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ORIGINAL ARTICLE

Plasma lactate and leukocyte mitochondrial DNA copy number as biomarkers of insulin sensitivity in non-diabetic women

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Abstract

High plasma lactate levels have been associated with reduced mitochondrial respiratory capacity and increased type 2 diabetes risk, while mitochondrial DNA (mtDNA) copy number has been proposed as a biomarker of mitochondrial function linked to glucose homeostasis. The aim of this study was to evaluate the association between circulating lactate levels and leukocyte mtDNA copy numbers with insulin secretion/sensitivity indexes in 65 Chilean non-diabetic women. mtDNA copy numbers were measured in leukocytes using qPCR and digital-droplet PCR. A 75-g Oral Glucose Tolerance Test (OGTT) was performed to calculate systemic and tissue-specific insulin sensitivity indexes, as well as insulin secretion surrogates based on plasma c-peptide. An intravenous glucose tolerance test (IVGTT; 0.3 g/kg) was also carried out. Disposition indexes were calculated as the product of insulin secretion × sensitivity. Plasma levels of leptin, adiponectin, TNF-α, MCP-1, and non-esterified fatty acids were also determined. Fasting plasma lactate shows a significant association with a wide range of insulin sensitivity/resistance indexes based on fasting plasma samples (HOMA-S, adipose IR index, Revised-QUICKI, leptin-adiponectin ratio, TyG index, McAuley index and TG-to-HDL-C ratio), as well as OGTT-based measures such as the Matsuda index, the hepatic insulin resistance index, and the disposition index. Fasting plasma lactate was also positively associated with the circulating adipokines TNF-α and MCP-1. We also detected a direct association between fasting plasma lactate with leukocyte mtDNA copy numbers. The above results support the use of fasting plasma lactate, and possibly leukocyte mtDNA copy numbers, as biomarkers of reduced oxidative mitochondrial capacity, decreased hepatic insulin sensitivity, and future diabetes risk.

Keywords Lactate · Mitochondrial DNA · Insulin sensitivity · Biomarker · Diabetes

Introduction

Plasma lactate has been identified as a circulating metabolite associated with cardiometabolic traits and type 2 diabetes (T2D) [33], with large epidemiologic studies supporting the hypothesis that fasting plasma lactate is positively associated with measures of homeostasis-model assessment insulin-resistance (HOMA-IR) index and a higher risk of T2D [11, 22, 23]. Lactatemia is also increased during the Oral Glucose Tolerance Test (OGTT) as a result of the effect of insulin in the glycolytic pathway [45, 55], in a magnitude that is possibly linked to insulin resistance and mitochondrial respiratory capacity [15, 20, 25, 41, 45]. Mitochondrial dysfunction is critically involved both in insulin secretion in β-cells and insulin action in insulin-sensitive tissues such as liver, muscle, and adipose tissue [30, 50]. Additionally, variable mitochondrial DNA (mtDNA) copy number (polyplasmy) in leukocytes

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might represent a biomarker of mitochondrial capacity and stress linked to insulin action and chronic diseases [5, 31].

In the past, lactate was only considered as an end-product of anaerobic glycolysis, which is abundantly and acutely generated from the muscle during episodes of intense physical activity and connected with glucose homeostasis through Cori's cycle as a gluconeogenic precursor in the liver. However, it has been shown that lactate is not only important in anaerobic glycolysis, but it is also produced and used continuously in diverse cell types under aerobic conditions, being the major carbon source to fuel the mitochondrial Krebs cycle [21]. Interestingly, it has been reported that oxamate (a pyruvate analogue that inhibits the lactate dehydrogenase—LDH) is able to improve glycemic control and insulin sensitivity in the obese *db/db* mice lacking leptin receptor, indicating that lactate production can be modulated systemically with detectable effects in energy metabolism and glucose homeostasis [56]. Lactate and other circulating monocarboxylic acids have their own membrane transporters (MCT1–4) [9]. Specifically, MCT1 is widely expressed in the adipose allowing transport of the monocarboxylic acids such as pyruvate, lactate, ketone bodies, acetate, and propionate. Interestingly, both LDH and the lactate/pyruvate transporter MCT1 are disallowed and repressed in pancreatic insulin-producing β-cells [40]. In this way, pancreatic β-cells eliminate divergent pathways apart from mitochondrial ATP production, which is critical for coupling glucose metabolism to insulin secretion. On the other hand, circulating lactate is also the ligand of the membrane receptor GPR81 expressed in adipose tissue. The effect of GPR81 activation by lactate seems to inhibit lipolysis in adipose tissue potentiating the anabolic effects induced by insulin [2]. Moreover, plasma lactate may interfere with insulin action leading to skeletal muscle resistance through affecting insulin signaling, including lower stimulation of IRS-1 and IRS-2, PI3K (phosphatidyl-inositol-3-kinase), and AKT/protein kinase B. Also, inactivation of muscle pyruvate dehydrogenase increases glucose oxidation leading to decreased lactate plasma levels [47]. Given the putative roles of circulating lactate in different organs, it has been proposed that circulating lactate may act on multiple cellular processes through autocrine-, paracrine-, and endocrine-like actions [7].

Diabetes is defined as a "group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both" [3]. In this context, a hyperbolic relation has been described for insulin secretion and sensitivity indexes derived from Intravenous Glucose Tolerance Test (IVGTT) in such a way that it is possible to calculate different Dispositions Indexes (DIs: insulin secretion × insulin sensitivity) [24]. The Disposition Index can be considered as a measure of insulin secretion adjusted by systemic insulin sensitivity. Our group has previously validated an OGTT-based DI (Oral Disposition Index; ODI), which reproduces the mathematical properties of DI from IVGTT [44].

There are no reports assessing the relation between plasma lactate levels and leukocyte mtDNA copy numbers in connection with tissue-specific insulin sensitivity indexes (hepatic, muscle, adipose tissue) and/or disposition indexes. Since mitochondrial function has been related both to insulin secretion and sensitivity, the aim of this study was to evaluate the associations between circulating lactate levels and mtDNA copy number with systemic and tissue-specific insulin secretion/sensitivity indexes derived from oral and intravenous glucose challenges.

