Running title: Serum metabolome and sperm parameters

Association of the serum metabolomic profile by nuclear magnetic resonance spectroscopy with sperm parameters: A cross-sectional study of 325 men.

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Capsule: Evaluation of 155 serum metabolites identifies several metabolites with potentially clinically relevant strength of association with the odds of a low total motile sperm count.

Objective: To determine whether 155 circulating metabolic measures relevant to lifestyle and metabolic health are associated with sperm parameters, as measured by concentration, motility and total motile sperm count (TMSC).

Study design: Cross-sectional.

Setting: University Hospital.

Patients: 325 men prospectively recruited between April 1, 2017 and March 31, 2019.

Intervention(s): Non-fasting serum lipids, lipoprotein subclasses, and low-molecular weight metabolites (including amino acids, glycolysis and inflammatory markers) were quantified by NMR spectroscopy. Detailed demographic, lifestyle, fertility and medical history and semen analysis.

Main Outcome Measure(s): Associations of serum metabolic profiles with sperm parameters.

Results: Participants were mean 37.2 (SD 5.7) years, with a median sperm concentration of 35 million/ml (IQR 15, 69) and median motility of 53% (IQR 42,67). 76% of men had a TMSC >15 Million, 10% 5-15 Million and 14% <5 Million. In both univariate and confounder adjusted analyses an extensive range of lipids and lipoproteins, glycolysis related metabolites, amino acids, ketone bodies, creatinine or albumin, did not show strong statistical evidence of associated with sperm concentration, motility, or the odds of having a reduced or low TMSC (all $P_{Bonferroni} > 0.0029$). Higher levels of glycolysis metabolites and ketone bodies were associated with increased odds of TMSC <15M compared with ≥15M (odds ratios of ~1.2 to 1.3), and several lipids/lipoprotein concentrations appeared to protect against very low TMSC (<5M compared with ≥5M) with odds ratios of ~0.8 or greater.

Conclusion: Several metabolites exhibit potentially clinically relevant strength of association with the odds of a low TMSC and warrant replication.

Keywords

semen analysis, sperm, metabolomics

Introduction

Despite increasing concern regarding the significant decline in sperm counts over the last few decades (1), therapeutic options for male infertility are limited (2). The significant body of data to support the concept that oxidative stress (3) and also potentially metabolic dysfunction (4) are associated with sperm dysfunction, have driven recent intervention trials to primarily focus on preconceptual optimisation of health (2, 5, 6).

Robust identification of additional modifiable and non-modifiable risk factors for poor semen quality have however had limited success. For example smoking (7, 8), adiposity (7, 9-12) and alcohol excess (7, 13, 14) have all shown non-consistent associations with the risk of poor semen parameters. Consequently, advice is primarily based on a "good health advice" model, rather than robust evidence for a detrimental effect on semen parameters (15). An alternative approach to identify risk factors for an abnormal semen analysis would be to examine circulating metabolites, which may provide insights into downstream factors as well as more upstream exposures (16). Detailed metabolic profiling has previously been used to explore the metabolic pathways that may underpin phenotypes, including adiposity (17), and has been widely used in epidemiological studies to identify novel risk factors for a variety of reproductive and pregnancy characteristics (18-22). The application of NMR spectroscopy to male infertility has however been limited to primarily small studies (n =29 to 80) which have assessed selected metabolites within the sperm metabolome (23-25) or seminal fluid (26-29). Alternative metabolomic approaches (liquid chromatography coupled to mass spectrometry (LC-MS) or high resolution mass spectrometry (LC-HRMS) or gas chromatography-mass spectrometry (GC-MS)) have been undertaken on plasma, serum, seminal plasma and urine, but studies have been of varying size (n=30 to 260), primarily used a case-control design with no adjustment for confounders, have variable thresholds for defining cases and have utilised

conventional discovery based metabolomic approaches that have not provided absolute quantities (30-33).

The aim of the current study was to undertake an exploratory analysis of the association of 155 circulating metabolic measures with sperm parameters. These measures were profiled by a high throughput cost efficient NMR platform, covering a range of metabolic pathways, predominantly a lipidome, including lipoprotein lipids, fatty acids, as well as some amino acids, ketone bodies, and glycaemic traits.

Material and methods

Study Design and Participants

Cross-sectional study of men aged 18 to 55 who presented to Glasgow Royal Infirmary, UK between 1 April 2017 and 31 March 2019 for assessment prior to assisted conception. A total of 326 men were recruited and of these 325 (99%) had complete data on semen parameters and were included in the analyses presented in this paper.

The study was conducted according to ICH Guideline for good clinical practice, the Declaration of Helsinki and the Convention of the Council of Europe. All men provided written informed consent. The study protocol was approved prior to study initiation by the relevant institutional review boards (see Supplementary Material).

Study procedures:

Demographic, lifestyle, fertility and medical history was obtained by self-reported questionnaire and clinical data by linkage to electronic medical records. Blood samples were taken for NMR analyses and immediately spun and frozen at −80 °C. All NMR assays completed for this study were undertaken within 1 year of storage and with no previous freeze/thaw cycles.

Semen analysis:

The semen sample was provided in the morning of the same day as the blood sample for NMR analyses. Semen analysis was completed utilizing the World Health Organisation guidelines (34). The men were advised to produce a semen sample after 2–3 days of sexual abstinence. The semen sample was collected in the hospital or at home in the morning (instructions to produce sample within one hour of semen analyses appointment which were

at 30 minute intervals from 0800 to 1000) of the same day as the blood sample for NMR analyses were taken (with blood samples taken between 1330 and 1600). Giving a range of 3.5 hours to 8 hours between ejaculation and the blood sample for NMR analysis. Semen volume was measured using a graded tube. The concentration was measured in an Improved Neubauer chamber in two replicates and at least 200 spermatozoa counted in each replicate, at a magnification of 200×. Where the difference between the counts was greater than acceptable based on the 95% confidence intervals, the first two values were discarded, and two fresh dilutions of semen prepared and assessed. Motility was scored manually, as percentages of (A) fast forward progressive, (B) slow forward progressive, (C) non-progressive and (D) immotile spermatozoa in 200 spermatozoa in at least five power fields per replicate, according to recommendations (34). The Glasgow Royal Infirmary clinic from which study participants were recruited, collaborates in a nationwide quality control system organized by the UK National External Quality Assessment Scheme (NEQAS). For those men where the initial and repeat sample exhibited azoospermia a surgical sperm retrieval was performed, and the sample evaluated prior to cryopreservation.

TMSC groups

The men were grouped according to the TMSC, calculated by multiplying the sample volume by the concentration and the percentage of A and B motility divided by 100%. Three groups were created: men with TMSC > 15 million (M); men with a TMSC of 5 to 15M; and men with TMSC of <5M (including those with azoospermia) (35-37)

NMR protocol

Profiling of 155 lipid and metabolite measures was performed by a high-throughput targeted NMR platform (Nightingale Health© (Helsinki, Finland)) at the University of Bristol. The

platform applies a single experimental setup, which allows for the simultaneous quantification of routine lipids, 14 lipoprotein subclasses and individual lipids transported by these particles, multiple fatty acids, glucose, various glycolysis precursors, ketone bodies, and amino acids in absolute concentration units. The NMR-based metabolite quantification is achieved through measurements of three molecular windows from each serum sample. Two of the spectra (LIPO and LMWM windows) are acquired from native serum and one spectrum from serum lipid extracts (LIPID window). The NMR spectra were measured using Bruker AVANCE III spectrometer operating at 600 MHz. Measurements of native serum samples and serum lipid extracts are conducted at 37^{O}C and 22^{O}C, respectively. Details of this platform have been published previously (16, 38, 39) and it has been widely applied in genetic and observational epidemiological studies (18, 19, 40-44). Further details of the platform are provided in the Supplemental Material (Text box; Table S1 and Figure S1).

