$ for all assays), and intra-assay variability was low, with coefficients of variation (CVs) below 10% for both standard curve points and samples measured in triplicate.

Insulin-like growth factor 1 (IGF-1), gamma-glutamyl transferase (GGT), cortisol, plasminogen activator inhibitor-1 (PAI-1), thyroid-stimulating hormone (TSH), free triiodothyronine (T3), reverse T3, free thyroxine (T4), ferritin, total cholesterol, direct high-density lipoprotein (HDL) cholesterol, and triglycerides were measured externally by SYNLAB. Gastrin, direct low-density lipoprotein (LDL) cholesterol, interferon-gamma (IFN- γ), tumour necrosis factor-alpha (TNF- α), interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-8 and IL-10 were measured externally by Randox. Serum iron was measured by Randox and Clinilabs as previously reported [1,2,4,31,32]. Serum sex hormone-binding globulin (SHBG), oestrogen, progesterone, testosterone, luteinising hormone (LH), and follicle-stimulating hormone (FSH) were measured by Clinilabs.

Serum alkaline phosphatase (ALP) was measured in 5 participants by SYNLAB and in 5 participants by HORIBA Pentra C400 Clinical Chemistry Analyser (Horiba Medical, Northampton, UK). Serum or plasma albumin, alanine transferase (ALT), aspartate transferase (AST), creatine kinase NAC (CK-NAC), total protein, and total and direct bilirubin were assayed in triplicate on HORIBA Pentra C400. Validation on the HORIBA Pentra C400 was conducted in-house to assess variance and precision between samples [2].

2.5. Extracellular Vesicle Isolation and Characterisation from Human Plasma

2.5.1. Isolation of Plasma EVs

Plasma EVs were prepared from 100 μ L plasma aliquots from each participant. Plasma samples were thawed on ice prior to EV isolation, which was performed with minor adaptations from previously standardised procedures including for small-scale EV samples [14,39,40]. For each participant, 100 μ L of EDTA plasma aliquot was diluted at a 1:5 ratio (100 μ L plasma plus 400 μ L DPBS) in ice-cold Dulbecco's phosphate-buffered saline (DPBS; ultrafiltered using a 0.22 μ m filter prior to use). Diluted plasma was then centrifuged at $4000 \times g$ for 20 min at 4 $^{\circ}$ C to remove cellular debris, apoptotic bodies, and aggregates. The resulting supernatant, containing plasma EVs, was collected and centrifuged at $25,000 \times g$ for 1 h at 4 $^{\circ}$ C using a microtube rotor (75003652) at maximum speed in a MultifugeTM X1R centrifuge (Thermo Electron LED GmbH, Osterode am Harz, Germany). We developed this small-scale isolation approach in comparison with EV isolations carried out on larger preparations using ultracentrifugation at $100,000 \times g$ (following the initial $4000 \times g$ step) and observed comparable EV size distribution curves. The supernatant was discarded, and the EV-enriched pellets were resuspended in 100 μ L of DPBS and stored at -80° C until analysis, which was carried out within one week of EV preparation. EV characterisation adhered to the MISEV guidelines [9,41] and included nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and Western blot analysis for two surface markers, CD63 and Flotillin-1 (Flot-1).

2.5.2. Nanoparticle Tracking Analysis

Plasma-EV quantification and size distribution profiles were established by NTA, based on Brownian motion of particles in suspension, using the NanoSight NS300 system (Malvern Panalytical Ltd., Malvern, UK). The individual participant EV samples were diluted 1:100 in DPBS (10 μ L of EV preparation diluted in 990 μ L of DPBS). The diluted EV samples were applied to the NanoSight NS300 equipped with a sCMOS camera and a 488 nm diode laser, using an automatic syringe pump to ensure even flow of the sample, set at speed 50. Particle numbers per frame were 40–60, camera settings were at level

12 for recording and for post-analysis the detection threshold was set at 5. NTA videos were recorded for 4×60 s, and the replicate histograms generated from the repeated measurements were averaged for each participant, representing the mean $\pm$ SEM of the four recordings for each sample.

2.5.3. Transmission Electron Microscopy

Plasma EVs were further assessed by morphological analysis using TEM. EVs were resuspended in 100 mM sodium cacodylate buffer (pH 7.4). One drop (3–5 μ L) of the EV suspension was placed onto a grid, which held a carbon support film which had been previously glow discharged. Following partial air drying of the EV suspension, the sample was fixed for 1 min at room temperature (RT) by placing the grid onto a drop of a fixative solution (2.5% glutaraldehyde; Agar Scientific Ltd., Stansted, UK) in 100 mM sodium cacodylate buffer (pH 7.4). The grid was applied to the surface of three drops of distilled water for washing of the EV sample, removing excess water using a filter paper. The EVs were then stained for 1 min with 2% aqueous uranyl acetate (Agar Scientific Ltd., Stansted, UK), removing excess stain with a filter paper and air drying the grid. TEM imaging of EVs was conducted using a JEOL JEM 1400 transmission electron microscope (JEOL, Tokyo, Japan), which was operated at 80 kV, using a magnification of $30,000\times$ to $60,000\times$. Recording of digital images was performed with an AMT XR60 CCD camera (Deben, Bury St Edmunds, UK).

2.5.4. Western Blot Analysis

The plasma EVs were assessed for the EV-specific markers CD63 and Flot-1 using Western blotting. EV samples were diluted 1:1 in denaturing $2 \times$ Laemmli sample buffer (containing 5% β -mercaptoethanol; Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) and heated for 5 min at 100°C . Protein separation was carried out at 165 V using 4–20% gradient TGX gels (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK), followed by Western blotting at 15 V for 1 h using a Trans-Blot[®] SD semi-dry transfer cell (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Membranes were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich, Gillingham, UK) in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T; Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) for 1 h at RT, and primary antibody incubation was carried out overnight at 4°C using the EV markers CD63 (ab216130, Abcam, Cambridge, UK) and Flot-1 (ab41927, Abcam, Cambridge, UK), diluted 1:1000 in TBS-T. The membranes were then washed at RT in TBS-T for 3×10 min and thereafter incubated with HRP-conjugated goat anti-rabbit IgG secondary antibodies (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK), diluted 1:3000 in TBS-T, for 1 h at RT. The membranes were then washed for 4×10 min in TBS-T and visualised using enhanced chemiluminescence (ECL; Amersham, UK) with digital images acquired using the UVP BioDoc-It[™] System (Thermo Fisher Scientific, Dartford, UK).

2.6. Statistical Analysis

Data were assessed for normality using the Shapiro–Wilk test, acknowledging its limited power to detect non-normality in small samples ($n = 10$). Differences between study phases (P1, P2, P3) were evaluated using either repeated measures (RM) one-way analysis of variance (ANOVA) with Tukey's correction for multiple comparisons or the Friedman test with Dunn's correction, depending on normality. When the assumption of sphericity (Mauchly's test) was not met, the Geisser–Greenhouse correction was applied to the degrees of freedom. Values were considered statistically significant if $p < 0.05$. Data are presented as mean $\pm$ standard deviation (SD), unless otherwise stated. Statistical analyses and graphical outputs were generated using GraphPad Prism (version 11.0.2;

GraphPad Software, Inc., San Diego, CA, USA), except for NTA curve generation, which was performed using NanoSight 3.0 software (Malvern Panalytical Ltd., Malvern, UK).

Additionally, mixed-effects models and sensitivity analyses were conducted in R (version 4.5.1). To account for the repeated-measures study design, linear mixed-effects models with a random intercept for each participant were used to assess associations between physiological variables and EV profiles across study phases. Models were implemented using the `lmerTest::lmer` function, with hypothesis testing based on Satterthwaite's method for estimating degrees of freedom within a frequentist framework.

A separate linear mixed-effects model was fitted for each biomarker as the independent variable and each EV outcome (concentration, mean size, and modal size) as the dependent variable, using both raw and log-transformed data. Given the large number of association tests (>300) performed, the reported unadjusted *p* values should be interpreted as exploratory. To convey effect-size uncertainty, all fixed-effects estimates (β) are reported together with their corresponding 95% confidence intervals (CIs), calculated using Wald-type, profile-likelihood, or parametric bootstrap methods, as appropriate. A sensitivity analysis using the Benjamini–Hochberg procedure for false discovery rate (FDR) correction is also reported alongside the unadjusted *p* values, with FDR-adjusted *p* < 0.10 considered statistically significant.

3. Results

3.1. Participant Characteristics

Participants had a mean BMI of 20.52 (± 1.39 kg/m²) and a mean fat mass of 14.21 (± 2.55 kg) at baseline (P1). Key findings from our analysis in KetoSAGE participants are presented, as previously reported in KetoSAGE studies [1,2,4,31,32]. Markers of BMI, fat mass, FFM, insulin, glucose, BHB, HOMA-IR, GKI, IGF-1, leptin, GLP-1, GGT, ALP, ALT, and ALT/AST ratio were statistically significant from P1 to P2, with this trend reversing following P3. In contrast, cortisol, AST, albumin, CK-NAC, iron, total protein, total bilirubin, and direct bilirubin did not show statistically significant changes across phases. A summary of key metabolic and liver biomarkers across all study phases is presented in Table 1.

Table 1. BMI, fat mass, fat-free mass, fasted insulin, glucose, BHB, HOMA-IR, GKI, IGF-1, leptin, GLP-1, cortisol, GGT, ALP, ALT, AST, ALT/AST ratio, albumin, CK-NAC, iron, total protein, total and direct bilirubin across all phases in KetoSAGE participants. 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. Blood samples were collected at 8 a.m. after a 12 h overnight fast. Data analysed by RM one-way ANOVA or Friedman's test (*n* = 10).

KetoSAGE (<i>n</i> = 10)							
	P1	P2	P3	ANOVA <i>p</i> Value	P1 vs. P2	P2 vs. P3	P1 vs. P3
BMI (kg/m²)	20.52 (± 1.39)	21.54 (± 1.29)	20.82 (± 1.46)	<0.0001 *	<0.0001 *	<0.0001 *	0.0734
Fat Mass (kg)	14.21 (± 2.55)	15.88 (± 2.23)	14.78 (± 2.20)	<0.0001 *	<0.0001 *	0.0018 *	0.1102
FFM (kg)	38.78 (± 3.64)	39.77 (± 3.75)	37.22 (± 8.22)	0.0179 *	0.0219 *	0.1325	>0.9999
Insulin (μIU/mL)	4.95 (± 1.24)	9.06 (± 2.13)	5.62 (± 1.83)	<0.0001 *	<0.0001 *	0.0001 *	0.5686
Glucose (mmol/L)	4.23 (± 0.50)	5.01 (± 0.70)	4.24 (± 0.28)	0.0005 *	0.0014 *	0.0016 *	0.9980

Table 1. Cont.

KetoSAge (n = 10)							
	P1	P2	P3	ANOVA p Value	P1 vs. P2	P2 vs. P3	P1 vs. P3
BHB (mmol/L)	2.43 (±1.28)	0.18 (±0.13)	2.31 (±0.71)	0.0002 *	0.0012 *	<0.0001 *	0.9638
HOMA-IR	0.93 (±0.26)	2.03 (±0.65)	1.07 (±0.40)	<0.0001 *	0.0010 *	0.0024 *	0.4719
GKI (Lab Day)	2.23 (±1.20)	49.68 (±42.62)	1.99 (±0.60)	0.0001 *	0.0024 *	0.0024 *	>0.9999
GKI (21D Av)	2.82 (±1.34)	56.30 (±30.01)	2.76 (±1.15)	<0.0001 *	0.0010 *	0.0052 *	>0.9999
IGF-1 (µg/L)	149.30 (±32.96)	273.40 (±85.66)	136.90 (±39.60)	0.0015 *	0.0045 *	0.0055 *	0.4124
Leptin (ng/mL)	4.50 (±3.66)	15.08 (±8.00)	4.57 (±3.48)	<0.0001 *	0.0010 *	0.0052 *	>0.9999
GLP-1 (pg/mL)	1383.18 (±911.36)	576.72 (±452.43)	1471.85 (±1066.75)	0.0075 *	0.0219 *	0.0219 *	>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
GGT (U/L)	9.60 (±3.13)	12.40 (±2.55)	9.70 (±2.50)	0.0021 *	0.0044 *	0.0059 *	0.9904
ALP (U/L)	52.98 (±11.43)	66.98 (±15.60)	56.39 (±15.28)	0.0160 *	0.0160 *	0.0742	0.7328
ALT (U/L)	13.71 (±3.64)	25.42 (±12.26)	13.16 (±2.69)	<0.0001 *	0.0010 *	0.0052 *	>0.9999
AST (U/L)	18.65 (±4.15)	26.18 (±8.77)	19.63 (±3.11)	0.0265 *	0.0570	0.0915	0.5481
ALT/AST ratio	0.74 (±0.14)	0.96 (±0.30)	0.69 (±0.17)	0.0017 *	0.0266 *	0.0047 *	0.2387
Albumin (g/L)	41.82 (±3.66)	40.49 (±2.08)	42.37 (±2.10)	0.1774	0.3881	0.1681	0.8480
CK-NAC (U/L)	55.03 (±24.15)	77.30 (±43.75)	61.30 (±24.24)	0.2223	0.2209	>0.9999	>0.9999
Iron (µmol/L)	16.62 (±7.27)	14.40 (±8.70)	11.76 (±11.78)	0.1873	>0.9999	0.2209	0.3526
Total Protein (g/L)	69.40 (±9.58)	66.73 (±6.36)	67.25 (±3.79)	0.8302	>0.9999	>0.9999	>0.9999
Total Bilirubin (µmol/L)	7.88 (±4.50)	6.72 (±1.67)	5.59 (±2.29)	0.4362	>0.9999	>0.9999	0.5391
Direct Bilirubin (µmol/L)	1.82 (±1.10)	1.69 (±0.59)	1.65 (±0.44)	0.7909	0.9290	0.9893	0.8352

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate amino-transferase; BHB, beta-hydroxybutyrate; BMI, body mass index; CK-NAC, creatine kinase NAC; FFM, fat-free mass; GGT, gamma-glutamyl transferase; GKI (21D Av), glucose ketone index (21-Day Average); GLP-1, glucagon-like peptide 1; HOMA-IR, homeostatic model assessment of insulin resistance; IGF-1, insulin-like growth factor 1. * $p < 0.05$ indicates a statistically significant difference; anthropometric and blood biomarker data have been presented previously in the KetoSAge cohort [1,2,4,31,32]; grey shading is used for visual distinction between alternating rows only.