Subjects and methods

Study design and subjects

Cross-sectional study of $n = 65$ Chilean women non-obese non-diabetic volunteers, without family history of diabetes, aged 27.4 ± 6.9 years and BMI of 23.8 ± 3.5 kg/m^2 (mean ± SD) (Table 1). The volunteers visited the UC Centre of

Table 1 Anthropometric and biochemical characteristics of Chilean of $n = 65$ non-diabetic women ([a])

	Mean ± SD
Age (years)	27.4 ± 6.9
Weight (kg)	60.1 ± 9.3
Height (m)	1.6 ± 0.07
BMI (kg/m^2)	23.8 ± 3.5
Fasting plasma glucose (mg/dl)	77.3 ± 6.6
Fasting plasma insulin (μIU/ml)	7.7 ± 3.5
Fasting plasma lactate (mmol/L)	1.3 ± 0.5
Fasting plasma TNF-α (pg/mL)	2.8 ± 1.8
ΔPlasma GIP (pg/mL) OGTT	216.8 ± 109.2
Δ Plasma NEFAs (μEq/L)	0.5 ± 0.2
Plasma total cholesterol (mg/dL)	179.9 ± 36.9
Plasma HDL cholesterol (mg/dL)	64.0 ± 14.6
Plasma triglycerides (mg/dL)	106.8 ± 60.8
Systolic arterial pressure (mmHg)	111.8 ± 10.1
Diastolic blood pressure (mmHg)	70.5 ± 8.1
HOMA-S index	77.9 ± 43.9
MATSUDA-ISICOMP index	5.7 ± 2.9
Acute insulin release (AIR) index	596.9 ± 336.2
CSi index	6.5 ± 4.0
Leptin-adiponectin ratio	1.9 ± 1.3
OGTT-based Disposition Index (ODI)	0.3 ± 0.2
IVGTT-based Disposition Index (IDI)	3372.3 ± 2024.3
Adipose IR index	46.9 ± 22.4
TyG index	175.7 ± 22.9

[a] Sample size varies from variable to variable depending on success in measurement and whether it was based on measurements in the OGTT day (total sample size $n = 65$) or IVGTT day (total sample size $n = 58$)

Clinical Research in three occasions 1 week apart approximately. In the first visit, measurements of weight, height, body mass index (BMI), and systolic/diastolic blood pressure were obtained, as well as biochemical determinations measured in the central laboratory of the Pontificia Universidad Católica de Chile (http://redsalud.uc.cl/ucchristus/laboratorio-clinico/) such as fasting plasma glucose, total cholesterol, HDL-cholesterol, triglycerides, glycated hemoglobin, and hemogram (Table 1). At this stage, subjects with diabetes, glucose intolerance, dyslipidemia, anemia, or in pregnancy were excluded from the study. In the second visit, participants were recruited for a standard 75 g oral glucose tolerance test (OGTT), after 8–12 h of fasting, drawing blood samples at times − 15, − 5, 15, 30, 60, 90, and 120 min after glucose administration, for the determination of plasma glucose, lactate, non-esterified fatty acids (NEFAs) and insulin levels. In fasting samples (− 15 min), an aliquot was separated for measuring leptin and adiponectin. In the third visit, a total of n = 58 participants of this group were enrolled for an abbreviated Minimal-Model Intravenous Glucose Tolerance Test (IVGTT) , while the other 7 participants were unavailable for the IVGTT visit. The abbreviated IVGTT protocol consisted in the intravenous administration of 0 .3g of glucose by kg of body weight, to measure plasma glucose, lactate, and insulin at times − 15, − 5, 2, 4, 6, 8, 10, 15, 20, 30, 40, and 50 min after glucose intravenous administration [52]. All participants signed written informed consent and the research protocol was approved by the Ethics Committee of the School of Medicine of the Pontificia Universidad Católica de Chile.

Anthropometry, blood pressure, and biochemical determinations

Anthropometric measurements were carried out by trained personnel in light clothing and without shoes, using a calibrated set of stadiometers, scales, and tapes. Weight, waist circumference, and height were used to calculate BMI (kg/m^2) and the waist-to-height ratio. Systolic and diastolic blood pressure were measured with digital sphygmomanometer as an average of three measurements. Venous blood samples were drawn by venipuncture in tubes containing sodium fluoride (for glucose and lactate measurements) and EDTA-tubes. Plasma and leukocytes were separated from whole blood by centrifugation at 3500 rpm, 5 °C, 15 min, and frozen immediately at − 80 °C until assay. Leukocyte DNA was extracted using QIAGEN Mini-blood kits. Plasma levels of insulin (μU/mL), glucose (mg/dL), and lactate (mmol/dL) were measured in the central laboratory of the Pontificia Universidad Católica de Chile by electro-chemiluminescence and colorimetric methods. NEFAs were quantified with a colorimetric method (Wako, code 993-35191). Leptin, adiponectin, and c-peptide concentrations were measured by radioimmunoassay (RIA) kits. Plasma levels of proinflammatory adipokines (TNF-α—tumor necrosis factor-α and MCP-I—monocyte chemoattractant protein-1) as well as the incretin GIP (gastric inhibitory polypeptide) were determined by a multiplex-ELISA kit measured in a MAGPIX equipment (Merck, code: HMHEMAG-34 K).

Insulin sensitivity and secretion indexes

The Calculated Sensitivity index (CSi) derived from the Abbreviated IVGTT-Minimal Model was computed using insulin and glucose levels measured from minutes 10 to 50 (min 10, 15, 20, 30, 40, and 50) using the website http://webmet.pd.cnr.it/csi/ [52]. The Matsuda-ISICOMP index was calculated from OGTT [32]. The hepatic insulin resistance index (HIRI) was calculated using the first part of the OGTT curve through the equation ($\text{glucose}_{0-30}[\text{AUC}] \times \text{insulin}_{0-30}[\text{AUC}]$). The Muscle Insulin Sensitivity Index (MISI) was calculated from the decline in plasma glucose concentration from its peak to its nadir, using the last part of the OGTT curve. Both HIRI and MISI were previously validated with a euglycemic insulin clamp in combination with tritiated glucose [1]. Surrogate measures of insulin sensitivity based on fasting plasma samples were also calculated, such as the HOMA-S index, which represents the inverse of the HOMA-IR index (HOMA-S = 1/HOMA-IR = (1/(fasting insulin (μUI/mL) × fasting glucose (mg/dL)/405), the adipose insulin resistance (Ad-IR) index (calculated from plasma basal insulin levels and NEFAs), the plasma leptin-adiponectin ratio, the TyG index (which uses plasma triglyceride and glucose levels), the TyG-BMI index, the McAuley index (uses fasting insulin and plasma triglyceride levels), the plasma TG-to-HDL-C ratio (uses plasma triglyceride levels and HDL-cholesterol levels), and the revised-QUICKI (Quantitative Insulin Sensitivity Check Index), which uses fasting plasma insulin, glucose, and NEFAs [34, 37].

The Acute Insulin Release (AIR) index was calculated from Abbreviated IVGTT-Minimal Model during the first 10 min after glucose administration. Based on the OGTT, the ratio of AUC-c-peptide divided by the AUC-Glucose during the 2 h-OGTT (AUC-Ratio-C-Peptide/Glu) was calculated as a global measure of insulin secretion [19, 51]. Disposition Indexes representing measures of insulin secretion adjusted by systemic insulin sensitivity were calculated as the product of secretion × insulin sensitivity measurements from IVGTT (IDI, Intravenous Disposition Index = AIR × CSi) or from OGTT (ODI, Oral Disposition Index = Matsuda-ISICOMP index × AUC-Ratio-C-Peptide/Glu).