Metabolite quantification and quality control

The NMR spectra were analysed for absolute metabolite quantification (molar concentration) in an automated fashion. For each metabolite, a ridge regression model was applied for quantification in order to overcome the problems of heavily overlapping spectral data. In the case of the lipoprotein lipid data, quantification models were calibrated using high performance liquid chromatography methods, and individually cross-validated against NMR-independent lipid data. Low-molecular-weight metabolites, as well as lipid extract measures, were quantified as mmol/l based on regression modelling calibrated against a set of manually fitted metabolite measures. The calibration data are quantified based on iterative line-shape fitting analysis using PERCH NMR software (PERCH Solutions Ltd., Kuopio, Finland). Absolute quantification cannot be directly established for the lipid extract measures due to

experimental variation in the lipid extraction protocol. Therefore, serum extract metabolites are scaled via the total cholesterol as quantified from the native serum LIPO spectrum.

With respect to controls, samples were analysed in batches of 96 containing two control samples; a pooled serum control and a synthetic control. Aliquots of these same controls were used for the whole analysis, as well as other analyses carried out in the same time frame. This allowed consistency of results to be monitored to check that no batch of sample had degraded overly compared to others, sample preparation was consistent and that the NMR instruments produced stable results.

Assessment of potential confounders

Age, BMI, educational attainment, ethnicity, family history of cardiovascular disease (defined as first degree relative affected), physical activity, alcohol intake, smoking status, and duration of infertility were considered as potential confounders because they plausibly influence both semen parameters and the NMR metabolites (45). All confounders were obtained by a questionnaire or from medical notes when the men were originally recruited.

Statistical analysis

All analyses were conducted using Stata (Version 15.1) and R version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria). An analysis plan was written in June 2019. Characteristics were summarized as number and percentage, total range, mean, standard deviation, median, and 25th and 75th quantiles (IQR) as appropriate. Multivariable linear regression was used to examine the associations of serum metabolic profiles (treated as exposures) with % progressive motility and sperm concentration (treated as outcomes). Robust standard errors were estimated for associations with continuous sperm outcomes. The

metabolic measures were scaled to standard deviation (SD) units (by subtracting the mean and dividing by the standard deviation of all men included in the analyses). This scaling allows easy comparison of multiple metabolic measures with different units or with large differences in their concentration distributions. Sperm concentration (natural log transformed) and progressive motility were also scaled to SD units in the same way as the metabolites. Multivariable logistic regression was used to examine the associations of the serum metabolic profiles (treated as exposures) with TMSC. Two analyses were undertaken: (i) exploring the odds of having a low TMSC (<15M) compared to a normal TMSC (i.e. >15M) and (ii) exploring the odds of having a very low TMSC (<5M) compared to a low or normal TMSC (i.e. ≥5M). We adjusted for all a priori selected confounders (age, ethnicity, education, family history of cardiovascular disease, BMI, physical activity, alcohol, smoking, and duration of infertility) in both the multivariable linear and logistic regression analyses.

Additional sensitivity analyses

In addition to presenting our main results as difference in mean metabolite in SD units per SD of concentration or motility, we also present the full results (confounder adjusted) in the metabolite, concentration and motility original units in Supplementary Material (Supplemental Tables 5 and 6). We repeated our main analyses on those men with a known male factor cause of infertility (N=66 (20% of the cohort)) and compared these associations to those men with an unknown or known female partner cause of infertility (N=258 (79%)) (Supplemental Figures 5 to 10). We wished to compare the point estimates in this group to the results of the whole cohort to provide some indication as to whether our results might be driven by the cause of infertility or potentially generalisable to men of reproductive age without infertility. We compared the magnitudes of the results in men who were going to undergo assisted conception because of the couple having unexplained or a known female

cause of infertility with those of the whole cohort using a scatterplot. As 99% of the men recruited had full data on semen parameters and at least one metabolite, with <1% of these having missing covariable data (see Table 1) we did not need to undertake any additional analyses to explore potential biases due to missing data.

Accounting for multiple testing

Due to the correlated nature of the metabolic biomarkers, over 95% of the variation in the 155 metabolic biomarkers was explained by 15 principal components. Therefore, multiple testing correction, accounting for 15 independent tests using the Bonferroni method, resulted in $P_{Bonferroni} < 0.0033$ (0.05/15) being denoted as statistically significant.

Results

Three hundred and twenty-five men (99% of the 326 recruited) with available semen analysis and metabolomics evaluation were included in the study. Characteristics of the participants are shown in Table 1. Mean (SD) age of the men was 37.2 (5.7) years with a mean (SD) BMI of 25.2 (3) Kg/m^2. The majority (92%) were white European, with over 50% having a university degree, 30% being ever smokers, 69% reporting exercising equal to or more than 3-4 times per week, 47% having a family history of cardiovascular disease and median alcohol consumption being 4 units per week. For 54% of the men the cause of them and their partner requiring referral for assisted conception was unexplained and for 26% the cause was related to their female partner; the source of requiring assisted conception was deemed due to sperm / male causes in 20% of the men included in this study. Sample production was by masturbation for 98%, with 6 men requiring surgical sperm retrieval. The median sperm concentration was 35 Million per ml (IQR 15, 69 Million per ml) and median progressive motility was 53% (IQR 42, 67 %) in the whole sample. These differed by cause of infertility, with the median sperm concentration in those with a male cause being 12 Million per ml

(IQR 1.4, 23 Million per ml) and the median progressive motility being 32.5% (IQR 15,58) and equivalent values in men where the cause of infertility was unknown or a known cause in their female partner being 42 Million per ml (IQR 23, 84) and 55% (IQR 46, 68).

The unadjusted associations of sperm concentration, motility and TMSC with confounders are shown in Supplemental Tables 2, 3, and 4 respectively. With the exception of established male factor infertility having a negative association with sperm concentration, motility and TMSC there was no clear evidence for associations of baseline demographic or lifestyle characteristics, including BMI, with semen parameters.

The adjusted associations between sperm concentration and progressive motility and the respective metabolomics measures are presented in Figure 1. With the unadjusted analyses for sperm concentration and progressive motility presented in Supplemental Figures S2. Histidine was the only metabolite with Bonferroni corrected statistical evidence of an association with motility in the unadjusted analyses and there was no statistical evidence of any metabolites associating with sperm concentration. Whilst all of the associations were imprecisely estimated (i.e. with wide confidence intervals), most of the point estimates for the lipids were very close to the null, except for very large, large and medium HDL particle concentrations and apolipoprotein A-1 which had inverse associations with point estimates of ~ -0.1SD with sperm motility per 1SD higher lipid. Creatinine also has a point estimate of 0.1SD, showing a positive association with sperm motility, though again with wide confidence intervals and not reaching the Bonferroni corrected statistical significance. In results adjusted for confounders (Figure 1) the associations showed very little difference to the unadjusted results. The adjusted association for histidine with motility was 0.12 SD per 1 SD higher histidine concentration (95%CI: 0.02, 0.22), though the p-value was slightly higher

than the Bonferroni corrected value at 0.02. In contrast, lactate showed a negative association with motility (-0.11 SD in sperm motility (95%CI -0.23, 0.01) per 1 SD increase in lactate concentration) but no association with concentration.