3.2. Extracellular Vesicle Profile

EV profiles (particle concentration, mean size and modal size) were assessed by NTA (a representative NTA sample curve is shown in Figures 2A and S1) and were characterised by Western blotting for two EV surface markers (CD63 and Flot-1; Figure 2B, and the full lane in Supplementary Figure S2), and by TEM for morphology (Figure 2C).

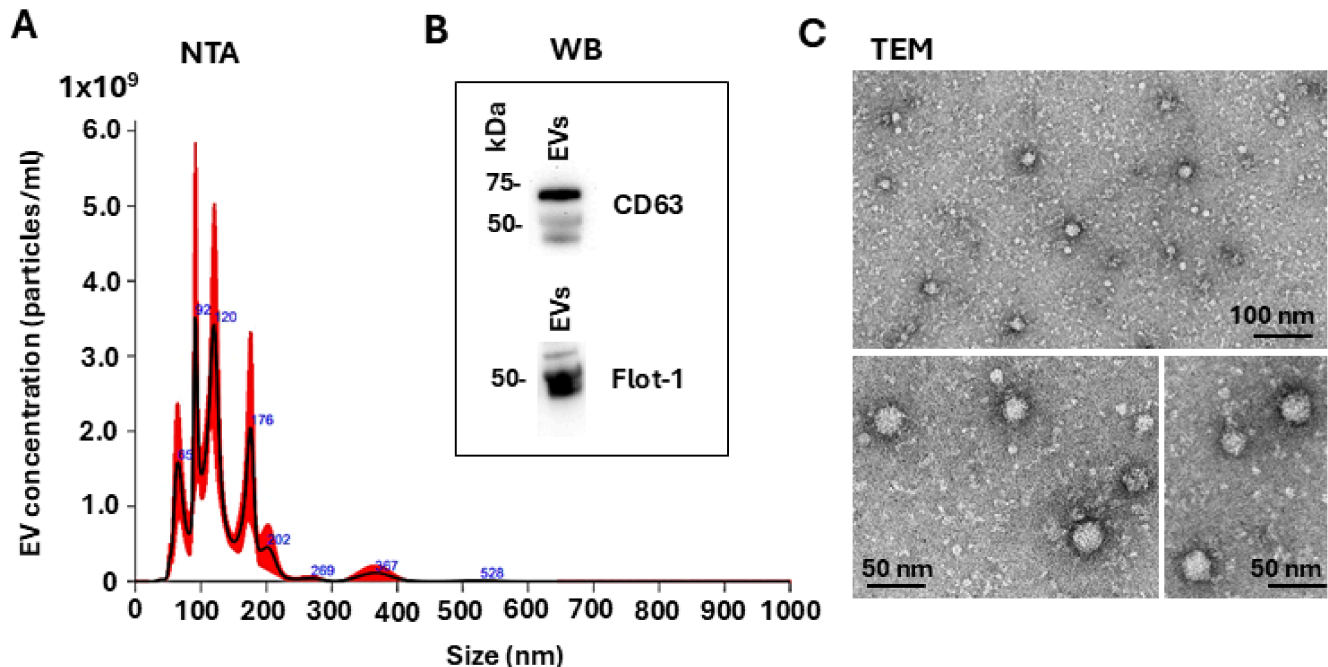


Figure 2. Characterisation of plasma EVs from KetoSage trial profiles. (A) Representative example of nanoparticle tracking analysis (NTA), mean is shown by the black line and SEM presented by the red line. (B) Western blotting of human plasma EVs showing positive for CD63 and Flot-1; size standard is presented in kDa. (C) Transmission electron microscopy (TEM) of human plasma EVs; scale bar is indicated at 50 and 100 nm, respectively.

3.3. Extracellular Vesicles Analysis in Suppression of Ketosis

Across the three study phases in the KetoSage participants, no statistically significant phase-level differences were detected in circulating EV concentration [P1, median (Md) = 3.54×10^9 (6.61×10^9) particles/mL vs. P2, Md = 7.11×10^9 (10.24×10^9) particles/mL vs. P3, Md = 3.93×10^9 (3.52×10^9) particles/mL; $p = 0.1352$, Figure 3A], EV mean size [P1, Md = 134.1 (43.9) nm vs. P2, Md = 129.7 (39.9) nm vs. P3, Md = 158.6 (42.3) nm; $p = 0.3675$, Figure 3B], or EV modal size [P1, mean (M) = 114.70 ± 34.33 nm vs. P2, M = 96.07 ± 15.41 nm vs. P3, M = 112.60 ± 26.96 nm; $p = 0.0704$, Figure 3C], although directional changes were observed (Figure 3).

3.4. Associations of Metabolic, Endocrine, Liver, Sex Hormone, Lipid, and Cytokine Biomarkers with EV Concentration

Table 2 summarises the relationships between key metabolic, endocrine, liver, lipid, and cytokine biomarkers and EV concentrations across the three study phases, while accounting for within-participant variability. Nominally significant positive associations were identified between EV concentration and adiponectin [effect estimate (β) = 6.07×10^8 (95% CI: 7.89×10^7 , 1.14×10^9)], free T3 [β = 2.84×10^9 (95% CI: 1.28×10^8 , 5.56×10^9)], total bilirubin [β = 9.46×10^8 (95% CI: 1.24×10^8 , 1.77×10^9)], direct bilirubin [β = 4.22×10^9 (95% CI: 7.66×10^8 , 7.67×10^9)], LH [β = 1.86×10^8 (95% CI: 2.52×10^7 , 3.46×10^8)], and EGF [β = 8.49×10^7 (95% CI: 4.52×10^6 , 1.65×10^8)]; all unadjusted $p < 0.05$. However,

none of these associations remained statistically significant after FDR correction (Table 2; Figure 4). No other biomarkers showed nominal associations with EV concentration.

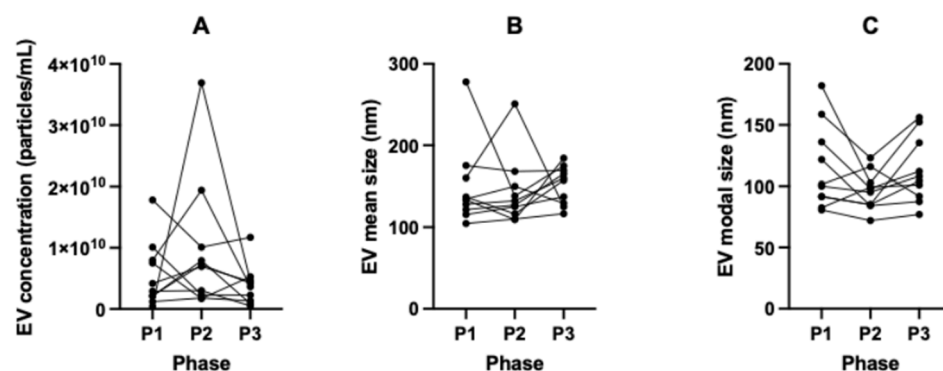


Figure 3. Nanoparticle tracking analysis of KetoSAge participants ($n = 10$) in three study phases. (A) EV concentration (particles/mL) between the three phases; (B) EV mean size of nanoparticles (nm) between the three phases; and (C) EV modal size (nm) between the three phases. Data analysed by Friedman test in graphs (A,B) and presented as median (Md) and interquartile range (IQR), and by repeated measures one-way ANOVA in graph (C) and presented as mean (M) $\pm$ SD.

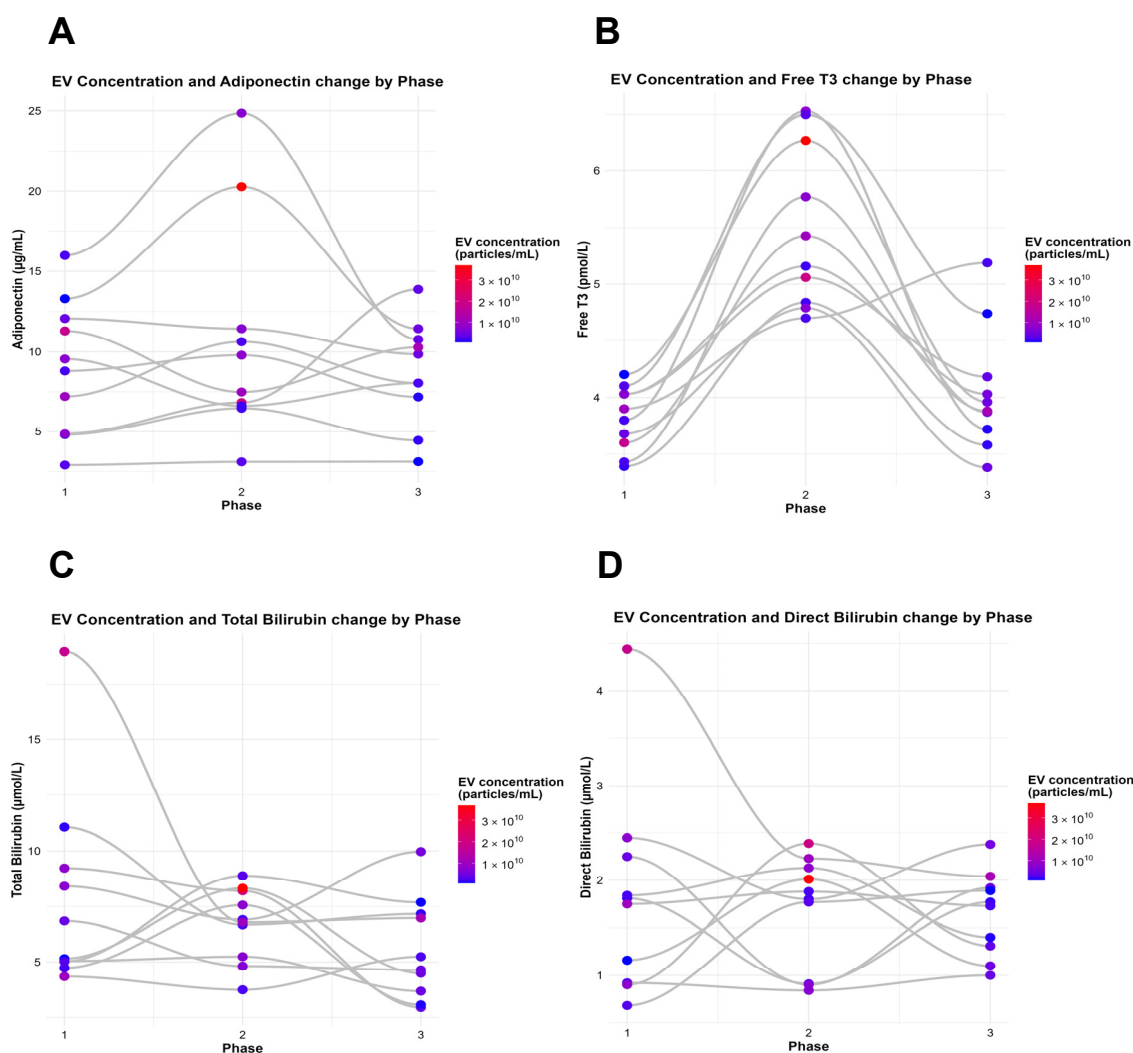


Figure 4. Cont.

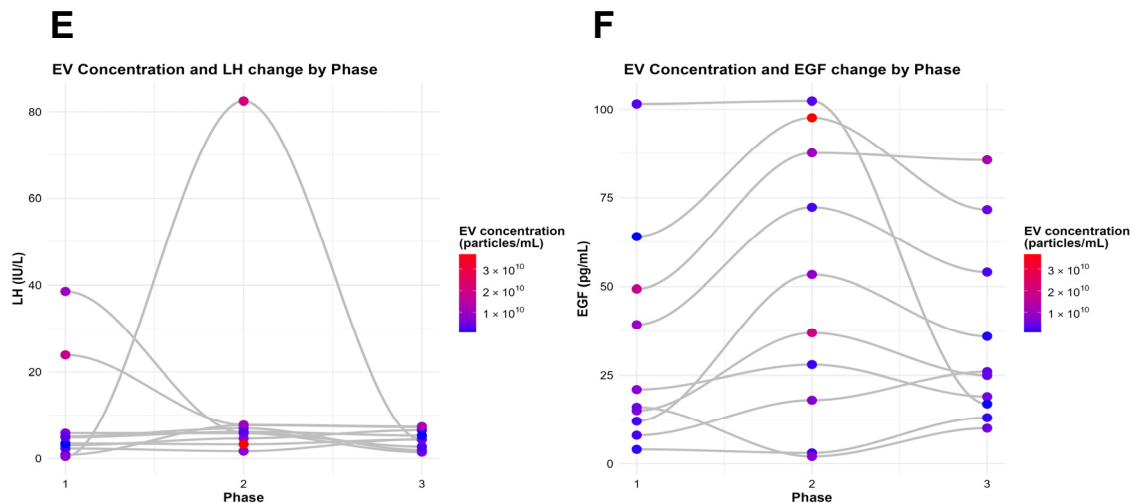


Figure 4. Associations between EV concentration and (A) adiponectin, (B) free T3, (C) total bilirubin, (D) direct bilirubin, (E) luteinising hormone (LH), and (F) epidermal growth factor (EGF) across all study phases. Analyses were performed in R, and graphs were generated using the ggplot2 package (version 4.0.0) in RStudio (version 2025.09.1+401).

Following log-transformation to account for scale differences across variables, a similar pattern of associations was observed. Positive associations were identified between EV concentration and GKI (21-Day Average) [$\beta = 2.51 \times 10^{-1}$ (95% CI: 0.025, 0.477)] and iron [$\beta = 6.79 \times 10^{-1}$ (95% CI: 0.049, 1.309)]; both unadjusted $p < 0.05$. However, neither association remained statistically significant after FDR correction (Table 2; Figure 5). No other nominal associations were identified.