Measurement of mtDNA copy numbers

The mtDNA copy number from peripheral blood leukocytes was determined using two different techniques: quantitative-PCR (qPCR) and digital droplet PCR (ddPCR) [35, 39]. The qPCR was performed in a Stratagene MX3000P equipment using Brilliant III Ultra-Fast SYBR® Green QPCR Master

Mix (Agilent Technologies, CA, USA). To determine the human mtDNA copy numbers, two previously reported set of primers were used [43]: one for the nuclear β2-microglobulin gene (β2M; forward: 5′-TGCTGTCTCCATGTTTGATGTATCT-3′ and reverse: 5′-TCTCTGCTCCCCACCTCTAAGT-3′) and the other set of primers for the MT-TL1 (forward: 5′-TGCTGTCTCCATGTTTGATGTATCT-3′ and reverse 5′-TGGCCATGGGTATGTTGTTA-3′). PCR assay conditions were previously optimized to obtain high efficiency and specificity (a single amplicon) of 86 and 107 base pairs respectively. Amplifications were carried out in triplicate and subsequently normalized to threshold cycles obtained with a commercial human genomic DNA as calibrator (Promega. Code: G304A, USA). mtDNA copy numbers were also quantified using the digital droplet PCR platform ddPCR QX200 System (BioRad). All PCR reactions were made in duplicate and assayed in 96-well plates. First, 100 ng of total genomic DNA was digested at 37 °C during 1 h with 5 units of HindIII restriction enzyme (Fermentas, code: ER0505), together with 1× buffer R and water to complete a final volume of 20 μL. The ddPCR assay mix was prepared in a 20-μl reaction volume adding 1× QX200 ddPCR EvaGreen Supermix (BioRad, code 1864033), 150 nM of forward and reverse primers, and 5 μl of digested total DNA (0.5 ng). As non-template control (NTC), 5 μl of double-distilled water was used instead of DNA sample. Two NTCs were included in each ddPCR assay plate. In addition, a commercial Human Genomic DNA (Promega. Code: G304A, USA) was used in every ddPCR plate as a calibrator DNA. Droplet emulsion formation was performed by mixing the 20-μl reaction with 70 μl of droplet generation oil (BioRad, code: 1864005) using a microfluidic droplet generation cartridge (BioRad, code: 1864008) and a Droplet Generator (QX200 ddPCR System, BioRad). Endpoint PCR amplifications were performed using a T100 Thermal Cycler (BioRad) following the next conditions: one denaturation cycle at 95 °C for 5 min, 40 two-step (denaturation, hybridization and elongation) cycles at 95 °C for 30 s and 61.5 °C for 1 min, followed by one step at 4 °C for 5 min, one step at 90 °C for 5 min and stored at 4 °C. The presence or absence of amplification per droplet was evaluated using a Droplet Reader (QX200 ddPCR System, BioRad) and analyzed using the QuantaSoft software. For both qPCR and ddPCR, results are expressed as the ratio of mtDNA/nDNA (copies of MT-TL1/copies of β2M) in samples DNA normalized with respect to the calibrator DNA.

Statistical analyses

Summary statistics are expressed as mean ± standard deviation. The Lin correlation coefficient was used to assess concordance between methods of measurement of mtDNA copy numbers [28]. A paired *t* test was performed to evaluate differences in plasma lactate measurements at − 15 and − 5 min before OGTT. We used Spearman correlation coefficients to assess the association between study variables. Use of logarithm transformations and Pearson correlation coefficients did not modify the main conclusions of this study. Charts of correlations of studied variables are shown with untransformed variables showing least-squares regression lines and confidence intervals only for reference. Considering a correlation coefficient of $r = -0.4$ between lactate vs common indexes of insulin sensitivity (HOMA-S or others) [10], a sample size of $n = 65$ provides a statistical power > 90% with a confidence of 95% to detect significant associations. After standard statistical testing, the Sidak correction for multiple comparisons was carried out. All statistical analyses were carried out with STATA 14.0 statistical software (http://www.stata.com).

Results

Significant differences were found between plasma lactate concentrations at min − 15 compared to min − 5 (Fig. 1). When comparing fasting plasma lactate measurements in the OGTT-day and the IVGTT-day, we estimated an inter-day standard deviation of 0.34 at time − 15 min and 0.2 at time − 5 min before either OGTT or IVGTT. Given these results, subsequent analysis using fasting plasma lactate were carried out with measurements at the time − 5 min before OGTT (for association with OGTT-derived indexes, hormone profiles and anthropometric variables) or − 5 min before IVGTT (only for assessment of IVGTT-derived indexes). We found a positive significant correlation between basal lactate levels with hematocrit, BMI, and waist-to-height ratio (Fig. 1), with no significant differences in correlation coefficients by insulin resistance status, defined as being above or below the median value of HOMA-S, Matsuda index or CSi. Figure 2 shows plasma lactate trajectories during either OGTT or IVGTT in insulin-sensitive and insulin-resistant according to the median of selected insulin sensitivity indexes. Both fasting plasma lactate levels and the total area under the curve (AUC) of lactate were significantly different when comparing insulin-sensitive versus insulin-resistant participants ($p < 0.0001$; Fig. 2). However, no differences were found in iAUC of plasma lactate in both groups.

Figures 3 and 4 show the relationship between basal plasma lactate levels with metabolic and insulin-related variables. Fasting plasma lactate levels showed an inverse significant association with different common indexes of insulin sensitivity measures such as HOMA-S and the Matsuda-ISICOMP indexes (Fig. 3a, b). No association was found with NEFAs changes during OGTT (Fig. 3c). Basal plasma lactate levels also showed a negative slope in relation with CSi (Fig. 3d), but without reaching statistical significance. A positive significant association was found between basal plasma lactate and the adipose IR index and the plasma leptin-adiponectin ratio (Fig. 3e, f). A significant association was also found between