Analyses of the associations of metabolites with odds of a low TMSC compared with a healthy count (Figure 2) and of odds of a very low TMSC compared to either a low or healthy count (Figure 3) did not have statistical (Bonferroni corrected) support for any associations, though these analyses have less power than with the continuously measured outcomes and wide confidence intervals. Point estimates for adjusted associations were close to the null for all lipids, lipoproteins and fatty acids. For glucose, lactate, pyruvate, both ketone bodies and glycoprotein acetyls point estimates suggested positive associations with reduced compared to a normal TMSC with odds ratios of ~1.2 and for glycerol a positive association with an odds ratio of ~1.3. There was also a point estimate of 0.8 for creatinine with low versus normal TMSC. When comparing TMSC of <5 million to ≥ 5 million the adjusted associations for most very small VLDL, IDL and large, medium and small LDL concentrations, and some of the cholesterols and triglycerides and phospholipids had point estimates of ~0.8 suggesting potentially important reduced odds of a very low sperm count with higher levels of these lipids but for which we had limited power to obtain precise estimates that excluded the null (Figure 3). Degree of fatty acid unsaturation had a point estimate OR of ~0.6 in these analyses and some other metabolites were associated with very low TMSC with OR suggestive of a relative increase or decrease in odds of 20% or more.

When analyses were repeated only in those undergoing assisted conception for male factor (n=66) as opposed to unexplained or female cause of infertility (n=258), most of the 155 associations were broadly consistent, with a tendency for those with male factor associations

to be stronger for both concentration and motility (Figures S3 and S4). In those with male factor infertility there was evidence of higher phenylalanine associating with higher sperm concentration and motility (0.44 SD (95%CI 0.09, 0.80) per 1 SD for both outcomes) (Figures S3 and S4). Comparing all point estimates across all 155 metabolites between those with male factor infertility and those with female or unknown causes showed poor consistency between the two groups (Figure 4; goodness of fit statistic $R^2 = 0.1$ for sperm concentration and 0.03 for motility). Indeed most of the associations with sperm concentration were in the opposite directions between the two groups (slope = -0.92 (95% CI –0.48, 1.36)), including for associations of phenylalanine and creatinine which associated positively with concentration in those with male cause infertility and weakly inversely in those without. For sperm motility many associations were also directionally inconsistent (weak positive slope 0.35 (95% CI 0.004, 0.70)); associations of both phenylalanine and creatinine were positively associated with motility, with a stronger association in those with male cause fertility than those without (Figure 4). Results were unchanged when the potential effects of seasons were considered.

Discussion

In this cross-sectional exploratory study of men attending a fertility clinic for evaluation we did not identify any associations between circulating metabolites and sperm concentration, sperm progressive motility or the risk of a low or very low TMSC on the basis of reaching conventional 5% levels of statistical significance after Bonferroni correction for multiple testing. However, point estimates suggested some potentially clinically important associations. Higher levels of glycolysis metabolites and ketone bodies were associated with increased odds of TMSC <15M compared with ≥15M (odds ratios of ~1.2 to 1.3), and several lipids/lipoprotein concentrations appeared to protect against very low TMSC (<5M compared with ≥5M) with odds ratios of ~0.8 or greater. That this study is unique in exploring these associations highlights the importance of funding to undertake larger studies that could precisely estimate associations and provide more robust evidence of how large any associations are likely to be. Based on a post-hoc power calculation we estimate that a sample size of 3,188 would be required at the Bonferroni corrected p-value used in this study to detect a 20% relative difference in odds (i.e. OR of 0.8 or 1.2) or larger with 80% power.

A potential role for lipids and semen parameters is plausible as cholesterol is the precursor of steroid hormones, including testosterone which is produced in the Leydig cells (46), testicular cholesterol has been shown contribute to normal spermatogenesis (47) and cholesterol-fed rats and rabbits showed reduced spermatid cell numbers, reduced seminiferous tubules' diameters, and smaller Leydig cell nuclear dimensions (48). However, the association of serum lipids and semen parameters has been inconsistent, with some (49) but not all studies (47, 50) finding an inverse association with lipids and sperm concentration and / or motility. The LIFE study (n=501) which quantified 35 different semen parameters, found that total and free cholesterol and phospholipid concentrations were negatively associated with several

sperm head morphology defects but not with concentration or motility (51). In the largest study to date of healthy individuals (n= 7,920) total cholesterol level was positively correlated with total sperm motility and progressive motility, but there was no association observed for HDL, LDL, Triglyceride or VLDL with concentration or motility (52). A similarly large study, with a comprehensive evaluation of the lipoprotein spectrum as per the current study combined with the extensive assessment of semen parameters as per the LIFE study would be useful. Further clarification of whether lipoproteins levels in the male reproductive tract are regulated locally as per the rat (53), and not through passive diffusion from serum would also be useful.

We observed a weak negative association of increasing lactate concentrations with reduced motility. Human sperm have been shown to be capable of metabolising 13C labelled glucose and pyruvate to lactate via lactate dehydrogenase (54), and that lactate oxidation by lactate dehydrogenase isoenzymes has a significant role in energy metabolism during the middle and later stages of spermatogenesis (55). Lactate has been shown to have an inhibitory effect on sperm motility, in part by acidification of the cytosol (56, 57), but also it has been suggested that lactate may inhibit the binding site of lactate dehydrogenase (56). It is possible that export of lactate from sperm leads to acidification of the extracellular medium and contributes to a reduction in sperm motility. Confirmation that circulating lactate concentrations may also have a negative association on sperm motility warrants replication given that lactate concentrations increase during acute illness, and other systemic insults but also during strenuous exercise (58).

The impact of supplementation with omega-3 fatty acids on semen parameters has been assessed in two small (n=74 and 138) randomised controlled trials (59). Supplementation

with docosahexaenoic (DHA) and eicosapentaenoic acids (EPA) (990 mg/d and 135 mg/d, respectively) for 10 weeks demonstrated no effect on sperm parameters but improved sperm DNA fragmentation (60). Although supplementation with higher amounts of DHA + EPA (0.72 g/d and 1.12 g/d, respectively) led to significant improvements in total sperm count and concentration, sperm motility, and morphology (61). However, we were unable to detect any robust association with either omega-3 or omega-6 fatty acids and sperm parameters; for these exposures all point estimates were close to the null (thought the degree of saturation of fatty acids did have a point estimate odds ratio of ~0.8 for TMSC< 5M compared with ≥5M). Similarly, although a variety of amino acids, including arginine and cysteine are frequently incorporated into anti-oxidant preparations and, have been suggested as a means of improving semen parameters and potentially live-birth rates (62), neither branched chain or aromatic amino acids were associated with semen parameters. Replication the association of histidine with motility would be useful, as it has been suggested that at ejaculation sperm chromatin is critically stabilized by salt bridges in which zinc interchelates between thiols and possibly the imidazole groups of histidine (63). Low phenylalanine concentrations in seminal plasma have previously been reported for oligospermic males (64), however whether supplementation is beneficial is unknown.