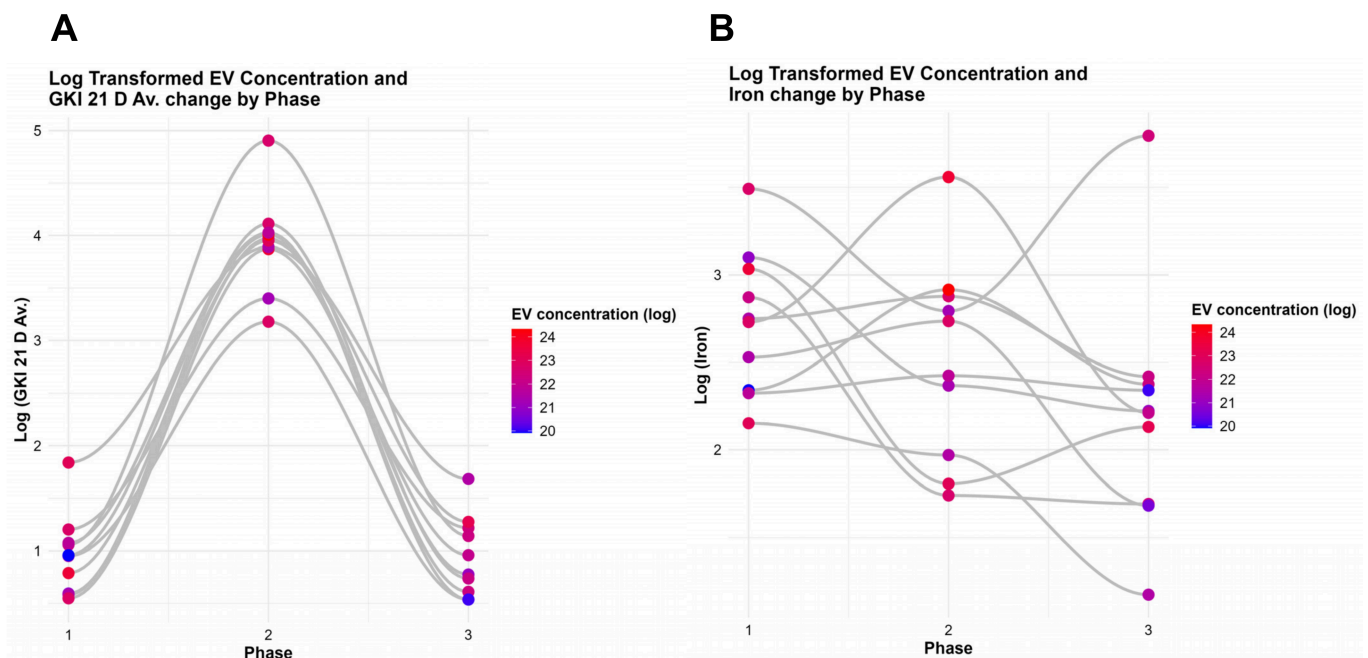


Figure 5. Associations between log-transformed EV concentration and (A) GKI (21-Day Average) and (B) iron across all study phases. Analyses were performed in R, and graphs were generated using the ggplot2 package (version 4.0.0) in RStudio (version 2025.09.1+401).

Table 2. Associations between metabolic, endocrine, liver, sex hormone, lipid, and cytokine biomarkers and EV concentration across the three study phases in KetoSAge participants. Measurements were taken following each study phase: baseline nutritional ketosis (NK; P1); intervention suppress ketosis (SuK; P2); and return to NK after SuK removal (P3). All samples were collected at 8 a.m. after a 12 h overnight fast ($n = 10$; § $n = 9$).

					Log-Transformed			
Model	Effect Estimate	95% CI	p Value	FDR Adjusted p Value	Effect Estimate	95% CI	p Value	FDR Adjusted p Value
Metabolic–Endocrine Biomarkers								
Insulin ~ EV concentration	6.64×10^8	$(-4.07 \times 10^8, 1.74 \times 10^9)$	0.2264	0.7369	7.27×10^{-1}	$(-0.232, 1.685)$	0.1485	0.5757
Glucose ~ EV concentration	1.59×10^9	$(-2.81 \times 10^9, 5.99 \times 10^9)$	0.4804	0.8211	1.22	$(-1.628, 4.068)$	0.4081	0.7754
BHB ~ EV concentration	-9.88×10^8	$(-2.97 \times 10^9, 9.99 \times 10^8)$	0.3301	0.7807	-1.84×10^{-1}	$(-0.422, 0.054)$	0.1454	0.5757
HOMA-IR ~ EV concentration	2.43×10^9	$(-1.57 \times 10^9, 6.43 \times 10^9)$	0.2349	0.7439	5.73×10^{-1}	$(-0.215, 1.361)$	0.1656	0.5757
GKI (Lab Day) ~ EV concentration	2.12×10^7	$(-6.11 \times 10^7, 1.03 \times 10^8)$	0.6141	0.8488	1.74×10^{-1}	$(-0.052, 0.401)$	0.1475	0.5757
GKI (21D Av) ~ EV concentration	6.79×10^7	$(-1.61 \times 10^7, 1.52 \times 10^8)$	0.1136	0.7369	2.51×10^{-1}	$(0.025, 0.477)$	0.0413 *	0.5502
IGF-1 ~ EV concentration	1.53×10^7	$(-1.63 \times 10^7, 4.70 \times 10^7)$	0.3425	0.7807	6.22×10^{-1}	$(-0.239, 1.483)$	0.1702	0.5757
Leptin ~ EV concentration	3.37×10^8	$(-1.90 \times 10^7, 6.94 \times 10^8)$	0.0657	0.7145	2.77×10^{-1}	$(-0.123, 0.677)$	0.1857	0.5757
GLP-1 ~ EV concentration	3.45×10^5	$(-2.66 \times 10^6, 3.35 \times 10^6)$	0.8226	0.9193	3.40×10^{-3}	$(-0.502, 0.509)$	0.9895	0.9990
Cortisol ~ EV concentration	5.38×10^7	$(-4.84 \times 10^8, 5.92 \times 10^8)$	0.8472	0.9256	-5.70×10^{-2}	$(-0.916, 0.802)$	0.8985	0.9495
Adiponectin ~ EV concentration	6.07×10^8	$(7.89 \times 10^7, 1.14 \times 10^9)$	0.0364 *	0.6298	7.40×10^{-1}	$(0.035, 1.445)$	0.0604	0.5502
Gastrin ~ EV concentration	-1.07×10^8	$(-6.10 \times 10^8, 3.96 \times 10^8)$	0.6817	0.8550	-3.57×10^{-2}	$(-0.799, 0.727)$	0.9282	0.9678
tOCN ~ EV concentration	-3.68×10^7	$(-1.62 \times 10^8, 8.80 \times 10^7)$	0.5635	0.8235	-3.48×10^{-1}	$(-1.092, 0.396)$	0.3720	0.7574
unOCN ~ EV concentration	1.06×10^8	$(-9.82 \times 10^8, 1.19 \times 10^9)$	0.8498	0.9256	-1.26×10^{-1}	$(-0.692, 0.441)$	0.6699	0.9273
cOCN ~ EV concentration	-4.64×10^7	$(-1.86 \times 10^8, 9.33 \times 10^7)$	0.5152	0.8211	-3.67×10^{-1}	$(-1.122, 0.388)$	0.3543	0.7574
TSH ~ EV concentration	1.87×10^9	$(-1.95 \times 10^9, 5.69 \times 10^9)$	0.3513	0.7807	2.32×10^{-1}	$(-0.044, 0.508)$	0.1345	0.5757
Free T3 ~ EV concentration	2.84×10^9	$(1.28 \times 10^8, 5.56 \times 10^9)$	0.0405 *	0.6298	1.53	$(-0.252, 3.319)$	0.1059	0.5736
Reverse T3 ~ EV concentration	1.25×10^{10}	$(-1.73 \times 10^{10}, 4.23 \times 10^{10})$	0.4125	0.8109	9.95×10^{-1}	$(-0.030, 2.019)$	0.0676	0.5502
Free T4 ~ EV concentration	8.30×10^8	$(-1.25 \times 10^9, 2.91 \times 10^9)$	0.4398	0.8211	1.04	$(-2.752, 4.831)$	0.5959	0.9151
Liver Biomarkers								
GGT ~ EV concentration	5.27×10^8	$(-3.91 \times 10^8, 1.45 \times 10^9)$	0.2711	0.7624	1.22×10^{-1}	$(-1.104, 1.348)$	0.8472	0.9495
ALP ~ EV concentration	-1.05×10^8	$(-2.84 \times 10^8, 7.48 \times 10^7)$	0.2529	0.7624	-7.57×10^{-1}	$(-2.322, 0.807)$	0.3524	0.7574
ALT ~ EV concentration	-4.67×10^6	$(-3.02 \times 10^8, 2.92 \times 10^8)$	0.9755	0.9838	1.57×10^{-1}	$(-0.767, 1.082)$	0.7413	0.9273
AST ~ EV concentration	-8.15×10^7	$(-4.98 \times 10^8, 3.35 \times 10^8)$	0.7020	0.8560	3.44×10^{-1}	$(-1.024, 1.712)$	0.6261	0.9151
ALT/AST ratio ~ EV concentration	4.31×10^9	$(-7.47 \times 10^9, 1.61 \times 10^{10})$	0.4782	0.8211	-3.90×10^{-3}	$(-1.427, 1.419)$	0.9958	0.9990
Albumin ~ EV concentration	-2.43×10^8	$(-1.25 \times 10^9, 7.61 \times 10^8)$	0.6390	0.8488	-4.49×10^{-1}	$(-6.508, 5.610)$	0.8856	0.9495
CK NAC ~ EV concentration	-7.13×10^6	$(-9.39 \times 10^7, 7.97 \times 10^7)$	0.8733	0.9333	3.41×10^{-1}	$(-0.484, 1.167)$	0.4285	0.7878

Table 2. Cont.

Model	Effect Estimate	95% CI	<i>p</i> Value	FDR Adjusted <i>p</i> Value	Log-Transformed			
					Effect Estimate	95% CI	<i>p</i> Value	FDR Adjusted <i>p</i> Value
Iron ~ EV concentration	2.94×10^8	$(4.87 \times 10^6, 5.83 \times 10^8)$	0.0515	0.6774	6.79×10^{-1}	(0.049, 1.309)	0.0437 *	0.5502
Ferritin ~ EV concentration	3.07×10^7	$(-9.59 \times 10^7, 1.57 \times 10^8)$	0.6366	0.8488	-3.00×10^{-4}	(-0.543, 0.543)	0.9990	0.9990
Total Protein ~ EV concentration	-1.11×10^8	$(-5.25 \times 10^8, 3.04 \times 10^8)$	0.6073	0.8488	-8.29×10^{-2}	(-4.413, 4.247)	0.9705	0.9990
Total Bilirubin ~ EV concentration	9.46×10^8	$(1.24 \times 10^8, 1.77 \times 10^9)$	0.0270 *	0.6298	9.02×10^{-1}	(-0.003, 1.808)	0.0609	0.5502
Direct Bilirubin ~ EV concentration	4.22×10^9	$(7.66 \times 10^8, 7.67 \times 10^9)$	0.0206 *	0.6298	7.26×10^{-1}	(-0.163, 1.615)	0.1212	0.5757
Sex Hormones								
SHBG ~ EV concentration	-4.10×10^7	$(-9.95 \times 10^7, 1.76 \times 10^7)$	0.1878	0.7369	-6.44×10^{-1}	(-1.295, 0.006)	0.0748	0.5736
Oestrogen ~ EV concentration	3.67×10^6	$(-1.12 \times 10^6, 8.46 \times 10^6)$	0.1368	0.7369	3.21×10^{-1}	(-0.101, 0.753)	0.1553	0.5757
Testosterone ~ EV concentration	-5.34×10^8	$(-5.66 \times 10^9, 4.59 \times 10^9)$	0.8413	0.9256	-1.38×10^{-1}	(-0.976, 0.700)	0.7516	0.9273
Progesterone ~ EV concentration	-5.78×10^6	$(-1.82 \times 10^8, 1.70 \times 10^8)$	0.9487	0.9838	1.14×10^{-1}	(-0.092, 0.319)	0.2908	0.7004
LH ~ EV concentration	1.86×10^8	$(2.52 \times 10^7, 3.46 \times 10^8)$	0.0247 *	0.6298	2.75×10^{-1}	(-0.086, 0.637)	0.1481	0.5757
FSH ~ EV concentration	5.40×10^8	$(-2.67 \times 10^8, 1.35 \times 10^9)$	0.1913	0.7369	4.92×10^{-2}	(-0.654, 0.752)	0.8920	0.9495
Lipids								
Total Cholesterol ~ EV concentration	-1.84×10^7	$(-7.72 \times 10^7, 4.04 \times 10^7)$	0.5454	0.8211	-1.26	(-3.073, 0.554)	0.1867	0.5757
HDL Cholesterol ~ EV concentration	8.46×10^7	$(-1.60 \times 10^8, 3.29 \times 10^8)$	0.5069	0.8211	1.42	(-1.003, 3.836)	0.2678	0.6811
LDL Cholesterol ~ EV concentration	-1.00×10^9	$(-2.87 \times 10^9, 8.71 \times 10^8)$	0.3029	0.7757	-8.11×10^{-1}	(-1.839, 0.218)	0.1345	0.5757
Triglycerides ~ EV concentration	1.36×10^7	$(-7.12 \times 10^7, 9.83 \times 10^7)$	0.7553	0.8781	-3.28×10^{-1}	(-1.331, 0.674)	0.5265	0.8827
Cytokines								
PAI-1 ~ EV concentration	1.52×10^8	$(-3.05 \times 10^8, 6.09 \times 10^8)$	0.5233	0.8211	4.53×10^{-2}	(-0.422, 0.513)	0.8507	0.9495
MCP-1 ~ EV concentration	2.31×10^7	$(-1.59 \times 10^7, 6.22 \times 10^7)$	0.2516	0.7624	3.08×10^{-1}	(-0.428, 1.043)	0.4202	0.7811
EGF ~ EV concentration	8.49×10^7	$(4.52 \times 10^6, 1.65 \times 10^8)$	0.0384 *	0.6298	2.52×10^{-1}	(-0.123, 0.628)	0.2052	0.5947
VEGF ~ EV concentration	1.00×10^7	$(-2.20 \times 10^7, 4.21 \times 10^7)$	0.5496	0.8211	-8.33×10^{-2}	(-0.644, 0.478)	0.7766	0.9286
§INF-γ ~ EV concentration	-6.04×10^8	$(-2.27 \times 10^9, 1.07 \times 10^9)$	0.4897	0.8211	-2.39×10^{-1}	(-0.648, 0.170)	0.2775	0.6878
§TNF-α ~ EV concentration	-1.27×10^8	$(-2.27 \times 10^9, 2.01 \times 10^9)$	0.9092	0.9597	1.63×10^{-1}	(-0.789, 1.115)	0.7424	0.9273
§IL-1α ~ EV concentration	-4.23×10^9	$(-1.36 \times 10^{10}, 5.12 \times 10^9)$	0.3978	0.8002	-8.17×10^{-2}	(-0.470, 0.307)	0.6903	0.9273
§IL-1β ~ EV concentration	-4.19×10^8	$(-1.57 \times 10^9, 7.27 \times 10^8)$	0.4902	0.8211	-6.11×10^{-2}	(-0.604, 0.481)	0.8303	0.9495
§IL-2 ~ EV concentration	-1.52×10^9	$(-3.62 \times 10^9, 5.74 \times 10^8)$	0.1545	0.7369	-2.15×10^{-1}	(-0.739, 0.309)	0.4422	0.7961
§IL-4 ~ EV concentration	-8.56×10^8	$(-3.83 \times 10^9, 2.12 \times 10^9)$	0.5854	0.8343	-2.54×10^{-1}	(-1.187, 0.679)	0.6051	0.9151

Table 2. Cont.