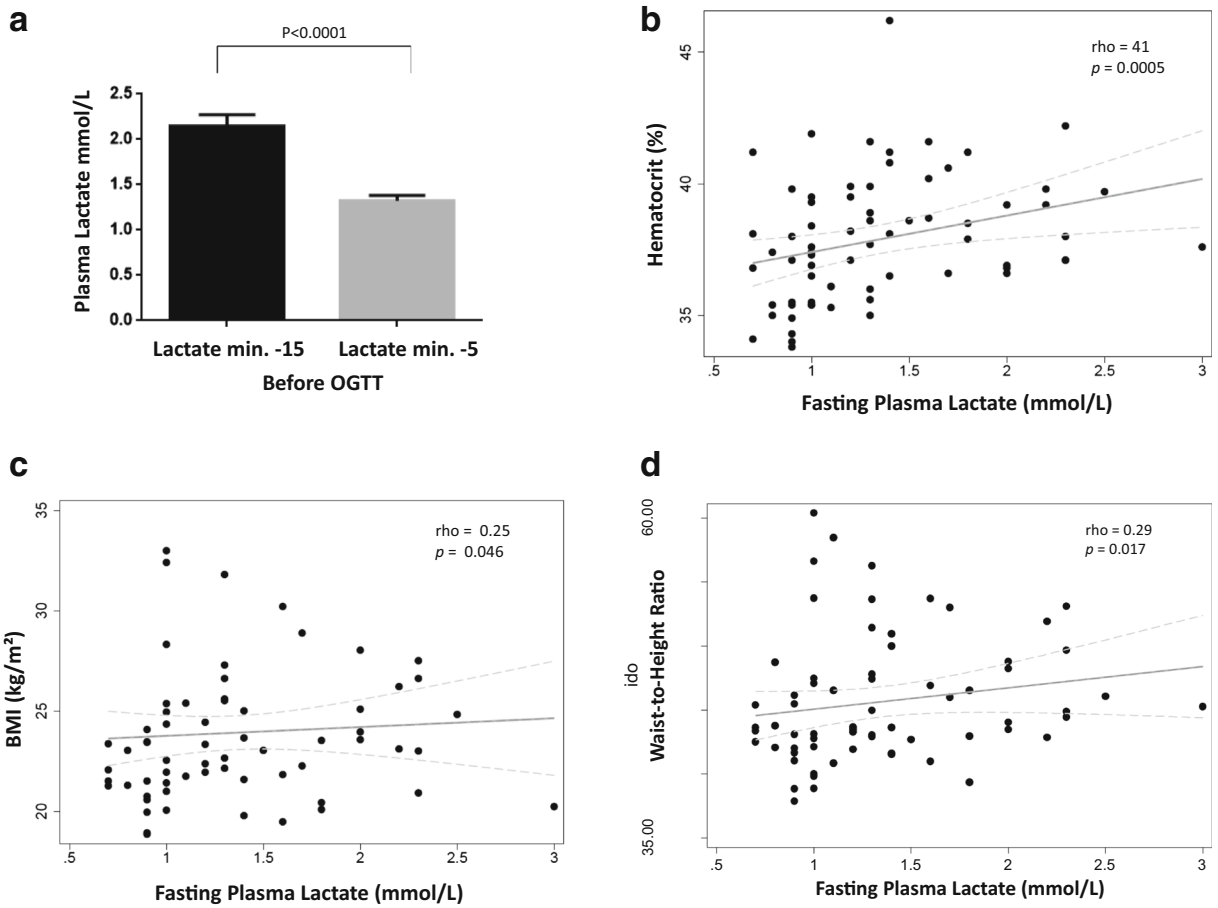

Fig. 1 Fasting plasma lactate in Chilean non-diabetic women: relation to the time of blood drawing, hematocrit, and anthropometric variables. **a** Significant differences ($p < 0.0001$) by paired student t between lactate levels (min − 15 and − 5 before glucose challenge, either OGTT or IVGTT). The time − 15 min coincides with the venipuncture. **b** Correlation between fasting plasma lactate and hematocrit. **c** Correlation between fasting plasma lactate and body mass index (BMI). **d** Correlation between fasting plasma lactate and waist-to-height ratio

fasting plasma lactate and HIRI (Fig. 4a), without finding associations between the basal plasma lactate with MISI (Fig. 4b). Near-significant associations were found between fasting plasma lactate and ΔGIP 0–120 min during OGTT (Fig. 4c). Significant associations were also found between basal circulating lactate with either fasting plasma TNF-α (Fig. 4d) or plasma MCP-1 levels (rho = 0.27; $p = 0.037$; data not shown). Plasma TNF-α and MCP-1 levels were strongly correlated (rho = 0.53; $p < 0.0001$; data not shown). Additionally, significant positive associations were found between fasting plasma lactate with surrogates of insulin resistance based on fasting blood samples and common biochemical measurements, such as TyG index (rho = 0.43; $p = 0.0004$), TyG-BMI index (rho = 0.45; $p = 0.0005$), and the TG-to-HDL-C ratio (rho = 0.25; $p = 0.04$). On the contrary, significant negative associations were found for insulin sensitivity indexes such as the McAuley index (rho = −0.50; $p < 0.001$), or the Revised-QUICKI (rho = − 0.28; $p = 0.03$) (data not shown).

A near-significant trend for association was found between fasting plasma lactate and the total insulin secretion during 2 h-OGTT measured as AUC-Ratio-C-Peptide/Glu (rho = 0.23; $p = 0.09$; data not shown). However, a negative significant association was found between fasting plasma lactate and the Oral Disposition Index (ODI) defined as MATSUDA-ISICOM index multiplied by AUC-Ratio-C-Peptide/Glu (Fig. 4e). No evidence of association was found for the relation between fasting plasma lactate levels and the AIR or the disposition index derived from IVGTT, defined as IDI = AIR × CSi (Fig. 4f). All significant associations between fasting plasma lactate levels with metabolic and insulin-related variables reported in Figs. 2, 3, and 4 were also achieved when replacing fasting plasma lactate by the total AUC of lactate during OGTT. In contrast, the correlations between the incremental AUC (iAUC) of plasma lactate during OGTT with insulin-related variables did not achieve statistical significance for any of the studied variables.