We did not observe any association with baseline demographics, including adiposity or alcohol intake and semen parameters. Previous larger population studies and meta-analyses have differed in their findings with respect to whether there is or is not an inverse association between adiposity and sperm concentration and motility (7, 9-12). Whether this reflects that BMI is a relatively crude marker for adiposity, although it is strongly related to other health outcomes including mortality (65), or whether metabolic health rather than adiposity per se underlies associations with semen parameter as recently suggested (66), is unclear. However,

the NMR platform used in the current study has previously detected differences for a range of metabolic disorders including adiposity (17), suggesting that adiposity or mild deviation of metabolic health may have a limited impact on crude semen parameters such as concentration and motility. Similarly, we did not observe a detrimental effect of alcohol on semen parameters, consistent with previous meta-analyses which have suggested that a detrimental effect of alcohol seemed to be limited to daily drinkers, whereas occasional drinkers or low intake as per our participants were apparently similar to never drinkers (67).

While our findings are of interest, we acknowledge some important limitations, the relatively small sample size, and lack of a replication cohort which is why we present the novel analyses we have undertaken as exploratory. As noted above we are unable to exclude some potentially clinically important associations and have estimated that a sample size of at least 3,188 would be required to obtain more precise and statistically robust results. Our cohort comprised of men presenting to a tertiary infertility service which on average are older than the mean age of fathers in the UK during this time period, irrespective of whether this was for becoming a farther to their, first, second or other child (37 vs 34 years) and may not be generalisable to men at the younger (e.g. < 20 years) or older (e.g. > 60 years) extremes of the reproductive lifespan. We included some men with extremely low sperm counts, azoospermia and surgical sperm retrieval and those with known female factor infertility and although we assessed whether the observed associations differed depending on the cause of infertility, our findings may not be generalisable to healthy individuals. Furthermore, that men were confirmed (through cotinine breath test) as non-smokers, were of a relatively restricted BMI range and largely white European and educated to degree level may have introduced a selection bias which have attenuated our results towards the null. A single semen sample was assessed by a single trained operator, consistent with recent guidance that

the intraclass coefficient of the parameters assessed is adequate to enable a single sample to be used for infertility referral and treatment (2). We used the TMSC thresholds derived by Tiegs and colleagues (37), as that study assessed 119,972 semen samples, rather than the 20 million threshold described by others in much smaller studies (35, 36). We did not assess other aspects of sperm function including sperm DNA fragmentation, however, at present there is no consensus on the optimal assay methodology or its contribution to prognosis. We acknowledge that at present it is unknown whether ejaculation (whether during sex or masturbation) alters blood metabolites levels, and future studies of paired pre and post ejaculation blood samples would be useful to clarify this. We also acknowledge that the metabolic composition of seminal fluid may have influenced sperm function, with several studies of seminal fluid suggesting associations of metabolites with semen parameters (26-29). Similarly repeat metabolite measures across the window of spermatogenesis may further inform a potential role of specific metabolites. Lastly, serum metabolites were measured on the day of the semen sample and therefore may not reflect the entire spermatogenic cycle, however, previous analyses of short- and long-term repeat ^{1}H NMR measures (<6 months and >3 years) have shown good intra-class coefficients supporting that they would be representative of exposures of the spermatogenic cycle (39).

Conclusions

This study provides preliminary data on a range of metabolic pathways and their association with semen parameters. While we found most point estimates to be close to the null and few associations that met conventional statistical thresholds after adjustment for multiple testing, we identified several metabolites that associated with odds of TMSC with potentially of important clinical effect sizes (differences in relative odds of 20%) which warrant further exploration and replication in a larger prospective study.

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Competing interests

No funding bodies had any role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. SMN has participated in Advisory Boards and received speakers or consultancy fees from Access Fertility, Beckman Coulter, Ferring, Finox, Merck, MSD, Roche Diagnostics and The Fertility Partnership. DAL has received grant funding for other studies not related to this one from government, charity and industry funders, including Roche Diagnostics and Medtronic.

Data availability

The data underlying this article will be shared on request to the corresponding author.

Author contributions

KA and SMN designed the study. KA recruited the cohort. KA, AT, DAL and SMN wrote the analysis plan and AT analysed the data, with NG producing the figures. SMN, DA, KA and AT drafted the initial manuscript. All authors contributed to data interpretation, critical revision, and final approval of the submitted manuscript.

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Figure 1. Associations of metabolites with sperm concentration and progressive motility in men undertaking assisted conception

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm concentration (red) and progressive motility (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity and duration of infertility.

Figure 2. Associations of metabolites with odds of having a TMSC less than 15 Million compared with greater or equal to 15 million.

Odds ratio for having a total motile sperm count less than 15 Million and respective 95% confidence intervals for each metabolic trait. Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity and duration of infertility.

Figure 3. Associations of metabolites with odds of having a TMSC less than 5 Million compared with 5 million or more.

Odds ratio for having a total motile sperm count less than 5 Million compared with 5 million or more and respective 95% confidence intervals for each metabolic trait. Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity and duration of infertility.

Figure 4: Scatterplot of associations between sperm concentration and motility and metabolites in those males with female or unknown cause of infertility (N = 398) and restricted to males with a reported male partner cause of infertility (N = 66).

Figure 4a shows associations with concentration by cause of infertility, Figure 4b shows associations with motility by cause of infertility. The green dots highlight the individual metabolites, with phenylalanine and creatinine highlighted. The grey dashed line reflects the reference line (slope 1, intercept 0) and the red dashed line is the best line of fit.

Supplemental Figure S1: Stages and methods used for NMR platform metabolic measures (adapted from Wurtz et al.(68))

Supplemental Figure S2. Association of metabolite levels with sperm concentration and motility (unadjusted)

Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm motility (red) and concentration (black).

Supplemental Figure S3. Association of metabolite levels with sperm concentration by cause of infertility