Model	Effect Estimate	95% CI	<i>p</i> Value	FDR Adjusted <i>p</i> Value	Log-Transformed			
					Effect Estimate	95% CI	<i>p</i> Value	FDR Adjusted <i>p</i> Value
§IL-6 ~ EV concentration	-1.02×10^9	$(-4.34 \times 10^9, 2.29 \times 10^9)$	0.5452	0.8211	-5.02×10^{-2}	$(-0.671, 0.571)$	0.8784	0.9495
§IL-8 ~ EV concentration	-7.08×10^7	$(-4.74 \times 10^8, 3.32 \times 10^8)$	0.7374	0.8773	9.15×10^{-2}	$(-0.465, 0.648)$	0.7545	0.9273
§IL-10 ~ EV concentration	-8.26×10^8	$(-8.61 \times 10^9, 6.95 \times 10^9)$	0.8360	0.9256	1.30×10^{-1}	$(-0.558, 0.817)$	0.7155	0.9273

Effect estimates (β) are reported in scientific notation; CI, confidence interval; FDR, false discovery rate; * $p < 0.05$ indicates nominal statistical significance based on the unadjusted p value; § biomarkers were measured in $n = 9$ participants.

3.5. Associations of Metabolic, Endocrine, Liver, Sex Hormone, Lipid, and Cytokine Biomarkers with EV Mean Size

Table 3 shows the relationships between key metabolic, endocrine, liver, lipid, and cytokine biomarkers and EV mean size across the three study phases, while accounting for within-participant variability. HDL cholesterol was the only biomarker nominally associated with EV mean size, showing positive associations in both the raw analysis [$\beta = 1.37$ (95% CI: 0.188, 2.558)] and the log-transformed analysis [$\beta = 5.55 \times 10^{-1}$ (95% CI: 0.066, 1.044)]; both unadjusted $p < 0.05$. However, neither association remained statistically significant after FDR correction (Table 3; Figure 6).

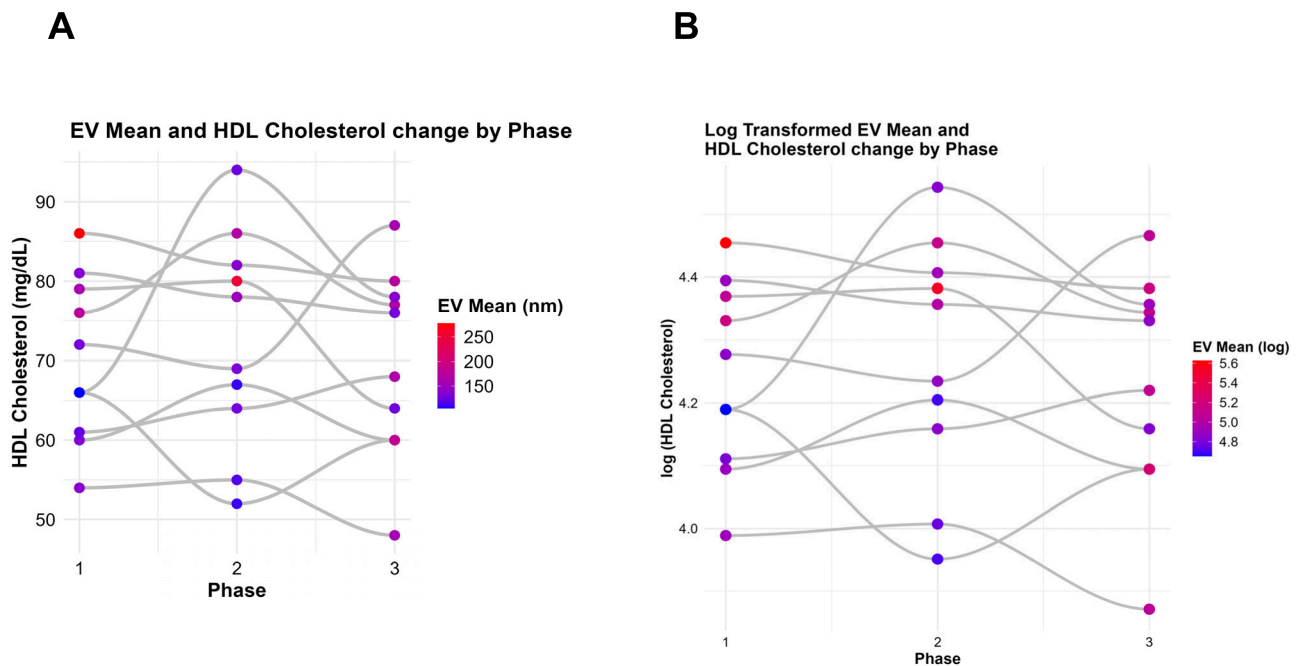


Figure 6. Associations between EV mean and HDL cholesterol across all study phases, using (A) raw data and (B) log-transformed data. Analyses were performed in R, and graphs were generated using the ggplot2 package (version 4.0.0) in RStudio (version 2025.09.1+401).

3.6. Associations of Metabolic, Endocrine, Liver, Sex Hormone, Lipid, and Cytokine Biomarkers with EV Modal Size

Table 4 presents the relationships between key metabolic, endocrine, liver, lipid, and cytokine biomarkers and changes in EV modal size across the three study phases, while accounting for within-participant variability. Nominal inverse associations were identified between EV modal size and insulin [$\beta = -3.24$ (95% CI: -6.207 , -0.265)], free T3 [$\beta = -9.11$ (95% CI: -16.651 , -1.566)], free T4 [$\beta = -7.98$ (95% CI: -14.572 , -1.38)], ALT [$\beta = -9.16 \times 10^{-1}$ (95% CI: -1.724 , -0.107)], and AST [$\beta = -1.33$ (95% CI: -2.497 , -0.153)]; all unadjusted $p < 0.05$. However, none of the associations remained statistically significant after FDR correction. No additional biomarkers were nominally associated with EV modal size in the raw analyses (Table 4; Figure 7).

Table 3. Associations between metabolic, endocrine, liver, sex hormone, lipid, and cytokine biomarkers and EV mean size across the three study phases in KetoSAGE participants. Measurements were taken following each study phase: baseline nutritional ketosis (NK; P1); intervention suppress ketosis (SuK; P2); and return to NK after SuK removal (P3). All samples were collected at 8 a.m. after a 12 h overnight fast ($n = 10$; § $n = 9$).

					Log-Transformed			
Model	Effect Estimate	95% CI	<i>p</i> Value	FDR Adjusted <i>p</i> Value	Effect Estimate	95% CI	<i>p</i> Value	FDR Adjusted <i>p</i> Value
Metabolic–Endocrine Biomarkers								
Insulin ~ EV mean size	−2.84	(−8.205, 2.518)	0.3085	0.7757	-1.18×10^{-1}	(−0.322, 0.086)	0.2667	0.6811
Glucose ~ EV mean size	−2.05	(−24.731, 20.639)	0.8607	0.9257	-6.30×10^{-2}	(−0.678, 0.552)	0.8426	0.9495
BHB ~ EV mean size	−2.23	(−12.322, 7.865)	0.6693	0.8488	1.07×10^{-2}	(−0.041, 0.062)	0.6884	0.9273
HOMA-IR ~ EV mean size	−9.12	(−29.329, 11.087)	0.3848	0.8002	-8.13×10^{-2}	(−0.248, 0.086)	0.3496	0.7574
GKI (Lab Day) ~ EV mean size	-1.32×10^{-1}	(−0.542, 0.279)	0.5356	0.8211	-1.02×10^{-2}	(−0.059, 0.039)	0.6879	0.9273
GKI (21D Av) ~ EV mean size	5.38×10^{-3}	(−0.432, 0.443)	0.9810	0.9838	-5.50×10^{-3}	(−0.056, 0.045)	0.8353	0.9495
IGF-1 ~ EV mean size	-6.05×10^{-2}	(−0.219, 0.098)	0.4614	0.8211	-9.30×10^{-2}	(−0.278, 0.092)	0.3339	0.7574
Leptin ~ EV mean size	8.47×10^{-1}	(−1.066, 2.761)	0.3928	0.8002	5.14×10^{-2}	(−0.035, 0.138)	0.2543	0.6794
GLP-1 ~ EV mean size	-4.71×10^{-3}	(−0.020, 0.011)	0.5511	0.8211	-2.13×10^{-2}	(−0.128, 0.086)	0.6998	0.9273
Cortisol ~ EV mean size	8.99×10^{-1}	(−2.031, 3.838)	0.5567	0.8211	8.20×10^{-2}	(−0.112, 0.276)	0.4200	0.7811
Adiponectin ~ EV mean size	7.42×10^{-1}	(−2.423, 3.907)	0.6506	0.8488	1.44×10^{-2}	(−0.171, 0.191)	0.8808	0.9495
Gastrin ~ EV mean size	-3.87×10^{-1}	(−3.109, 2.336)	0.7839	0.8937	-3.87×10^{-2}	(−0.213, 0.135)	0.6672	0.9273
tOCN ~ EV mean size	-4.32×10^{-1}	(−1.100, 0.237)	0.2171	0.7369	-8.10×10^{-2}	(−0.254, 0.092)	0.3683	0.7574
unOCN ~ EV mean size	−3.92	(−9.486, 1.642)	0.1785	0.7369	-8.07×10^{-2}	(−0.211, 0.049)	0.2377	0.6662
cOCN ~ EV mean size	-4.73×10^{-1}	(−1.225, 0.278)	0.2284	0.7369	-7.85×10^{-2}	(−0.254, 0.097)	0.3902	0.7754
TSH ~ EV mean size	-1.15×10^1	(−31.591, 8.558)	0.2762	0.7624	-4.77×10^{-2}	(−0.115, 0.019)	0.1954	0.5761
Free T3 ~ EV mean size	−1.32	(−15.956, 13.307)	0.8607	0.9257	-7.76×10^{-2}	(−0.472, 0.317)	0.7039	0.9273
Reverse T3 ~ EV mean size	-8.07×10^1	(−232.781, 71.394)	0.3073	0.7757	-1.59×10^{-1}	(−0.382, 0.065)	0.1761	0.5757
Free T4 ~ EV mean size	−7.69	(−18.293, 2.922)	0.1676	0.7369	-6.89×10^{-1}	(−1.476, 0.097)	0.0980	0.5736
Liver Biomarkers								
GGT ~ EV mean size	8.49×10^{-1}	(−4.187, 5.885)	0.7439	0.8773	7.15×10^{-2}	(−0.197, 0.340)	0.6065	0.9151
ALP ~ EV mean size	-3.30×10^{-1}	(−1.304, 0.645)	0.5134	0.8211	-8.98×10^{-2}	(−0.438, 0.258)	0.6177	0.9151
ALT ~ EV mean size	-2.77×10^{-1}	(−1.782, 1.228)	0.7210	0.8683	-4.94×10^{-2}	(−0.245, 0.147)	0.6251	0.9151
AST ~ EV mean size	−1.05	(−3.152, 1.051)	0.3357	0.7807	-1.38×10^{-1}	(−0.421, 0.154)	0.3621	0.7574
ALT/AST ratio ~ EV mean size	1.44×10^1	(−46.986, 75.735)	0.6498	0.8488	2.63×10^{-2}	(−0.286, 0.339)	0.8701	0.9495
Albumin ~ EV mean size	−1.03	(−6.335, 4.270)	0.7059	0.8560	-2.32×10^{-1}	(−1.543, 1.080)	0.7320	0.9273
CK NAC ~ EV mean size	-1.61×10^{-1}	(−0.619, 0.297)	0.4978	0.8211	-3.38×10^{-2}	(−0.223, 0.156)	0.7296	0.9273
Iron ~ EV mean size	−1.11	(−2.605, 0.381)	0.1571	0.7369	-1.24×10^{-1}	(−0.260, 0.011)	0.0846	0.5736
Ferritin ~ EV mean size	-2.71×10^{-1}	(−0.919, 0.376)	0.4183	0.8129	-4.07×10^{-2}	(−0.158, 0.077)	0.5044	0.8593
Total Protein ~ EV mean size	−1.22	(−3.467, 1.024)	0.2995	0.7757	-5.48×10^{-1}	(−1.499, 0.403)	0.2709	0.6811

Table 3. Cont.