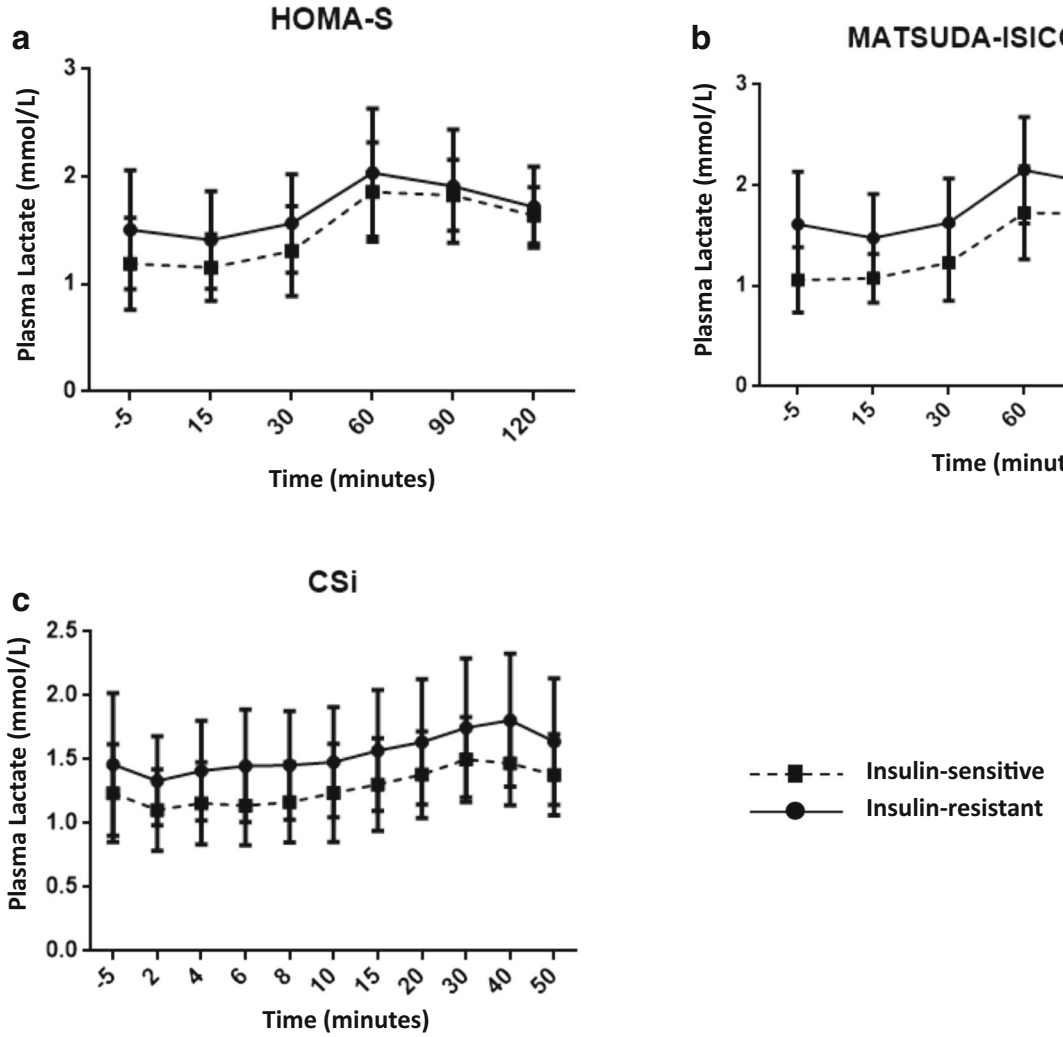

Fig. 2 Plasma lactate trajectories during glucose challenges according insulin sensitivity status in Chilean non-diabetic women. **a** Plasma lactate trajectories during OGTT. Insulin sensitivity was classified using the median of HOMA-S index (above the median: insulin-sensitive subjects; below the median: insulin-resistant). **b** Plasma lactate trajectories during OGTT. Insulin sensitivity was classified using the median of Matsuda-ISICOMP index (above the median: insulin-sensitive subjects; below the median: insulin-resistant). **c** Plasma lactate trajectories during the abbreviated IVGTT-minimal model. Insulin sensitivity was classified using the median of CSi index (above the median: insulin-sensitive subjects; below the median: insulin-resistant)

Figure 5a shows a positive correlation between the measurement of mtDNA copy number with qPCR versus the ddPCR technique (rho = 0.52; $p < 0.0001$). However, the concordance between these two measurements was low (Lin concordance coefficient = 0.30; 95% CI 0.17–0.42). Figure 5b–e shows the correlation between mtDNA copy numbers (measured through ddPCR) with metabolic and insulin-related variables. The mtDNA copy number shows positive significant associations plasma fasting lactate (Fig. 5b) and plasma TNF-α levels (Fig. 5d) and inverse associations with Matsuda-ISICOMP index and the Oral Disposition index (Fig. 5c, e). Additionally, mtDNA copy number shows positive significant associations with the adipose IR index (rho = 0.27; $p = 0.04$) and plasma MCP-1 levels (rho = 0.27; $p = 0.036$) (data not shown). No significant correlations were detected between mtDNA copy number with CSi or the Disposition Index derived from IVGTT. Figure 5f shows a significant positive correlation between mtDNA copy number with platelet count.

Multiple statistical comparisons were carried out in this study, with the simultaneous assessment of the relation between fasting plasma lactate or mtDNA copy numbers on one hand, with multiple insulin sensitivity or resistance indexes on the other hand. After stringent correction by Sidak and Bonferroni methods, the association of fasting plasma lactate levels remained significant for HOMA-S, HIRI, Matsuda-ISICOMP, the McAuley index, and the Oral Disposition Index ODI. Given the positive association between fasting plasma lactate and mtDNA copy number (Fig. 5b), a similar pattern was obtained for the association between leukocyte mtDNA copy numbers with insulin sensitivity indexes. However, in the case of mtDNA copy number as biomarker for insulin sensitivity, no statistical significance was achieved

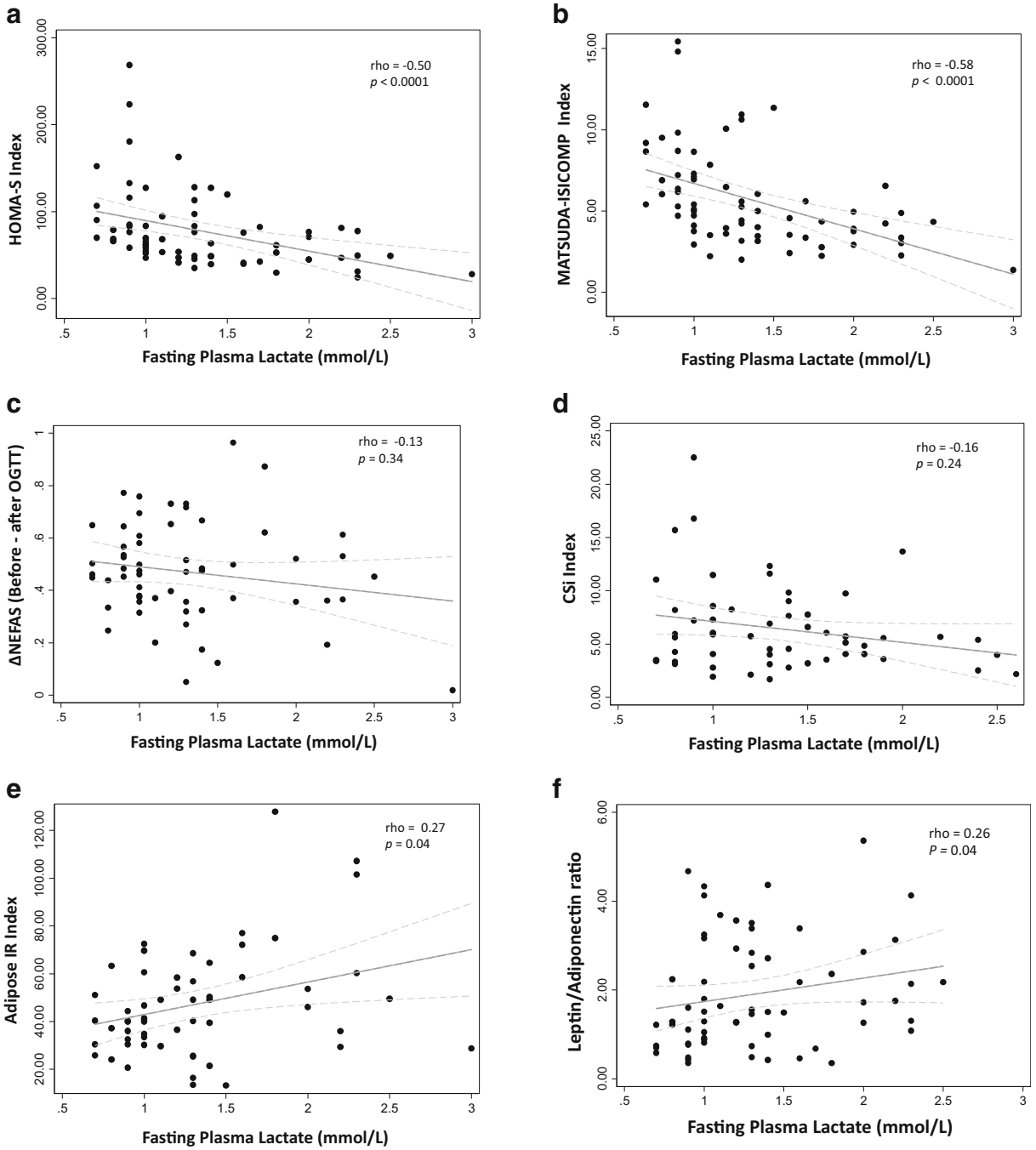

Fig. 3 Association between fasting plasma lactate levels with systemic sensitivity insulin indexes in Chilean non-diabetic women. **a** HOMA-S index. **b** Matsuda-ISICOMP index from OGTT. **c** Δ-NEFAs index (Levels before–after OGTT). **d** CSi from IVGTT. **e** Adipose IR index. **f** Plasma leptin-adiponectin ratio

after statistical correction for multiple comparisons by the Sidak or Bonferroni methods.