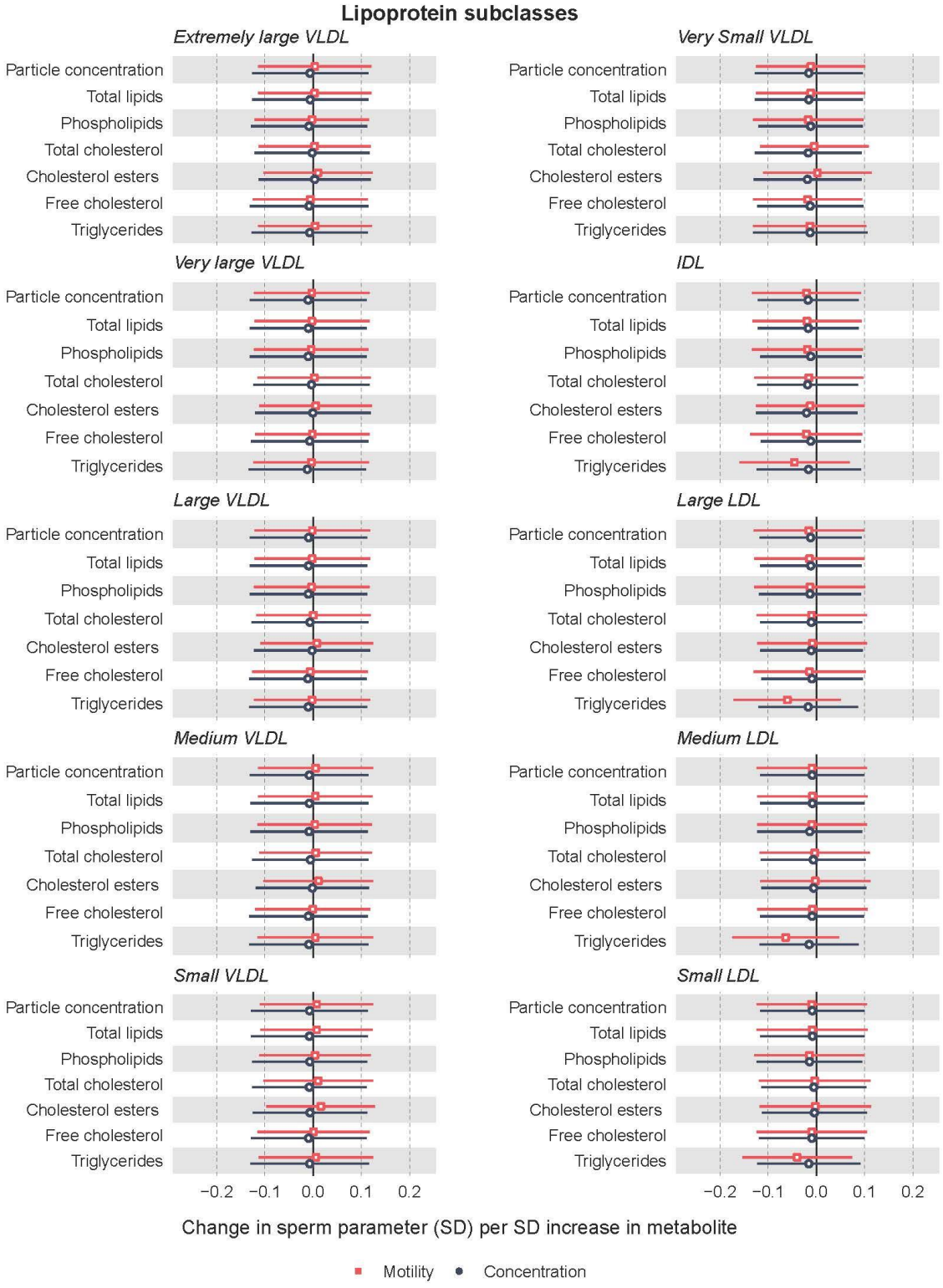
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm motility concentration for male factor infertility (red) and female / unexplained infertility (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, and primary/secondary infertility.

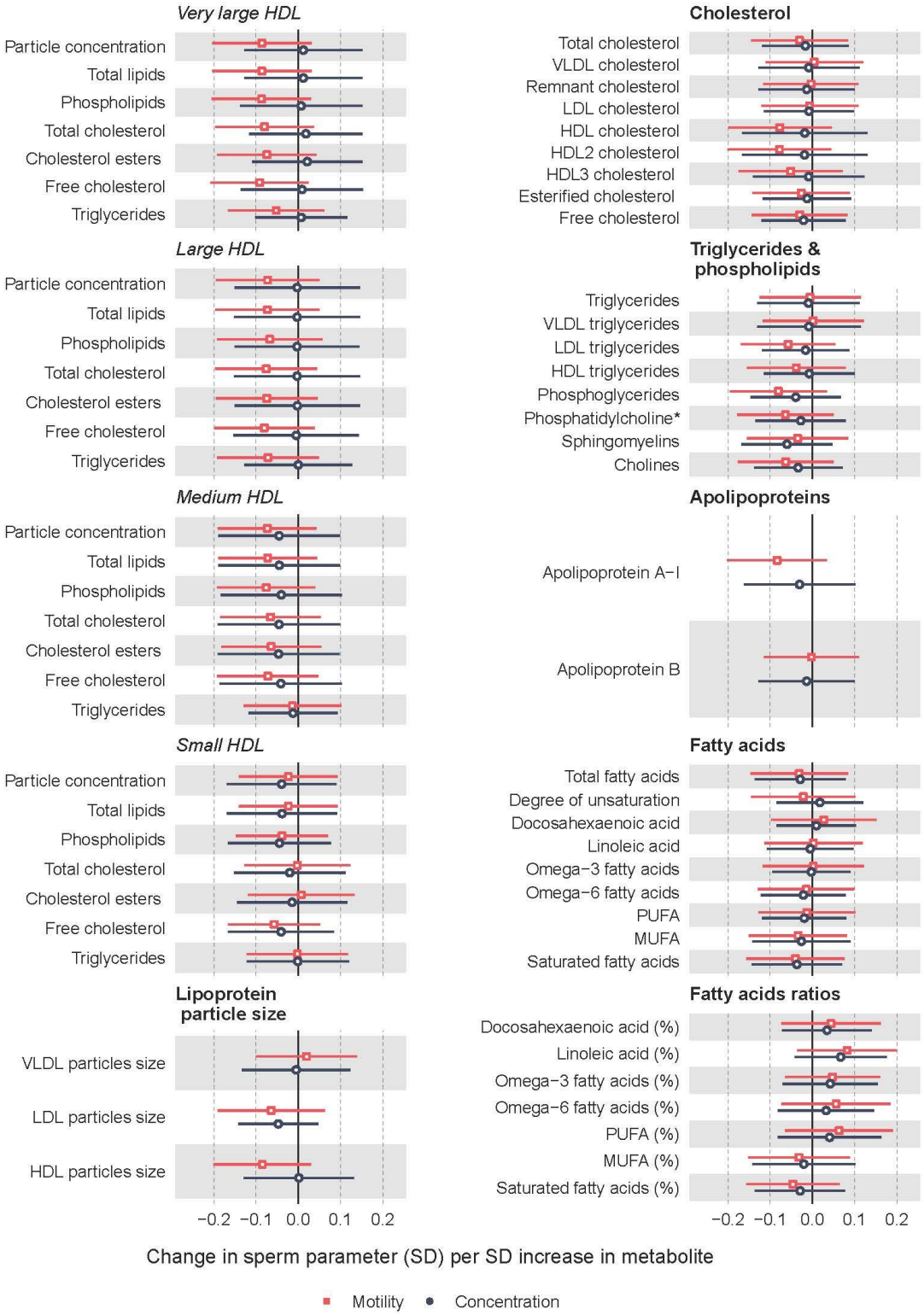
Supplemental Figure S4. Association of metabolite levels with sperm motility by cause of infertility