Model	Effect Estimate	95% CI	<i>p</i> Value	FDR Adjusted <i>p</i> Value	Log-Transformed			
					Effect Estimate	95% CI	<i>p</i> Value	FDR Adjusted <i>p</i> Value
Total Bilirubin ~ EV mean size	−2.03	(−6.555, 2.493)	0.3864	0.8002	-1.22×10^{-1}	(−0.323, 0.071)	0.2460	0.6786
Direct Bilirubin ~ EV mean size	−4.58	(−24.177, 15.019)	0.6505	0.8488	-2.97×10^{-2}	(−0.231, 0.172)	0.7743	0.9286
Sex Hormones								
SHBG ~ EV mean size	5.74×10^{-3}	(−0.317, 0.328)	0.9725	0.9838	4.13×10^{-2}	(−0.117, 0.199)	0.6159	0.9151
Oestrogen ~ EV mean size	6.33×10^{-3}	(−0.019, 0.031)	0.6248	0.8488	3.32×10^{-2}	(−0.062, 0.128)	0.4987	0.8593
Testosterone ~ EV mean size	−8.31	(−37.016, 20.403)	0.5786	0.8314	-3.83×10^{-2}	(−0.231, 0.154)	0.7010	0.9273
Progesterone ~ EV mean size	-3.17×10^{-1}	(−1.166, 0.532)	0.4722	0.8211	-9.50×10^{-3}	(−0.052, 0.033)	0.6691	0.9273
LH ~ EV mean size	3.81×10^{-1}	(−0.487, 1.249)	0.3976	0.8002	3.57×10^{-2}	(−0.041, 0.112)	0.3684	0.7574
FSH ~ EV mean size	2.76	(−1.266, 6.788)	0.1911	0.7369	6.99×10^{-2}	(−0.074, 0.213)	0.3502	0.7574
Lipids								
Total Cholesterol ~ EV mean size	-4.89×10^{-2}	(−0.359, 0.261)	0.7600	0.8781	-2.63×10^{-2}	(−0.438, 0.385)	0.9012	0.9495
HDL Cholesterol ~ EV mean size	1.37	(0.188, 2.558)	0.0368 *	0.6298	5.55×10^{-1}	(0.066, 1.044)	0.0404 *	0.5502
LDL Cholesterol ~ EV mean size	−2.71	(−12.444, 7.028)	0.5904	0.8344	-4.19×10^{-2}	(−0.273, 0.189)	0.7255	0.9273
Triglycerides ~ EV mean size	9.61×10^{-3}	(−0.435, 0.454)	0.9665	0.9838	-4.42×10^{-2}	(−0.263, 0.175)	0.6952	0.9273
Cytokines								
PAI-1 ~ EV mean size	1.98	(−0.387, 4.349)	0.1202	0.7369	6.75×10^{-2}	(−0.031, 0.165)	0.1885	0.5757
MCP-1 ~ EV mean size	5.28×10^{-2}	(−0.154, 0.251)	0.6211	0.8488	6.95×10^{-2}	(−0.089, 0.228)	0.3994	0.7754
EGF ~ EV mean size	9.59×10^{-2}	(−0.385, 0.577)	0.7002	0.8560	-5.40×10^{-3}	(−0.093, 0.083)	0.9050	0.9495
VEGF ~ EV mean size	7.32×10^{-2}	(−0.102, 0.249)	0.4251	0.8168	6.27×10^{-2}	(−0.065, 0.191)	0.3561	0.7574
§INF-γ ~ EV mean size	−2.17	(−11.799, 7.449)	0.6629	0.8488	-8.50×10^{-3}	(−0.122, 0.105)	0.8855	0.9495
§TNF-α ~ EV mean size	−2.77	(−15.233, 9.698)	0.6701	0.8488	-1.59×10^{-2}	(−0.250, 0.218)	0.8955	0.9495
§IL-1α ~ EV mean size	-2.00×10^1	(−77.131, 37.193)	0.5083	0.8211	-4.01×10^{-2}	(−0.141, 0.061)	0.4593	0.8098
§IL-1β ~ EV mean size	−2.52	(−9.440, 4.391)	0.4888	0.8211	-9.21×10^{-2}	(−0.226, 0.042)	0.2124	0.6054
§IL-2 ~ EV mean size	−4.13	(−17.379, 9.128)	0.5570	0.8211	-6.08×10^{-2}	(−0.197, 0.075)	0.4046	0.7754
§IL-4 ~ EV mean size	−3.08	(−20.969, 14.817)	0.7422	0.8773	-3.99×10^{-2}	(−0.286, 0.206)	0.7562	0.9273
§IL-6 ~ EV mean size	-1.03×10^1	(−28.310, 7.786)	0.2785	0.7624	-1.12×10^{-1}	(−0.256, 0.033)	0.1535	0.5757
§IL-8 ~ EV mean size	7.88×10^{-2}	(−2.325, 2.483)	0.9499	0.9838	-2.30×10^{-3}	(−0.149, 0.145)	0.9761	0.9990
§IL-10 ~ EV mean size	-3.07×10^1	(−69.820, 8.429)	0.1360	0.7369	-1.39×10^{-1}	(−0.2920, 0.014)	0.0899	0.5736

Effect estimates (β) are reported in scientific notation; CI, confidence interval; FDR, false discovery rate; * *p* < 0.05 indicates nominal statistical significance based on the unadjusted *p* value; § biomarkers were measured in *n* = 9 participants.

Table 4. Associations between metabolic, endocrine, liver, sex hormone, lipid, and cytokine biomarkers and EV modal size across the three study phases in KetoSage participants. Measurements were taken following each study phase: baseline nutritional ketosis (NK; P1); intervention suppress ketosis (SuK; P2); and return to NK after SuK removal (P3). All samples were collected at 8 a.m. after a 12 h overnight fast ($n = 10$; § $n = 9$).

					Log-Transformed			
Model	Effect Estimate	95% CI	<i>p</i> Value	FDR Adjusted <i>p</i> Value	Effect Estimate	95% CI	<i>p</i> Value	FDR Adjusted <i>p</i> Value
Metabolic–Endocrine Biomarkers								
Insulin ~ EV modal size	−3.24	(−6.207, −0.265)	0.0442 *	0.6299	-1.82×10^{-1}	(−0.351, −0.012)	0.0468 *	0.5502
Glucose ~ EV modal size	−8.64	(−22.096, 4.815)	0.2201	0.7369	-4.11×10^{-1}	(−0.931, 0.117)	0.1400	0.5757
BHB ~ EV modal size	4.48	(−0.991, 9.943)	0.1240	0.7369	4.21×10^{-2}	(0.003, 0.081)	0.0494 *	0.5502
HOMA-IR ~ EV modal size	-1.13×10^1	(−22.524, −0.125)	0.0603	0.7145	-1.46×10^{-1}	(−0.282, −0.010)	0.0468 *	0.5502
GKI (Lab Day) ~ EV modal size	-1.54×10^{-1}	(−0.387, 0.078)	0.2063	0.7369	-4.05×10^{-2}	(−0.078, −0.003)	0.0468 *	0.5502
GKI (21D Av) ~ EV modal size	-1.57×10^{-1}	(−0.398, 0.084)	0.2171	0.7369	-3.84×10^{-2}	(−0.077, 0.0004)	0.0674	0.5502
IGF-1 ~ EV modal size	-8.42×10^{-2}	(−0.169, 0.001)	0.0668	0.7145	-1.49×10^{-1}	(−0.299, 0.0005)	0.0643	0.5502
Leptin ~ EV modal size	-8.17×10^{-1}	−1.913, 0.279)	0.1579	0.7369	-3.00×10^{-2}	(−0.108, 0.048)	0.4569	0.8098
GLP-1 ~ EV modal size	2.11×10^{-3}	(−0.007, 0.011)	0.6600	0.8488	2.99×10^{-2}	(−0.064, 0.124)	0.5377	0.8927
Cortisol ~ EV modal size	6.87×10^{-1}	(−1.414, 2.788)	0.5272	0.8211	9.01×10^{-2}	(−0.115, 0.295)	0.3983	0.7754
Adiponectin ~ EV modal size	−1.50	(−3.575, 0.584)	0.1697	0.7369	-1.10×10^{-1}	(−0.298, 0.079)	0.2655	0.6811
Gastrin ~ EV modal size	-5.49×10^{-2}	(−1.965, 1.855)	0.9555	0.9838	1.35×10^{-2}	(−0.161, 0.188)	0.8805	0.9495
tOCN ~ EV modal size	-2.23×10^{-1}	(−0.663, 0.217)	0.3288	0.7807	-4.82×10^{-2}	(−0.219, 0.123)	0.5843	0.9151
unOCN ~ EV modal size	−2.30	(−5.896, 1.296)	0.2207	0.7369	-6.78×10^{-2}	(−0.198, 0.063)	0.3176	0.7544
cOCN ~ EV modal size	-2.39×10^{-1}	(−0.735, 0.256)	0.3515	0.7807	-4.52×10^{-2}	(−0.218, 0.128)	0.6130	0.9151
TSH ~ EV modal size	-1.25×10^1	(−26.056, 1.151)	0.0861	0.7369	-8.68×10^{-2}	(−0.155, −0.019)	0.0314 *	0.5502
Free T3 ~ EV modal size	−9.11	(−16.651, −1.566)	0.0279 *	0.6298	-3.73×10^{-1}	(−0.673, −0.073)	0.0240 *	0.5502
Reverse T3 ~ EV modal size	-8.15×10^1	(−171.681, 8.764)	0.0891	0.7369	-2.00×10^{-1}	(−0.390, −0.010)	0.0502	0.5502
Free T4 ~ EV modal size	−7.98	(−14.572, −1.383)	0.0248 *	0.6298	-8.88×10^{-1}	(−1.613, −0.163)	0.0232 *	0.5502
Liver Biomarkers								
GGT ~ EV modal size	−1.58	(−4.853, 1.686)	0.3508	0.7807	-6.82×10^{-2}	(−0.329, 0.193)	0.6126	0.9151
ALP ~ EV modal size	-2.47×10^{-1}	(−0.871, 0.385)	0.4499	0.8211	-1.47×10^{-1}	(−0.475, 0.181)	0.3868	0.7754
ALT ~ EV modal size	-9.16×10^{-1}	(−1.724, −0.107)	0.0370 *	0.6298	-1.98×10^{-1}	(−0.350, −0.046)	0.0181 *	0.5502
AST ~ EV modal size	−1.33	(−2.497, −0.153)	0.0368 *	0.6298	-2.79×10^{-1}	(−0.524, −0.035)	0.0346 *	0.5502
ALT/AST ratio ~ EV modal size	-2.80×10^1	(−65.766, 9.841)	0.1592	0.7369	-2.40×10^{-1}	(−0.521, 0.041)	0.1057	0.5736
Albumin ~ EV modal size	−1.61	(−4.981, 1.763)	0.3577	0.7843	-4.91×10^{-1}	(−1.706, 0.724)	0.4352	0.7917
CK NAC ~ EV modal size	-2.45×10^{-1}	(−0.538, 0.047)	0.1117	0.7369	-1.42×10^{-1}	(−0.324, 0.039)	0.1355	0.5757
Iron ~ EV modal size	-6.55×10^{-1}	(−1.640, 0.331)	0.2036	0.7369	-1.24×10^{-1}	(−0.252, 0.003)	0.0663	0.5502
Ferritin ~ EV modal size	1.57×10^{-1}	(−0.239, 0.553)	0.4443	0.8211	1.95×10^{-2}	(−0.092, 0.131)	0.7340	0.9273
Total Protein ~ EV modal size	−1.31	(−2.828, 0.217)	0.1043	0.7369	-7.77×10^{-1}	(−1.691, 0.146)	0.1104	0.5736
Total Bilirubin ~ EV modal size	−1.40	(−4.179, 1.374)	0.3317	0.7807	-1.49×10^{-1}	(−0.327, 0.028)	0.1107	0.5736

Table 4. Cont.

Model	Effect Estimate	95% CI	<i>p</i> Value	FDR Adjusted <i>p</i> Value	Log-Transformed			
					Effect Estimate	95% CI	<i>p</i> Value	FDR Adjusted <i>p</i> Value
Direct Bilirubin ~ EV modal size	−5.97	(−18.181, 6.241)	0.3467	0.7807	-1.07×10^{-1}	(−0.287, 0.073)	0.2537	0.6794
Sex Hormones								
SHBG ~ EV modal size	1.89×10^{-1}	(−0.024, 0.403)	0.0940	0.7369	1.47×10^{-1}	(−0.013, 0.307)	0.0851	0.5736
Oestrogen ~ EV modal size	1.08×10^{-2}	(−0.004, 0.025)	0.1569	0.7369	9.20×10^{-2}	(0.014, 0.170)	0.0298 *	0.5502
Testosterone ~ EV modal size	-2.17×10^{-1}	(−20.987, 20.552)	0.9838	0.9838	5.40×10^{-2}	(−0.140, 0.248)	0.5906	0.9151
Progesterone ~ EV modal size	1.87×10^{-2}	(−0.479, 0.517)	0.9420	0.9838	-4.17×10^{-4}	(−0.037, 0.036)	0.9824	0.9990
LH ~ EV modal size	2.93×10^{-1}	(−0.215, 0.801)	0.2702	0.7624	4.38×10^{-2}	(−0.020, 0.108)	0.1945	0.5761
FSH ~ EV modal size	6.98×10^{-1}	(−1.719, 3.115)	0.5772	0.8314	1.46×10^{-2}	(−0.110, 0.139)	0.8207	0.9495
Lipids								
Total Cholesterol ~ EV modal size	1.55×10^{-1}	(−0.044, 0.354)	0.1377	0.7369	2.86×10^{-1}	(−0.092, 0.664)	0.1500	0.5757
HDL Cholesterol ~ EV modal size	5.99×10^{-1}	(−0.294, 1.491)	0.2016	0.7369	3.91×10^{-1}	(−0.139, 0.921)	0.1629	0.5757
LDL Cholesterol ~ EV modal size	4.15	(−2.054, 10.349)	0.2010	0.7369	1.08×10^{-1}	(−0.104, 0.320)	0.3267	0.7574
Triglycerides ~ EV modal size	1.48×10^{-1}	(−0.124, 0.421)	0.2959	0.7757	4.45×10^{-2}	(−0.160, 0.249)	0.6734	0.9273
Cytokines								
PAI-1 ~ EV modal size	1.18	(−0.548, 2.907)	0.1935	0.7369	6.32×10^{-2}	(−0.028, 0.154)	0.1866	0.5757
MCP-1 ~ EV modal size	-8.38×10^{-3}	(−0.139, 0.123)	0.9012	0.9572	4.52×10^{-2}	(−0.107, 0.198)	0.5663	0.9151
EGF ~ EV modal size	-4.57×10^{-2}	(−0.381, 0.281)	0.7915	0.8964	-5.45×10^{-3}	(−0.093, 0.082)	0.9039	0.9495
VEGF ~ EV modal size	-3.18×10^{-2}	(−0.160, 0.097)	0.6322	0.8488	2.27×10^{-2}	(−0.121, 0.166)	0.7606	0.9273
§INF-γ ~ EV modal size	−3.08	(−9.646, 3.488)	0.3666	0.7935	-3.56×10^{-2}	(−0.155, 0.084)	0.5677	0.9151
§TNF-α ~ EV modal size	−6.31	(−15.359, 2.732)	0.1856	0.7369	-1.32×10^{-1}	(−0.366, 0.103)	0.2828	0.6909
§IL-1α ~ EV modal size	-2.01×10^1	(−64.198, 24.082)	0.3882	0.8002	-6.02×10^{-2}	(−0.179, 0.058)	0.3438	0.7574
§IL-1β ~ EV modal size	−3.02	(−8.249, 2.218)	0.2759	0.7624	-1.42×10^{-1}	(−0.296, 0.013)	0.1023	0.5736
§IL-2 ~ EV modal size	−1.34	(−12.174, 9.495)	0.8132	0.9149	-3.91×10^{-2}	(−0.200, 0.122)	0.6452	0.9273
§IL-4 ~ EV modal size	2.01	(−11.581, 15.608)	0.7753	0.8897	4.17×10^{-2}	(−0.227, 0.310)	0.7646	0.9273
§IL-6 ~ EV modal size	-1.13×10^1	(−23.406, 0.796)	0.0785	0.7369	-1.77×10^{-1}	(−0.331, −0.024)	0.0358 *	0.5502
§IL-8 ~ EV modal size	-3.60×10^{-1}	(−2.157, 1.437)	0.7000	0.8560	-4.62×10^{-2}	(−0.205, 0.113)	0.5798	0.9151
§IL-10 ~ EV modal size	-1.77×10^1	(−42.425, 6.954)	0.1713	0.7369	-1.36×10^{-1}	(−0.288, 0.017)	0.0924	0.5736

Effect estimates (β) are reported in scientific notation; CI, confidence interval; FDR, false discovery rate; * $p < 0.05$ indicates nominal statistical significance based on the unadjusted *p* value; § biomarkers were measured in $n = 9$ participants.