Discussion

Several studies have previously proposed plasma lactate levels as a marker of insulin resistance based on fasting levels of this metabolite and differential lactatemia responses to glucose loads or hyperinsulinemic euglycemic clamps [6, 20, 29]. In these studies, insulin resistance has been associated with either a blunted [20, 29] or a trend for enhanced [6] plasma lactate in response to oral glucose challenges. In our research, fasting plasma lactate was elevated in insulin-sensitive versus insulin-resistant subjects. However, it was also clearly visualized that the evolution of plasma lactate trajectories during

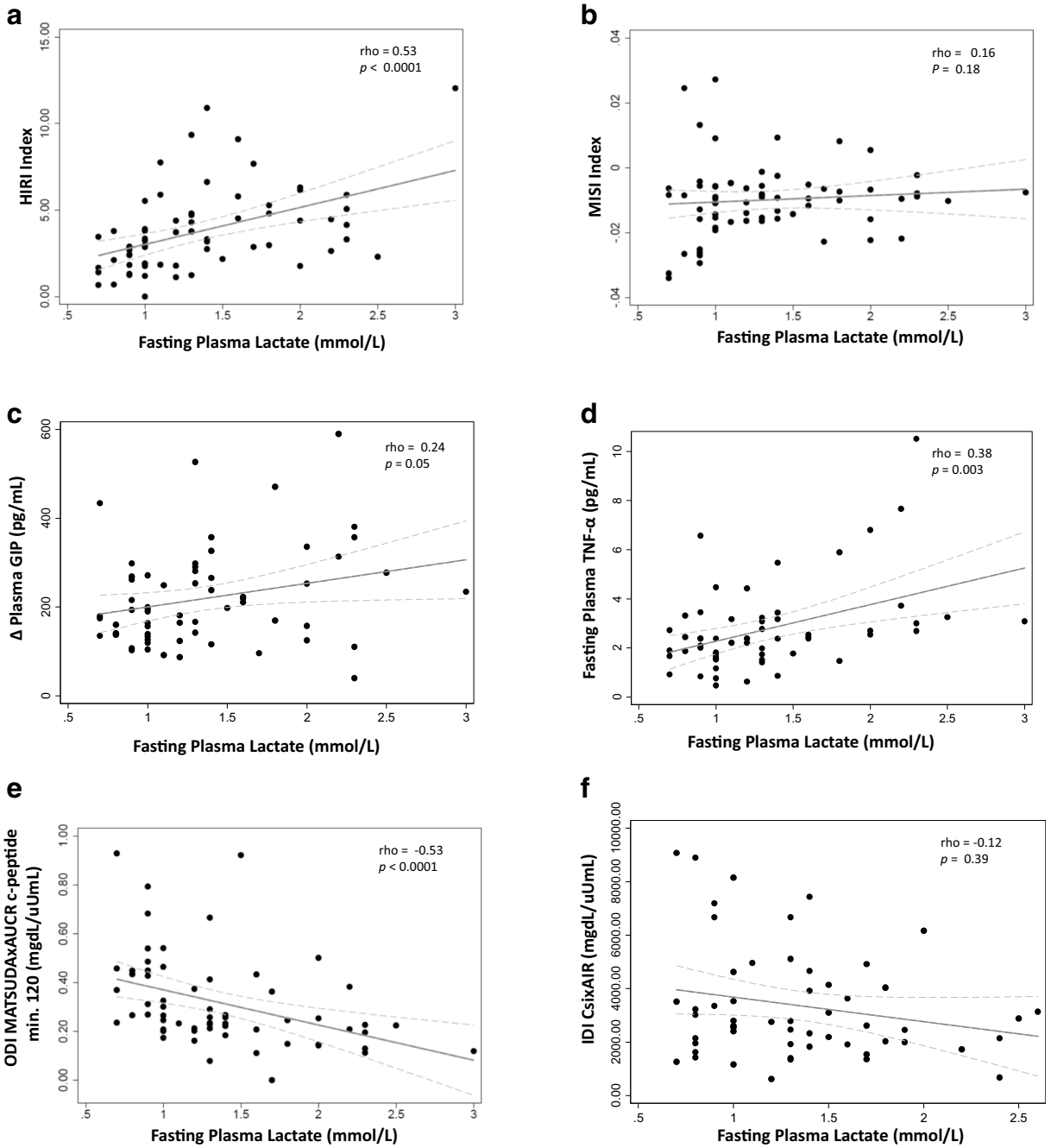

Fig. 4 Association between basal lactate with metabolic and insulin-related variables in Chilean non-diabetic women. **a** HIRI (hepatic insulin resistance index). **b** MISI (Muscle Insulin Sensitivity Index). **c** ΔGIP (difference in GIP after-before OGTT). **d** Plasma TNF-α. **e** ODI (Oral Disposition Index from OGTT) computed as Matsuda-ISICOMP Index × Ratio of AUC-c-peptide/AUC-glucose (AUCR-PC^{120min}). **f** IDI (Intravenous Disposition Index) computed as CSi × Acute Insulin Release (AIR) index was from IVGTT during the first 10 min after glucose administration

oral or intravenous glucose challenges were very similar in both groups, apart from the basal differences in plasma lactate. This observation indicates that fasting rather than postprandial circulating lactate level is the most adequate biomarker of insulin sensitivity. Interestingly, it has been reported that increased insulin sensitivity after weight loss programs is accompanied by reductions in plasma lactate levels [10]. In contrast, no significant association was found in our study between fasting plasma lactate and IVGTT-based indexes of insulin sensitivity. This lack of association was probably due to the additional effect of incretins on insulin secretion that only occur in OGTT, as well as the reduced amount of glucose