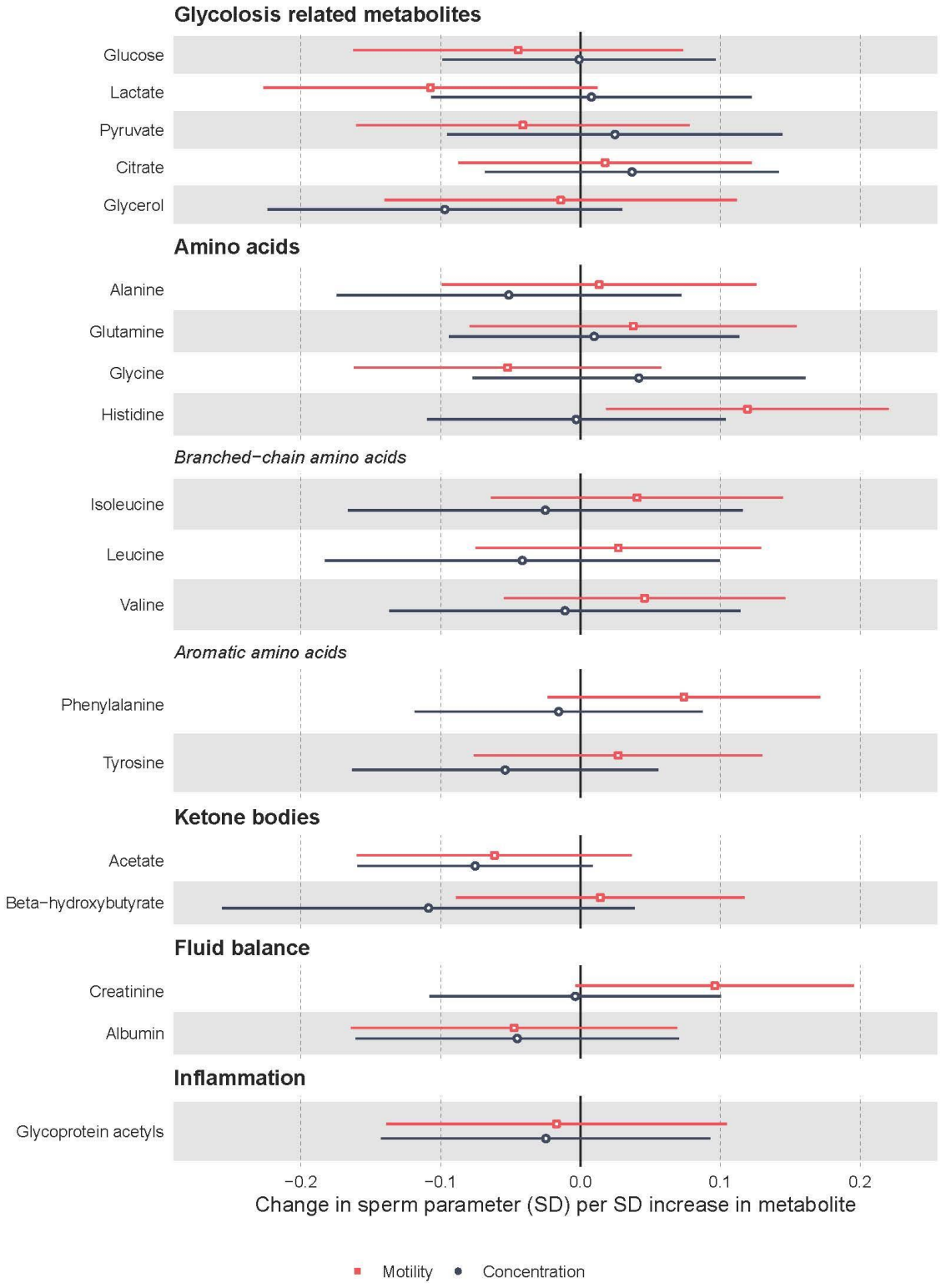
Effect sizes per 1 SD in metabolite concentrations and respective 95% confidence intervals are shown for sperm concentration for male factor infertility (red) and female / unexplained infertility (black). Adjusted for age, education, family history of CVD, BMI, physical activity, alcohol (units per week), ever smoking, ethnicity, duration of infertility, and primary/secondary infertility.

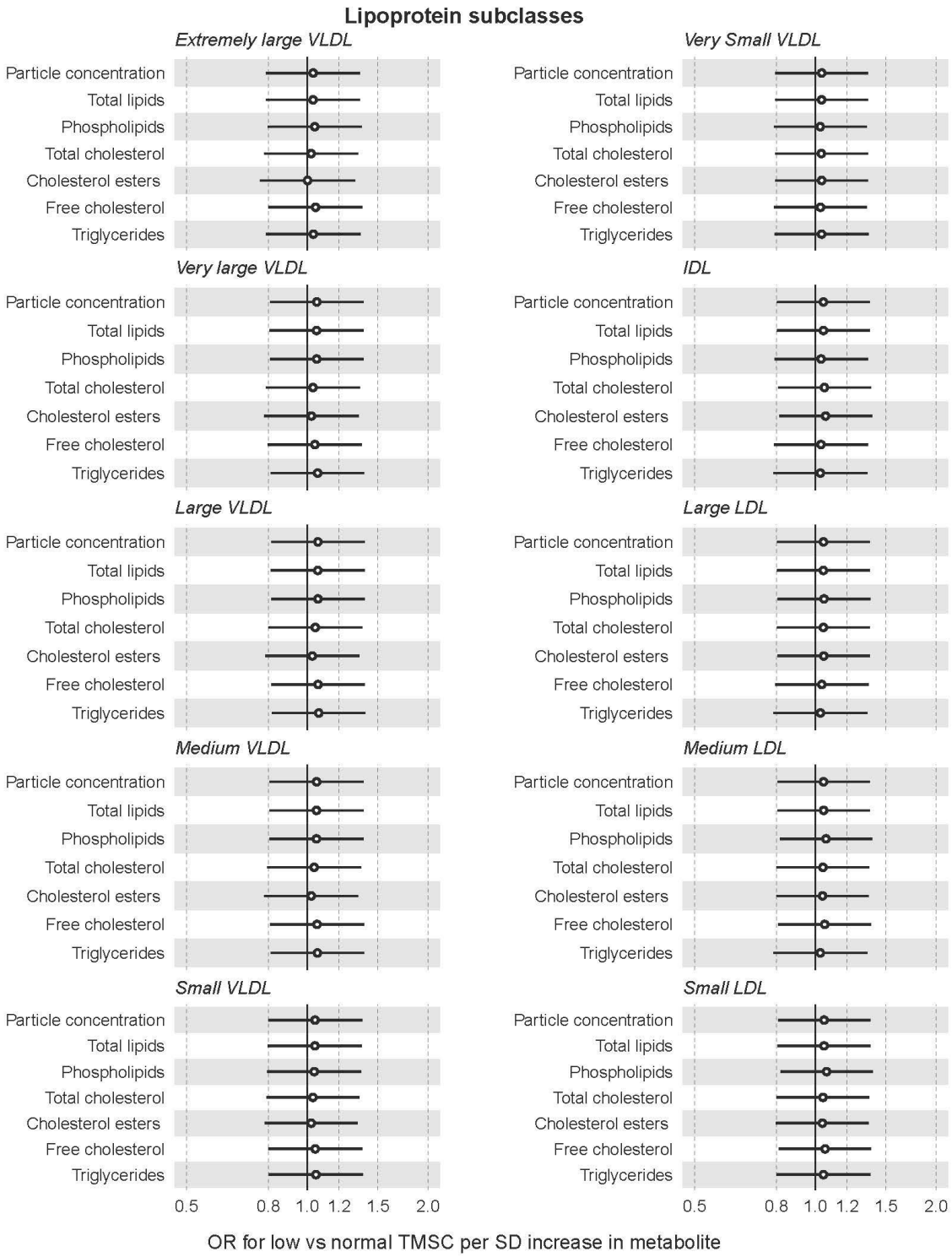
Table 1: Baseline characteristics of the study population (N=325)