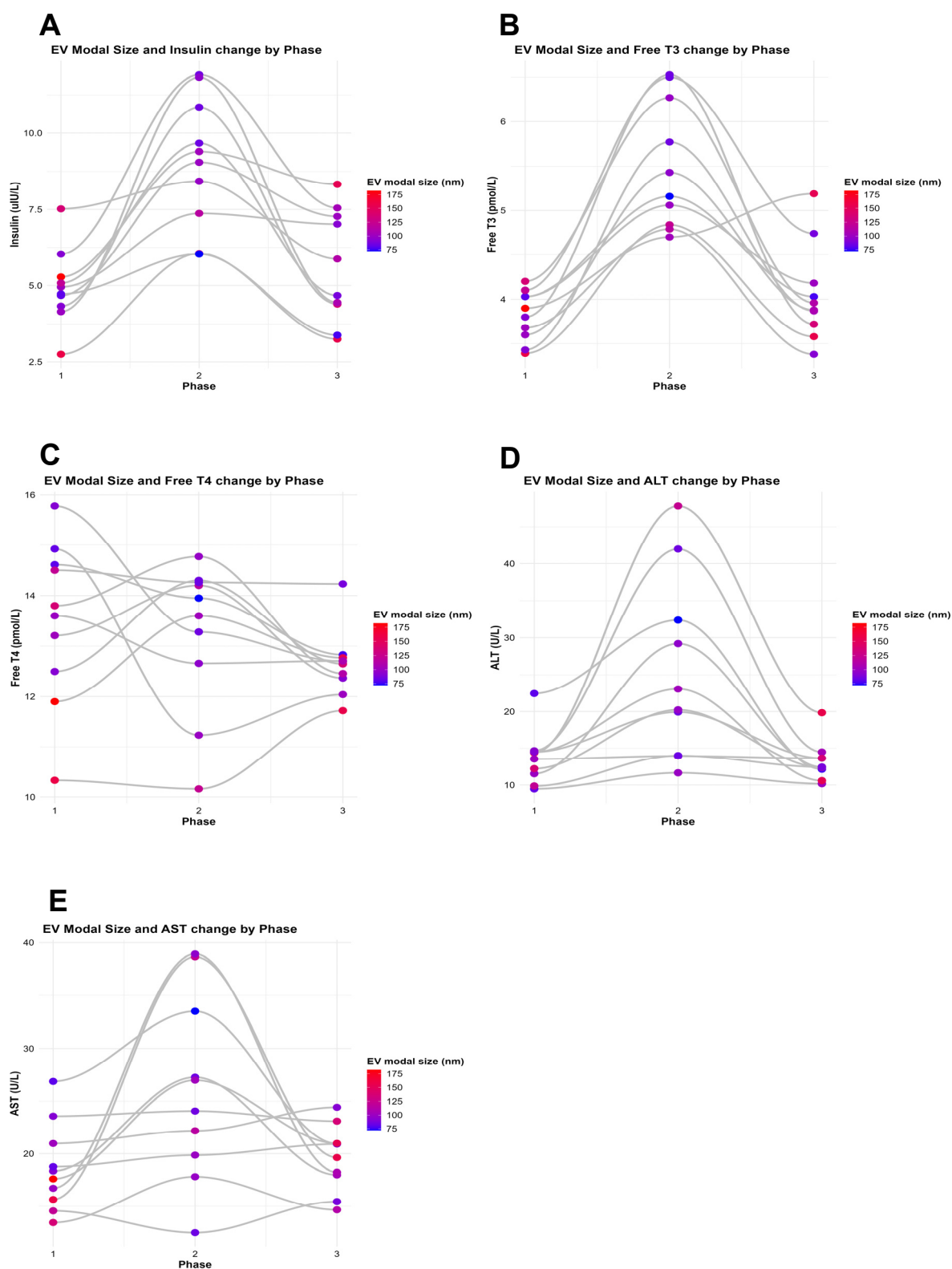


Figure 7. Associations between EV modal size and (A) insulin, (B) free T3, (C) free T4, (D) ALT, and (E) AST across all study phases. Analyses were performed in R, and graphs were generated using the ggplot2 package (version 4.0.0) in RStudio (version 2025.09.1+401).

Following log-transformation to account for differences in scale across variables, a similar pattern of associations was observed. Nominally significant inverse associations were identified between EV modal size and insulin [$\beta = -1.82 \times 10^{-1}$ (95% CI: $-0.351, -0.012$)], HOMA-IR [$\beta = -1.46 \times 10^{-1}$ (95% CI: $-0.282, -0.010$)], GKI (Lab Day) [$\beta = -4.05 \times 10^{-2}$

(95% CI: -0.078 , -0.003), TSH [$\beta = -8.68 \times 10^{-2}$ (95% CI: -0.155 , -0.019)], free T3 [$\beta = -3.73 \times 10^{-1}$ (95% CI: -0.673 , -0.073)], free T4 [$\beta = -8.88 \times 10^{-1}$ (95% CI: -1.613 , -0.163)], ALT [$\beta = -1.98 \times 10^{-1}$ (95% CI: -0.350 , -0.046)], AST [$\beta = -2.79 \times 10^{-1}$ (95% CI: -0.524 , -0.035)], and IL-6 [$\beta = -1.77 \times 10^{-1}$ (95% CI: -0.331 , -0.024)]; all unadjusted $p < 0.05$. In contrast, nominal positive associations were identified between EV modal size and BHB [$\beta = 4.21 \times 10^{-2}$ (95% CI: 0.003 , 0.081)] and oestrogen [$\beta = 9.20 \times 10^{-2}$ (95% CI: 0.014 , 0.170)]; both unadjusted $p < 0.05$. However, none of these associations remained statistically significant after FDR correction. No other biomarkers were nominally associated with EV modal size (Table 4, Figures 8–10).

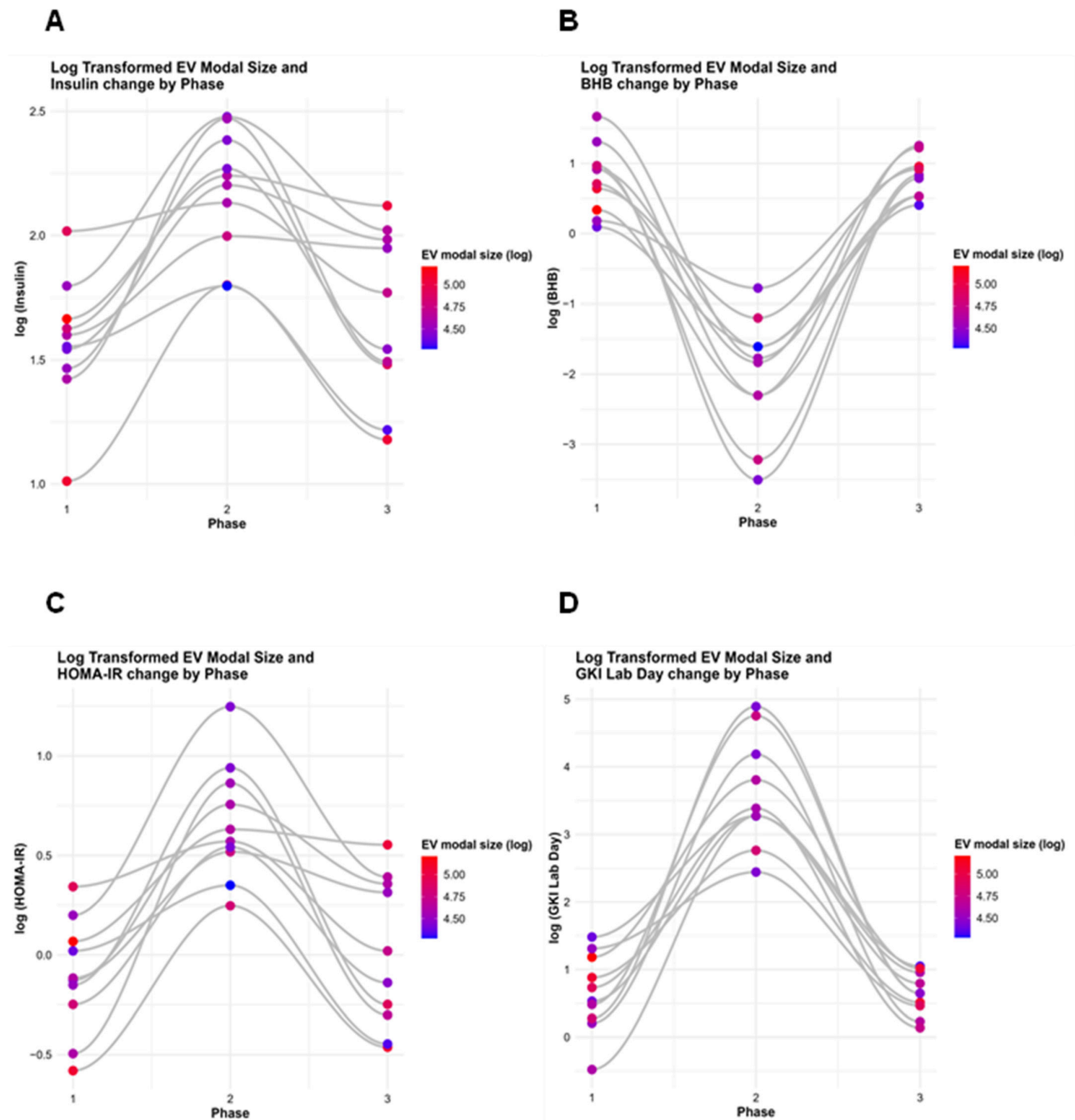


Figure 8. Associations between log-transformed EV modal size and (A) insulin, (B) BHB, (C) HOMA-IR, and (D) GKI (Lab Day) across all study phases. Analyses were performed in R, and graphs were generated using the ggplot2 package (version 4.0.0) in RStudio (version 2025.09.1+401).

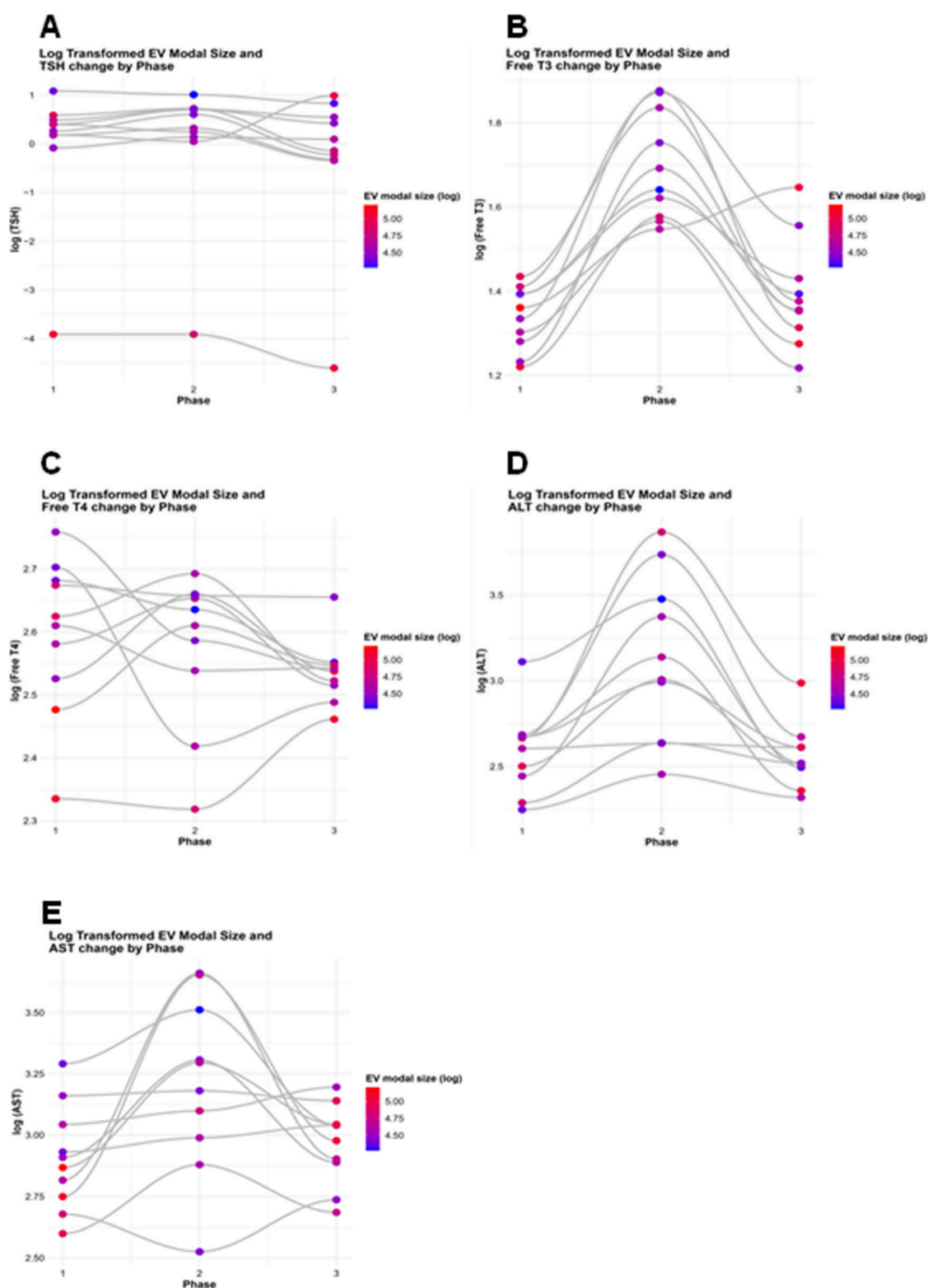


Figure 9. Associations between log-transformed changes in EV modal size and (A) TSH, (B) free T3, (C) free T4, (D) ALT, and (E) AST across all study phases. Analyses were performed in R, and graphs were generated using the ggplot2 package (version 4.0.0) in RStudio (version 2025.09.1+401).