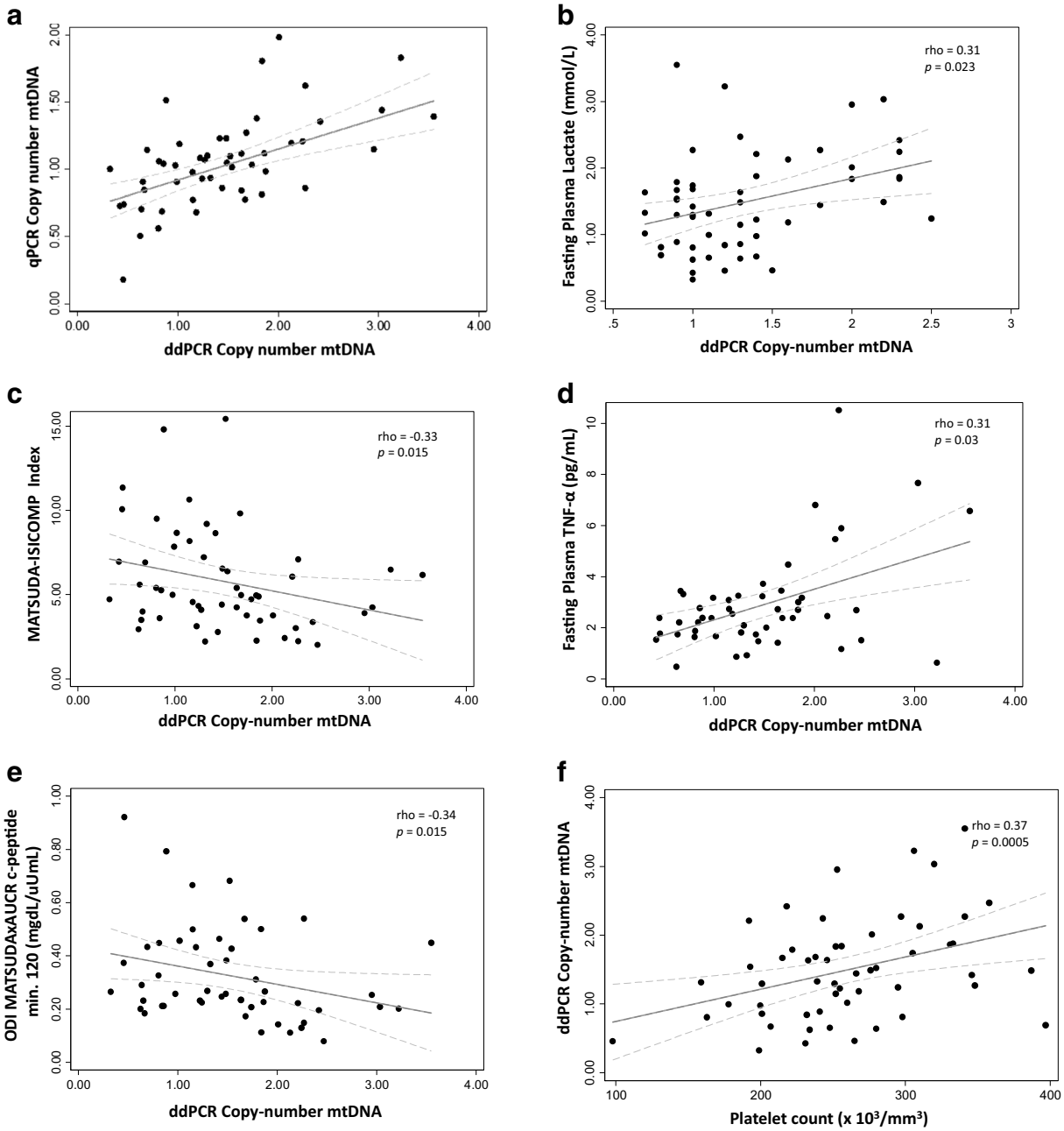

Fig. 5 Association between mtDNA copy number with metabolic variables and insulin-related indexes. **a** Measurement of mtDNA copy numbers with qPCR and ddPCR techniques. **b** Association between mtDNA copy number (ddPCR) with fasting plasma lactate. **c** Association between mtDNA copy number (ddPCR) with the Matsuda-ISICOMP index. **d** Association between mtDNA copy number (ddPCR) with fasting plasma TNF-α levels. **e** Association between mtDNA copy number (ddPCR) with the Oral Disposition Index computed as Matsuda-ISICOMP Index x Ratio of AUC-c-peptide/AUC-glucose (AUCR-PC^{120min}). **f** Association between platelet count and leukocyte mtDNA copy number (ddPCR)

administered during IVGTT compared to OGTT. In any case, the weak relation between fasting plasma lactate levels and CSi is still consistent in terms of directionality with the correlations shown in Fig. 3a, b.

In our study, fasting plasma lactate levels have been also associated with a validated OGTT-based liver-specific insulin sensitivity index [1]. It is important to mention that among metabolites that have been related to insulin resistance from metabolomic studies, lactate is distinctive since it is an endogenous metabolite that is directly linked to glucose oxidation [51] and can be simultaneously used by the liver as a gluconeogenic precursor, which may increase endogenous

glucose production and then exacerbate hyperglycemia [10]. In contrast, no association was found between plasma lactate levels and the degree of decline of circulating NEFAs during OGTT, indicating a weak role of plasma lactate in determining plasma NEFA levels through action on adipocytes expressing GPR81 [2]. Interestingly, we report herein a strong correlation between fasting plasma lactate levels with circulating cytokines (TNF-α and MCP-1) that play relevant roles in adipose tissue inflammation and insulin resistance. Our results also support the use of fasting plasma lactate levels as a biomarker of insulin sensitivity and future diabetes risk, in agreement with the Atherosclerosis Risk in Communities (ARIC) cohort, where fasting plasma lactate levels were revealed as an important risk factor of T2D after a follow-up of more than 12 years [22, 23].

Studies on induced pluripotent stem cells (iPSCs) from patients with severe insulin resistance due to insulin receptor mutations support the hypothesis that impaired oxidative capacity and mitochondrial dysfunction precedes increased circulating lactate levels and insulin resistance. Such iPSC cells show a higher number of mitochondria, similar mtDNA copy numbers, reduced mitochondrial size, reduced respiratory activity and energy production, decreased citrate synthase activity, spare respiratory capacity, and strong increased (80% higher) lactate production compared to controls [8]. In this situation, lactate production in the insulin-resistant state can be promoted from impairment in the function of the electron transport chain, a preference in the derivation of pyruvate toward lactate, reduction of the acetyl-coenzyme-A flux or interconversion with alanine, and other related metabolites [33, 45]. Interestingly, LDH was included in the Mitocarta database (http://www.broadinstitute.org/pubs/MitoCarta/index.html) as a mitochondrial enzyme, apart from its cytoplasm location, suggesting additional roles of this enzyme in mitochondrial function. Then, our data concerning the associations between fasting plasma lactate and insulin sensitivity indexes is concordant with the idea of the involvement of mitochondrial function as a common pathway affecting both insulin sensitivity and plasma lactate levels. On the other hand, high lactate levels have been also linked to an increased in oxidative stress associated to mitochondrial dysfunction [17, 18].

A number of studies have reported an association between blood mtDNA content and insulin sensitivity, showing either no association or positive correlations between these two variables [16, 27, 38, 46, 49, 54, 58]. According to these studies, reduced mtDNA copy numbers would be found in insulin-resistant status, and possibly in pre-diabetes and diabetes compared to insulin-sensitive normoglycemic subjects. Moreover, reduced leukocyte mtDNA copy numbers have been also associated with chronic diseases such as cardiovascular disease or even with all-cause mortality [4, 5]. A different view proposes that increased mtDNA copy numbers represent an increased mitochondrial biogenesis as a compensatory response to hyperglycemia and/or oxidative stress [31] which may cause mitochondrial dysfunction [57]. In our study, we have found that mtDNA copy number in leukocytes of non-diabetic women is inversely related to insulin sensitivity and positively associated with fasting plasma lactate levels. Although it has been described that insulin receptor is expressed in several types of blood cells and platelets, it is unlikely that these cells play a relevant role in determining whole-body glucose homeostasis. However, our results are concordant with the hypothesis that considers mtDNA copy number in leukocytes as biomarkers of low mitochondrial oxidative capacity deriving glucose mainly to the anaerobic branch of glycolysis leading to mitochondrial hyperproliferation [14] and increased derivation of glucose to lactate. It is important to note here that white blood cell energy requirements derive at a similar proportion from glycolysis or mitochondrial oxidative phosphorylation, at least lymphocytes [17].