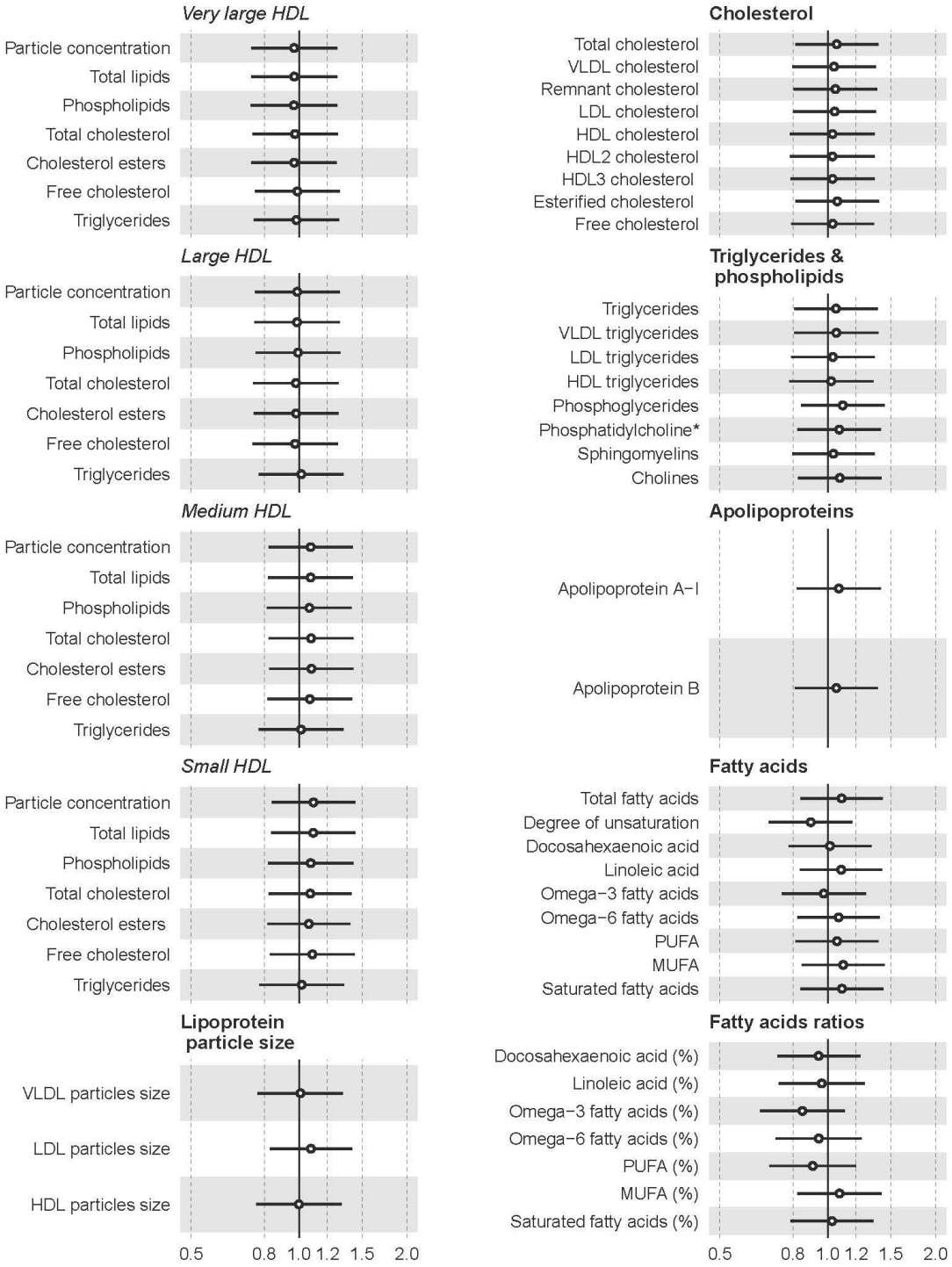
Age (years): Mean, SD, Range	37.2 (5.7) 25-55
Ethnicity: N(%)	
White	299 (92%)
Asian	19 (6%)
Other	7 (2%)
Ever smoked: N(%)	97 (30%)
Alcohol (units per week) : Median, IQR, Range	4 (2,9) 0-40
BMI: Mean, SD, Range	25.2 (3.0) 19.4-35.1
Highest Education N(%)	
School	147 (45%)
Undergraduate	138 (42%)
Postgraduate	40 (12%)
Physical activity (times per week) N(%)	
Never/Once	29 (9%)
Twice	73 (22%)
3-4 times	176 (54%)
5-7 times	35 (11%)
7+ times	12 (4%)
Family history of cardiometabolic disease: N(%)	153 (47%)
Cause of infertility (missing data N=1)	
Azoospermia	11 (3%)
Oligozoospermia	55 (17%)
Female factor	84 (26%)
Other/Unexplained/Avoidance of genetic disorder	174 (54%)
Sperm production method	
Masturbation	319 (98%)
MESA/TESA/Other	6 (2%)
Total motile sperm count	
< 5 Million	45 (14%)
5- 15 Million	33 (10%)
>15 Million	247 (76%)
% Motile : Median, IQR, Range	53 (42,67) 0-91
Sperm concentration (M per ml): Median, IQR, Range	35 (15,69) 0-230