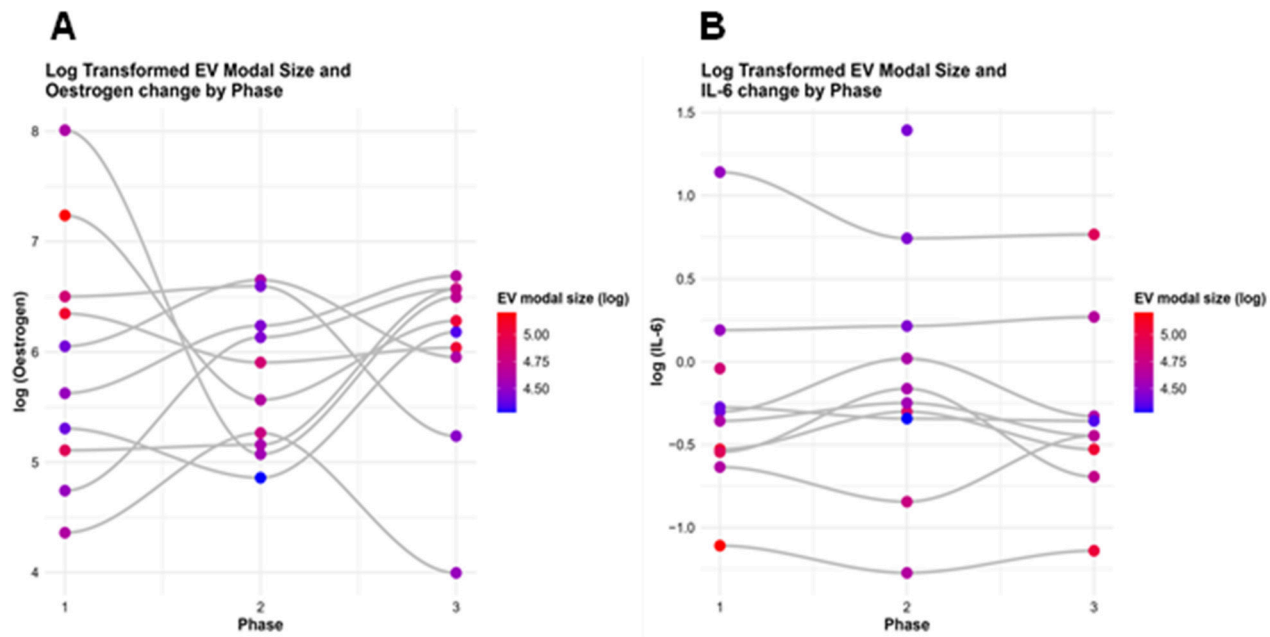


Figure 10. Associations between log-transformed changes in EV modal size and (A) oestrogen and (B) IL-6 across all study phases. Analyses were performed in R, and graphs were generated using the ggplot2 package (version 4.0.0) in RStudio (version 2025.09.1+401).

4. Discussion

4.1. Extracellular Vesicles as Metabolic State Conveyors in Hypoketonaemia-ICE (Insulin-Compensated Euglycaemia)

EVs present in blood circulation may serve as systemic biomarkers of nutrient status, reflecting and affecting both physiological and pathophysiological states, particularly in hyperinsulinaemia, insulin resistance, MetS, obesity, and ageing [1,2,31,32,42,43]. Our study assessed, for the first time, circulating plasma EVs in healthy premenopausal women maintaining long-standing euketonaemia, MP1, and examined the effects of 21 days of euketonaemia suppression resulting in hypoketonaemia with insulin-compensated euglycaemia (ICE). No significant grouped differences were observed in mean EV concentration, mean size or modal size across the three study phases (P1, P2, and P3) of the KetoSAGE trial, although a directional increase in EV concentration was observed during ketosis suppression (P2), suggesting a shift in EV release dynamics under altered metabolic conditions. Linear mixed-effects modelling (LMER), which provides greater sensitivity to within-participant variation, identified nominally significant associations between EV concentration, mean size and modal size with endocrine, metabolic, thyroid, liver, sex hormone, and inflammatory biomarkers across the three study phases.

The present findings extend the KetoSAGE dataset into the domain of EV biology, suggesting that suppression of euketonaemia in healthy premenopausal women is accompanied by coordinated EV-biomarker relationships involving endocrine-metabolic, liver biomarkers, and inflammatory pathways. The consistency of several biologically coherent patterns across related metabolic and endocrine pathways provides a rational for considering their potential mechanistic implications. Although these associations were exploratory and did not remain statistically significant after FDR correction, their overall pattern is consistent with the hypothesis of an insulin-responsive, metabolically plastic EV signalling network involved in systemic bioenergetic regulation rather than EVs acting solely as passive by-products of cellular stress. When interpreted alongside the expanding EV literature, these findings are consistent with the concept that euketonaemia maintains a distinct inter-organ signalling environment that may become disrupted under conditions of

chronic insulin elevation and ketogenesis suppression, resulting in ICE, where basal insulin levels remain persistently maintained above an individual's personal-hyperinsulinaemia threshold (PIT) leading to chronic hypoketonaemia (BHB < 0.5 mmol/L).

Metabolic dysfunction is associated with both altered circulating EV abundance and modifications in EV cargo composition. Given their role as mediators between originating and target cells, EVs may play a central role in intercellular and inter-organ communication in metabolic disorders [8]. EV number correlates positively with blood pressure, BMI, HOMA-IR, and HOMA-B [44], whilst plasma EV levels have also been shown to be higher in individuals with impaired glucose tolerance and in men compared with women, regardless of MetS status [8]. Higher BMI is also associated with impaired glucose tolerance [45], and increased EV concentrations have been reported in obesity, MetS, and animal models receiving high-fat with high-carbohydrate insulinogenic diets that suppress ketosis (HFD) [21,46]. Elevated fasting insulin has similarly been associated with higher EV concentrations, although acute insulin infusion in adults with MetS has been shown to decrease medium-sized EVs, suggesting context-dependent regulation [26]. Previous research further supports a role for EVs in fuel substrate utilisation and metabolic flexibility [19,24,25,42,44,47–52]. In hepatocellular models, small EVs present a higher concentration of glycolytic enzymes and can contribute to dysregulation of mitochondrial oxidative phosphorylation, supporting their role as active regulators of cellular bioenergetics [53].

EV size distribution is also altered in obesity, with circulating EV populations in obese women demonstrating a shift toward smaller vesicle diameters relative to non-obese controls [54]. EVs originating from white adipose tissue have been shown to play a critical role in the development of insulin resistance, potentially through the transfer of microRNAs such as miR-155 and miR-27a, which alter peroxisome proliferator-activated receptor gamma (PPAR γ) signalling [49]. PPAR γ activation promotes free fatty acids (FFA) release from circulating lipoproteins and enhances lipid uptake, contributing to adiposity and metabolic dysfunction. PPAR γ gene expression is increased in circulating blood cells and adipose tissue in obesity and T2DM, with further elevation detected in individuals with concurrent obesity and diabetes compared with lean or non-obese diabetic phenotypes [55–58].

Hyperinsulinaemia, MetS and obesity lead to an increased risk of ageing-related diseases [59–65]. EVs have been increasingly implicated in ageing-associated diseases including osteoporosis, osteoarthritis, Alzheimer's disease, Parkinson's disease, CVD, T2DM, and reproductive dysfunction [66–68]. These studies highlight the importance of senescent cell accumulation and the development of the senescence-associated secretory phenotype (SASP), through which EV release contributes to chronic inflammation and disease progression. In murine models, sarcopenia has been associated with increased circulating EV concentrations compared with age-matched controls, further supporting altered EV dynamics as a hallmark of metabolic ageing [66]. In addition to changes in EV abundance, ageing is also associated with selective remodelling of EV cargo composition, with aged mice exhibiting elevated expression of specific microRNAs within circulating EV populations [17,19,66–69].

4.2. Extracellular Vesicles as Dynamic Mediators of Insulin-Dependent Metabolic Signalling

A central insight from recent EV research is that EV release, cargo selection, and tissue targeting are tightly regulated by insulin signalling and nutrient availability. Early work by Martínez and Andriantsitohaina (2017) and Akbar et al. (2019) established EVs as integral components of MetS pathophysiology, linking EV concentration and phenotype to insulin resistance, endothelial dysfunction, and inflammatory tone [8,23]. More recent studies

have refined this model, demonstrating that EVs actively participate in the regulation of insulin sensitivity and glucose homeostasis across organs; for example, hepatocyte-derived EVs can exert insulin-sensitising effects through defined microRNA and protein cargo, improving whole-body glycaemic control via inter-organ communication [50,70]. These effects are context-dependent. In early metabolic adaptation, hepatocyte-derived EVs appear to act as compensatory signals that support insulin sensitivity. However, as hyperinsulinaemia becomes sustained, EV cargo composition shifts toward the propagation of insulin resistance, inflammatory signalling, and metabolic dysfunction [25,43]. The pattern of EV–biomarker associations observed across the three-phase KetoSage open-label crossover trial is consistent with a transition away from a euketonaemic, insulin-sparing signalling environment towards an insulin-dominant EV phenotype. Although exploratory, these findings raise the possibility that the suppression of euketonaemia does not simply alter circulating metabolite concentrations but may also influence EV-mediated endocrine communication across tissues.

4.3. Ketosis, EV Cargo, and Mitochondrial Signalling

Euketonaemia represents a distinct mitochondrial signalling state characterised by enhanced fatty acid oxidation, preserved NAD⁺ availability, and reduced insulin demand and thus exposure [1,7,32,33]. EVs generated under these conditions likely reflect mitochondrial health and well-regulated redox homeostasis. EV cargo contains mitochondrial proteins, mitochondrial DNA fragments, and redox-sensitive signalling molecules that reflect mitochondrial function and ageing trajectories [17,19]. Within this framework, suppression of ketosis resulting in hypoketonaemia with ICE would be expected to alter EV cargo in ways that signal reduced mitochondrial beta-oxidation capacity and increased reliance on glycolytic and insulin-dependent metabolic pathways. Evidence from dietary intervention studies supports this interpretation. Balestra et al. (2024) demonstrated that a very-low-calorie ketogenic diet modifies EV-mediated effects on hepatocyte viability and reactive oxygen species (ROS) handling, consistent with improved mitochondrial redox control [51]. Similarly, a modified Mediterranean-ketogenic diet alters plasma small EV profiles in individuals with mild cognitive impairment, reflecting systemic metabolic reprogramming in addition to isolated tissue effects [52]. The present findings are compatible with the hypothesis that suppression of ketosis (hypoketonaemia-ICE) may partially modify EV-mediated mitochondrial signalling phenotype, reflecting a broader shift in systemic bioenergetic signalling away from mitochondrial fat oxidation and toward glucose-dependent metabolic regulation.

4.4. Extracellular Vesicles as Amplifiers of Insulin Exposure and Endocrine Crosstalk

EVs actively amplify insulin exposure, and adipocyte-derived EVs transport insulinotropic protein cargo that increases pancreatic β -cell insulin secretion, indicating a direct EV-mediated mechanism linking adipose tissue signalling to systemic insulin dynamics [49]. This finding reframes adipose tissue EVs as contributory drivers of hyperinsulinaemia. In this context, suppression of ketosis (SuK)/hypoketonaemia may promote an EV environment that reinforces insulin secretion and insulin signalling through positive feedback loops, contributing to sustained hyperinsulinaemia, even in the absence of overt metabolic disease such as those with hypoketonaemia-ICE. Exercise-induced modulation of EV release further supports the sensitivity of EV networks to bioenergetic demand. Kyriakidou et al. (2021) showed that muscle damage and recovery responses alter systemic EV release across different age populations, highlighting skeletal muscle as a key contributor to EV-mediated metabolic communication [14]. Euketonaemia, by altering substrate utilisation and insulin exposure in muscle, is likely to influence these EV signals.

Suppression of ketosis/hypoketonaemia therefore removes an important modulator of muscle-derived EV signalling, with potential downstream effects on whole-body insulin demand, exposure and subsequent sensitivity.

4.5. Implications for Ageing, Disease Risk, and Translational Relevance

Ageing is increasingly recognised as a process shaped by the redox management capacity of cumulative metabolic signalling ROS exposure and not chronological time alone. EV remodelling has been proposed as an emerging hallmark of ageing, reflecting shifts in inflammatory tone, mitochondrial function, and endocrine communication [19,67]. The present study further suggests that even short-term suppression of ketosis in healthy individuals may induce patterns of EV remodelling resembling those previously reported in hyperinsulinaemia, insulin-resistant, or ageing populations. If confirmed, these observations may provide a plausible mechanistic link between chronic hyperinsulinaemia, loss of metabolic flexibility, and accelerated ageing trajectories.

From a translational perspective, these findings support the use of EV profiling as a sensitive biomarker of metabolic state, as advocated by Rohm et al. (2025) [47]. Unlike static measures of glucose or lipids, EVs integrate signals from liver, adipose tissue, muscle, immune cells, and endothelium, providing a systems-level readout of metabolic signalling. Adherence to MISEV2023 guidelines (Welsh et al., 2024a) [9] ensures that such analyses are interpretable and reproducible, strengthening the clinical relevance of EV-based assessments.

4.6. Integration with the KetoSAge Framework

Taken together, these exploratory findings support the concept that EVs may participate in the integration of ketosis, insulin signalling, mitochondrial function, and systemic ageing biology. Euketonaemia may therefore favour an EV signalling environment associated with insulin sensitivity, mitochondrial competence, and a regulated homeodynamic endocrine bioenergetic phenotype [71]. Suppression of ketosis shifts this balance toward an insulin-dominant EV phenotype with potential implications for long-term metabolic health, a decline in ageing quality (subjective and quantitative), and disease susceptibility. These observations extend the KetoSAge paradigm beyond classical biomarkers and endocrine measures by incorporating EV biology into the metabolic signalling architecture influenced by dietary carbohydrate exposure and insulin dynamics.