Mitochondrial dysfunction impairs ATP production and insulin secretion in β-cells [38]. It has been reported that a reduction in β-cell mtDNA in mice results in a decrease in glucose-mediated insulin secretion [48] and related to the age-dependent decline in β-cell secretory capacity [12, 35]. Park et al. [38] found that there was a negative correlation between mtDNA content in leukocytes and hyperglycemic clamp-based indexes of insulin secretion in healthy young adults. In our study, we have not found significant associations between mtDNA copy numbers in leukocytes and crude insulin secretion indexes derived from OGTT and IVGTT. Then, the non-significant trend for a positive association between fasting plasma lactate and the total insulin secretion during OGTT (defined as AUC-Ratio-C-Peptide/Glu) is possibly secondary to the inherent inverse hyperbolic relation previously described for insulin secretion and sensitivity indexes [44]. It is very unlikely that plasma lactate levels may affect directly insulin secretion, since MCT1 transporters for lactate and other monocarboxylic acids are selectively disallowed in pancreatic β-cells [40] unless aberrant MCT1 expression occurs, as it happens in gain-of-function mutations of *MCT1* causing inappropriate insulin secretion in the rare disorder "Exercise-induced hypoglycemia and hyperinsulinism" [36]. Interestingly, both fasting plasma lactate and leukocyte mtDNA copy numbers are negatively associated with the oral disposition index (ODI) based on c-peptide measurements across OGTT, which represents an adjusted insulin secretion index by systemic insulin sensitivity. It is worth to mention that different types of ODIs have been previously reported to predict the development of future diabetes above and beyond fasting and 2-h glucose levels [42, 53].

There are strengths and limitations that must be recognised in the interpretation of our results. It is important to consider that our study is mainly composed by a relatively

homogeneous group in terms of age, weight, and glycemic status (non-obese young adult normoglycemic women). Although homogeneity and selection restriction are desirable properties in considering the internal validity of our conclusions, it may affect negatively in terms of representativity of the population in the whole range of values of these variables or disease status. It is also necessary to recognize that discordances in previous studies regarding the association of plasma metabolites/hormones, mtDNA copy number, and insulin secretion/sensitivity indexes are also possibly due to the use of diverse biological samples (blood or other tissues), differences in age, diabetes status (diabetes, pre-diabetes, normoglycemic), or body weight (obese versus non-obese). We did not consider possible insulin-related metabolic fluctuations due to differences in phases of the menstrual cycle in our study. On the other hand, several technical factors may affect mtDNA copy number determination such as co-amplification of nuclear pseudogenes and dilution bias given by differences in genome size (nuclear versus mitochondrial) [14]. We have found in our study a clear association between leukocyte mtDNA copy number and platelet count, indicating that this latter variable is important in determining artifactual elevations in mtDNA copy numbers due to the lack of nuclear DNA in platelets [26]. Regarding the measurement of plasma lactate, another technical limitation relates to possible spurious elevations of such circulating metabolite due to the blood drawing procedure used or the occurrence of recent physical activity episodes [18]. For this reason, volunteers were specifically instructed to avoid physical activity before the analysis. Despite this, plasma lactate measurement at time −5 min was used instead of those obtained at time −15 min before either OGTT or IVGTT (Fig. 1a). In support of this, there were a lower inter-day standard deviation for plasma lactate measurement at time −5 min compared to measurements obtained at time - 15 min before glucose challenges.

In summary, we have found that fasting plasma lactate shows a significant association with a wide range of different insulin sensitivity/resistance indexes based on fasting plasma samples (HOMA-S, adipose IR index, Revised-QUICKI, leptin-adiponectin ratio, TyG index, McAuley index and TG-to-HDL-C ratio), the Matsuda index (and related indexes) from OGTT, and the OGTT-based disposition index. [13]. Special mention deserves the positive association between fasting plasma lactate and the hepatic insulin resistance index based on OGTT. Moreover, fasting plasma lactate was directly associated with circulating proinflammatory adipokines (TNF-α and MCP-1), with an interesting near-significant positive association with ΔGIP during OGTT that also deserves further investigation. We also detected a direct association between fasting plasma lactate with leukocyte mtDNA copy numbers. However, it was not achieved statistical significance between leukocyte mtDNA copy number with insulin sensitivity-related variables after stringent criteria for multiple comparison correction. As a graphical corollary, Fig. 6 shows a diagram with the proposed relations between fasting plasma lactate and leukocyte mtDNA copy numbers in relation to insulin-related variables, and their possible impact in increasing the risk of type 2 diabetes in non-obese, non-diabetic women. The above results support the use of fasting plasma lactate, and possibly leukocyte mtDNA copy numbers, as biomarkers of reduced oxidative mitochondrial capacity, decreased whole-body and hepatic insulin sensitivity, and future increased diabetes risk.

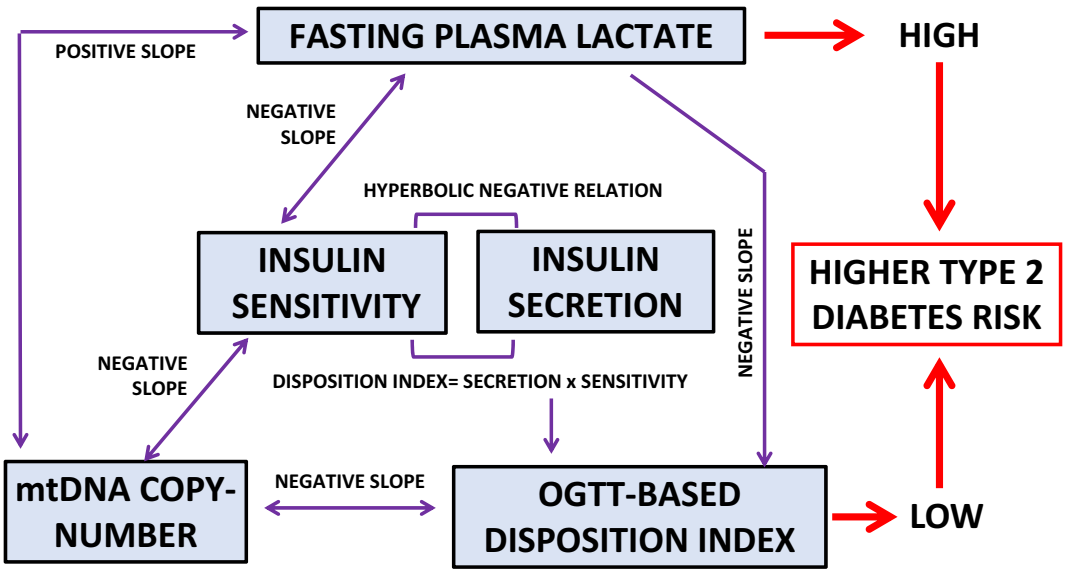

Fig. 6 Proposed relations between fasting plasma lactate, mtDNA copy numbers and insulin-related variables in increasing the risk of type 2 diabetes

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Conflict of interest The authors declare that they have no conflict of interest.

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