4.7. Integration of EV Findings with SHBG, Sex Hormones, Cancer Risk, and Liver Health

The present EV findings integrate directly with the central endocrine axis established across the KetoSAge trial, namely insulin-regulated hepatic signalling, with downstream effects on SHBG synthesis and sex steroid bioavailability. In this KetoSAge-EV trial, phase-level differences in EV concentration were not statistically detectable, although a directional increase was observed during ketosis suppression, followed by a return towards baseline after restoration of euketonaemia. Linear mixed-effects modelling identified nominally significant associations between EV concentration and LH, free T3, adiponectin and bilirubin fractions, including total and direct bilirubin. These biologically coherent relationships are consistent with potential mechanistic links between metabolic signalling state, hepatic endocrine regulation, and sex steroid bioavailability. Bilirubin reflects hepatobiliary handling and hepatic redox status, LH reflects hypothalamic-pituitary-gonadal axis activity, and free T3 reflects thyroid-driven energy flux [4].

SHBG is synthesised in hepatocytes and is highly sensitive to insulin signalling, with elevated basal insulin suppressing hepatic SHBG production and lowering circulating SHBG in both clinical and experimental contexts [4]. Lower SHBG concentrations are associated with increased incidence of several cancers [72–77]. Euketonaemia interven-

tions have been shown to support higher SHBG concentrations through reductions in basal insulin signalling [4]. EV biology strengthens this framework because liver-derived EVs participate in inter-organ metabolic communication and can modulate whole body glycaemic control and insulin sensitivity, with context-dependent shifts across metabolic states, as highlighted by Ji et al. (2021) and Miotto et al. (2024), and the systems synthesis by Lee et al. (2025) [43,50,70]. Within this mechanistic framework, suppression of ketosis may plausibly influence hepatic EV output and cargo selection in parallel with rising insulin exposure (hypoketonaemia-ICE), shifting liver endocrine outputs in a direction that favours lower SHBG synthesis and a more proliferative steroid signalling environment in peripheral tissues such as adipogenesis [1,32].

The observed nominal associations between EV concentration and bilirubin fractions observed in the KetoSAGE cohort are consistent with the concept that EV release may reflect hepatocyte metabolic handling and hepatobiliary processing rather than reflecting generalised cellular stress. Bilirubin is tightly coupled to hepatic redox balance, haem metabolism, and clearance systems, all of which are modulated by substrate selection and insulin sensitivity. Although the associations did not remain statistically significant following FDR correction, its direction and biological plausibility are consistent with a liver-centred interpretation whereby the state of hyperinsulinaemia–hypoketonaemia may influence hepatic signalling state and EV communication, in agreement with the altered SHBG dynamics reported across the KetoSAGE trial [2,4].

4.8. EV Mediated Amplification of Insulin Signalling and SHBG Regulated Sex Hormone Intracellular Signal

Adipose-derived EV signalling provides an additional mechanistic pathway linking ketosis suppression to hepatic SHBG regulation and downstream sex steroid signalling. Kulaj et al. (2023) demonstrated that adipocyte-derived EVs increase insulin secretion through insulinotropic protein cargo, establishing EVs as active drivers of hyperinsulinaemia [49]. This is highly relevant to the KetoSAGE trial, because insulin is the dominant regulator of ketogenesis suppression and hepatic SHBG downregulation. EV networks that augment insulin secretion or propagate insulin resistance may therefore indirectly reshape the sex hormone milieu by lowering SHBG concentrations, increasing cell signalling with free fractions of testosterone and oestradiol, and strengthening mitogenic intracellular signalling cascades in hormone-responsive tissues. This is consistent with established associations between low SHBG, insulin resistance, and cancer risk [72–86].

The nominally positive association between EV concentration and LH observed in the present study is consistent with a model of multi-axis endocrine integration, in which the EV signalling state may relate to pituitary output. LH is responsive to energy availability, metabolic signalling, and sex steroid feedback, while chronic hyperinsulinaemia can interact with gonadotrophin dynamics through ovarian steroidogenesis and altered hepatic steroid clearance [73,74,83,87–89]. These mechanisms are clinically relevant in female-specific endocrine conditions, where insulin-driven suppression of SHBG contributes to altered androgen and oestrogen signalling dynamics [73,74,83,88].

4.9. Cancer Risk Relevance: EVs as Drivers of Metabolic Reprogramming in Tumour Ecosystems

Cancer risk and progression are influenced by both endocrine signalling and metabolic substrate availability [4,65,72,75,76,90–129]. Lower SHBG increases bioavailable sex steroids and changes intracellular signalling behaviour in hormone-sensitive tissues, with relevance to breast, endometrial, ovarian, prostate, pancreatic, lung, and brain tumours [4,75,76,130]. EV biology adds a second dimension, since tumour microenvironment-derived EVs can reprogramme recipient cell metabolism by suppressing oxidative metabolism and promoting glycolytic and anabolic phenotypes, as re-

ported in Zhao et al. (2016) and reinforced by broader cancer EV frameworks, including Urabe et al. (2020) [11,131]. This forms a coherent model where ketosis suppression increases insulin signalling, lowers hepatic SHBG synthesis and output, increases permissive sex steroid signalling, and alters intracellular signalling in hormone-sensitive tissues. In parallel, EV-mediated intercellular communication shifts toward a growth-permissive metabolic phenotype that supports proliferation and tumour progression.

Hepatocellular carcinoma provides direct mechanistic evidence for selective metabolic cargo loading within EVs. Motile hepatocellular carcinoma cells demonstrate preferential enrichment and secretion of sugar metabolism regulatory proteins within exosomes, including enzymes governing glycolysis, gluconeogenesis, and pentose phosphate pathway flux, reflecting coordinated metabolic reprogramming across cellular and secreted vesicular systems [53]. This targeted export indicates that EVs function as active conveyors of metabolic instruction, extending glucose-centred bioenergetic signalling across the tumour microenvironment. These observations integrate with the broader metabolic–endocrine framework in which sustained insulin signalling drives proliferative activity and reinforces glycolysis-dependent growth phenotypes.

4.10. Liver Health and Metabolic Ageing Implications

Liver health is central to this integrated hypoketonaemia-ICE driven model because the liver coordinates ketogenesis, SHBG synthesis, lipoprotein handling, bile acid metabolism, and systemic inflammatory signalling [1,2,4,32]. EV research in metabolic syndrome and metabolic disease positions EVs as both biomarkers and mediators of hepatic steatosis, insulin resistance, endothelial dysfunction, and inflammatory activation [8,23,43]. Ageing frameworks further support EV remodelling as a hallmark process integrating mitochondrial signalling, inflammaging, and endocrine regulation across tissues [17,19,67]. Within this context, the present exploratory associations between EVs, thyroid signalling, bilirubin, and LH are consistent with the hypothesis that even short-term ketosis suppression engages a metabolic–endocrine–hepatic–EV network that maps directly onto mechanisms implicated in metabolic ageing and age-related disease progression.

4.11. Strengths and Limitations

This study provides novel insights into EV profiles in relation to the suppression of ketosis in metabolically healthy, long-standing euketonaemic women. A key strength is the controlled within-participant design, enabling the assessment of EV dynamics across defined metabolic states (euketonaemia, hypoketonaemia, and return-to-euketonaemia), thereby reducing inter-individual variability. Furthermore, the integration of EV profiling with detailed metabolic, endocrine, thyroid, liver, and inflammatory biomarkers provides a systems-level perspective on bioenergetic signalling.

However, several limitations should be considered. Validation in larger and more diverse cohorts is required to determine the generalisability of these findings. Future studies should incorporate more detailed characterisation of EV cargo to identify specific EV-related biomarkers in hypoketonaemia and MetS phenotypes. In particular, multi-omics approaches, including RNA sequencing, proteomics, and metabolomics, are warranted to define EV cargo adaptations in response to short-term suppression of ketosis and across broader hypoketonaemic populations. Although EV cargo has been implicated in inflammatory disorders, hypoxia and cancer, its role in metabolically healthy populations remains insufficiently characterised.

Additionally, several statistical considerations should be acknowledged. First, the sample size ($n = 10$) limited statistical power, particularly for the between-phase comparisons performed using RM ANOVA or the Friedman test and may have reduced the ability to

detect modest but biologically meaningful effects. Second, the linear mixed-effects analyses (Tables 2–4) involved a large number of unadjusted association tests. Accordingly, the reported *p* values should be interpreted as exploratory, as some statistically significant findings may reflect chance associations arising from multiple testing. Third, the open-label, non-randomised crossover design, together with the homogeneous study population (lean, premenopausal, long-term euketonaemic women), may limit the generalisability of the findings to broader populations.

Taken together, this study provides preliminary evidence that EV profiles may be sensitive to metabolic states characterised by elevated basal insulin signalling and reduced ketone availability, consistent with hypoketonaemia-ICE phenotype.

5. Conclusions

In conclusion, our study characterised EV profiles in healthy premenopausal women with long-standing euketonaemia (KetoSAge), who underwent a 21-day suppression of ketosis (hypoketonaemia-ICE) followed by a return to euketonaemia. Linear mixed-effects analysis identified within-participant nominally significant associations between EV concentration, mean size and modal size and key metabolic, endocrine, hepatic, and inflammatory biomarkers, including insulin, BHB, HOMA-IR, GKI, thyroid hormones (TSH, free T3, free T4), liver markers (ALT, AST, bilirubin), sex hormones (oestrogen and LH), and inflammatory markers (EGF and IL-6) across the study phases. However, these associations did not remain statistically significant after correction for multiple testing and should therefore be regarded as exploratory. Nevertheless, these findings extend the KetoSAge dataset into EV biology, suggesting that suppression of euketonaemia, resulting in hypoketonaemia-ICE, is associated with coordinated relationships between EV profiles and metabolic–endocrine, hepatic, and inflammatory pathways. This overall pattern is consistent with the hypothesis of an insulin-responsive EV signalling network linked to systemic bioenergetic state. Collectively, these findings are consistent with the concept that euketonaemia maintains a distinct inter-organ signalling environment that may become disrupted under conditions of chronic insulin elevation inducing ketogenesis suppression, resulting in insulin-compensated euglycaemia (ICE), where basal insulin concentrations remain persistently maintained above an individual's personal-hyperinsulinaemia threshold (PIT), leading to chronic hypoketonaemia (BHB < 0.5 mmol/L). If confirmed in larger studies, EV profiles may represent early indicators of metabolic–endocrine adaptation during changes in ketone-body availability. Future studies incorporating larger cohorts, together with EV proteomics, RNA sequencing, and metabolomic profiling are warranted to validate the presented findings and further clarify the role of EVs as biomarkers of metabolic phenotype and metabolic–endocrine adaptation across different metabolic–endocrine states.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/clinbioenerg2040016/s1>: Figure S1: Extracellular vesicles (EV) profile comparison of Phase 1 versus Phase 2 in Representative Participants. Representative nanoparticle tracking analysis (NTA) profiles from two participants are shown. The mean is shown by the black line and the standard error of the mean (SEM) by the red line. Panels A–B correspond to Participant 1 in euketonaemia (Phase 1) and hypoketonaemia (Phase 2), respectively. Panels C–D correspond to Participant 2 in the same phases. These examples illustrate a representative directional increase in EV concentration observed during ketosis suppression. Figure S2: Showing the full lane for the Western blots for CD63 and Flot-1.

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and L.P. designed tables and graphs. I.D.C., Y.K., N.C., A.S.-M., L.P., K.E., S.L. and I.K. contributed to writing and reviewed and edited the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

AD	Alzheimer's disease
ALP	Alkaline phosphatase
ALT	Alanine transferase
ANOVA	Analysis of variance
AST	Aspartate transferase
BHB	Beta-hydroxybutyrate
BIA	Bioelectrical impedance
BMI	Body mass index
BSA	Bovine serum albumin
CIs	Confidence intervals
CK-NAC	Creatine kinase-NAC
cOCN	Carboxylated osteocalcin
CR	Carbohydrate restriction
CV	Coefficient of variation
CVD	Cardiovascular disease
DPBS	Dulbecco's phosphate-buffered saline
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EVs	Extracellular vesicles
FDR	False discovery rate
FFA	Free fat acids
FFM	Fat free mass
Flot-1	Flotillin-1
FMD	Fasting mimicking diets
Free T3	Free triiodothyronine
FSH	Follicle-stimulating hormone
GGT	Gamma-glutamyl transferase
GKI	Glucose ketone index
GLP-1	Glucagon-like peptide 1
HDL	High-density lipoprotein cholesterol

HFD	High-fat diet
HOMA-IR	Homeostasis model assessment for insulin resistance
ICE	Insulin-compensated euglycaemia
IFN- γ	Interferon-gamma
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IQR	Interquartile range
IR	Insulin resistance
KetoSAge	Ketosis Suppression and Ageing
KMT	Ketogenic metabolic therapy
LDL	Low-density lipoprotein cholesterol
LH	Luteinising hormone
LMER	Linear mixed-effect modelling
M	Mean
MASLD	Metabolic dysfunction-associated steatotic liver disease
MCP-1	Monocyte chemoattractant protein-1
Md	Median
MetS	Metabolic syndrome
MP1	Metabolic phenotype 1
NAD ⁺	Nicotinamide adenine dinucleotide
NK	Nutritional ketosis
NTA	Nanoparticle tracking analysis
OGTT	Oral glucose tolerance test
ROS	Reactive oxygen species
PAI-1	Plasminogen activator inhibitor-1
PIT	Personal-hyperinsulinaemia threshold
PPAR γ	Peroxisome proliferator-activated receptor gamma
P1	Phase 1 of the study
P2	Phase 2 of the study
P3	Phase 3 of the study
RM	Repeated measures
RQ	Respiratory quotient
RT	Room temperature
SASP	Senescence-associated secretory phenotype
SD	Standard deviation
SEM	Standard error mean
SHBG	Sex hormone-binding globulin
SuK	Suppression of ketosis
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
TEM	Transmission electron microscopy
TNF- α	Tumour necrosis factor-alpha
tOCN	Total osteocalcin
TSH	Thyroid stimulating hormone
T2DM	Type 2 diabetes mellitus
T3	Triiodothyronine
T4	Thyroxine
unOCN	Uncarboxylated osteocalcin
VEGF	Vascular endothelial growth factor
β	Effect estimate

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