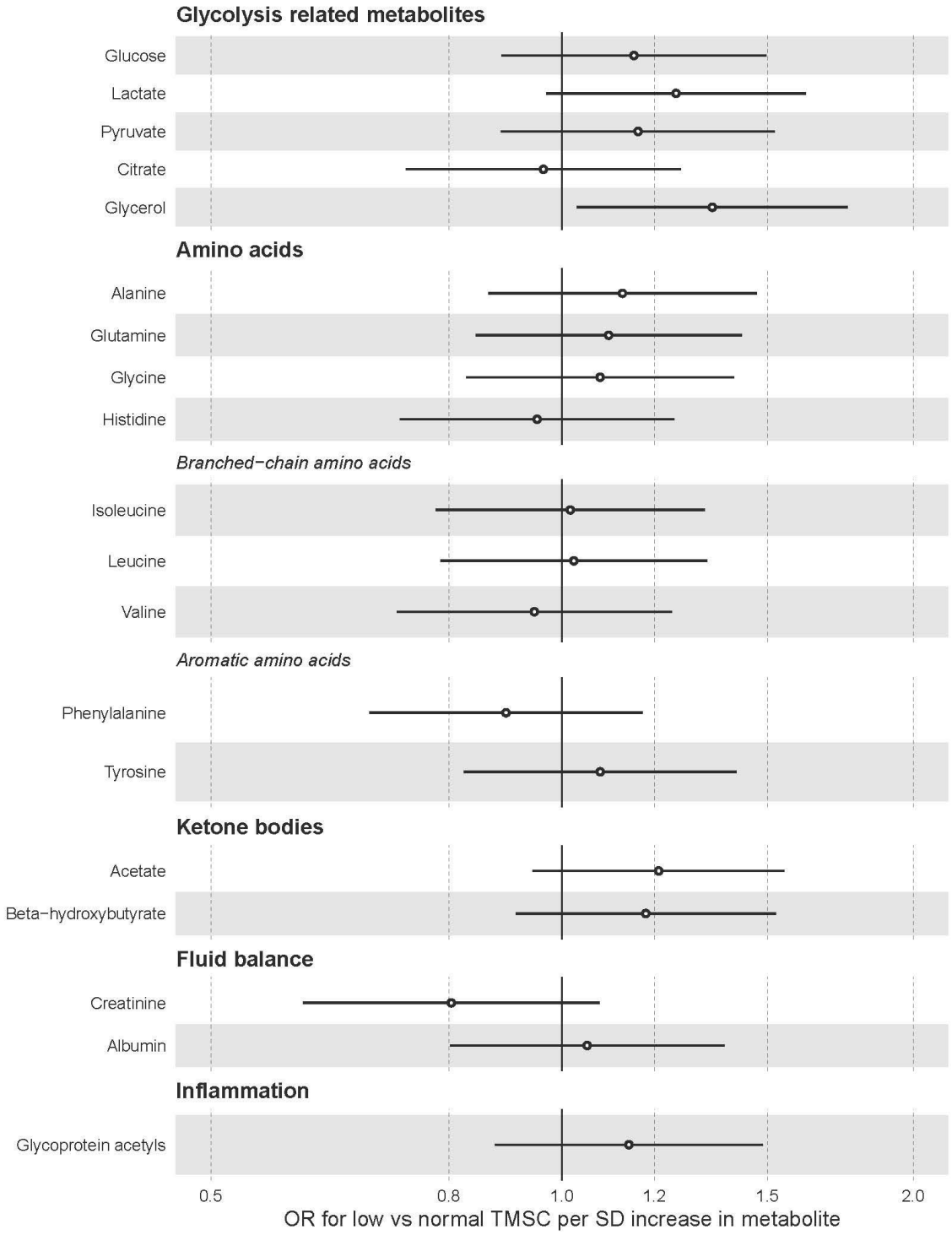

Lipoprotein subclasses
Extremely large VLDL
Very Small VLDL
Very large VLDL
IDL
Large VLDL
Large LDL
Medium VLDL
Medium LDL
Small VLDL
Small LDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
−0.2
−0.1
0.0
0.1
0.2
Change in sperm parameter (SD) per SD increase in metabolite
Motility
Concentration

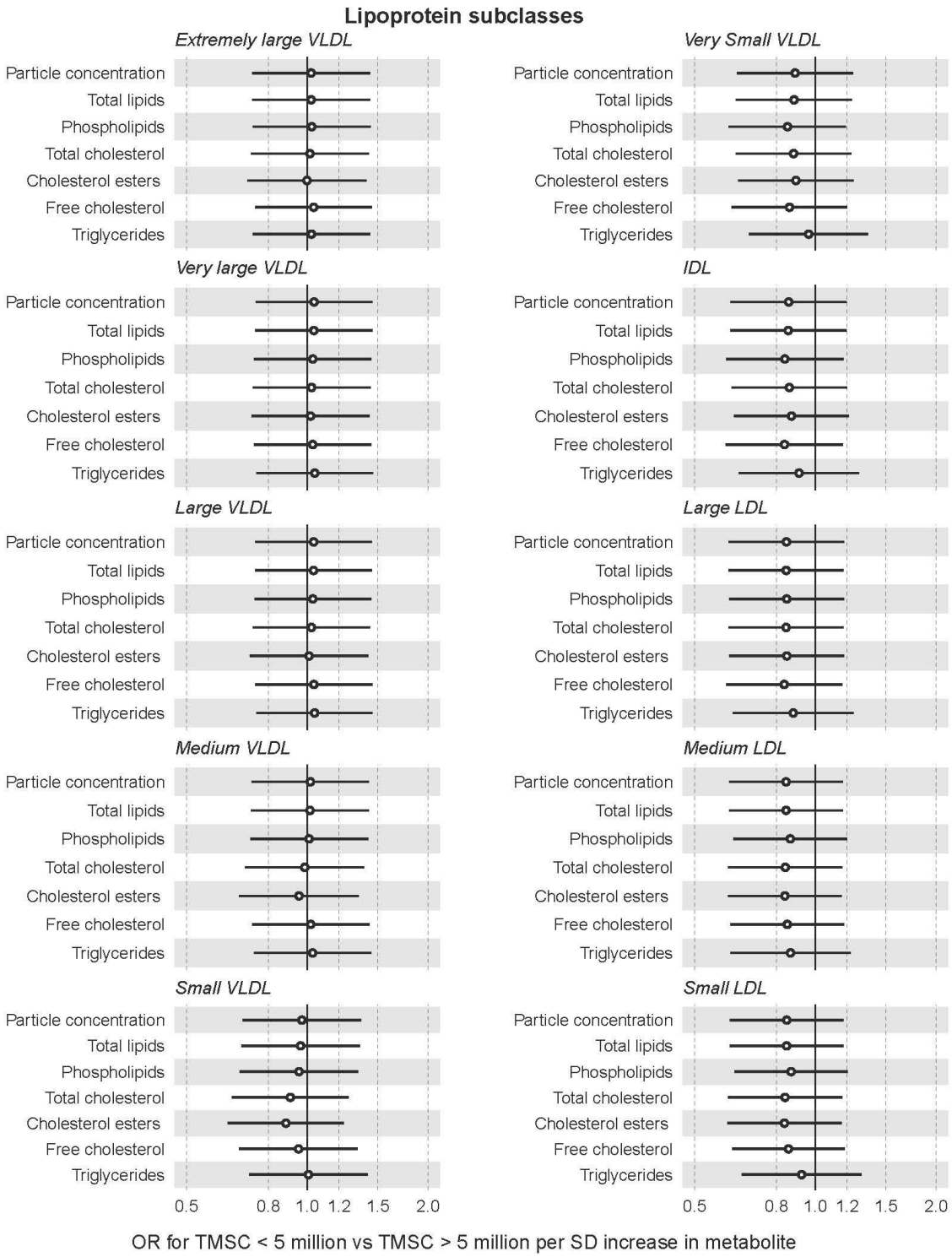

Very large HDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
Large HDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
Medium HDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
Small HDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
Lipoprotein particle size
VLDL particles size
LDL particles size
HDL particles size
−0.2 −0.1 0.0 0.1 0.2
Cholesterol
Total cholesterol
VLDL cholesterol
Remnant cholesterol
LDL cholesterol
HDL cholesterol
HDL2 cholesterol
HDL3 cholesterol
Esterified cholesterol
Free cholesterol
Triglycerides & phospholipids
Triglycerides
VLDL triglycerides
LDL triglycerides
HDL triglycerides
Phosphoglycerides
Phosphatidylcholine*
Sphingomyelins
Cholines
Apolipoproteins
Apolipoprotein A−I
Apolipoprotein B
Fatty acids
Total fatty acids
Degree of unsaturation
Docosahexaenoic acid
Linoleic acid
Omega−3 fatty acids
Omega−6 fatty acids
PUFA
MUFA
Saturated fatty acids
Fatty acids ratios
Docosahexaenoic acid (%)
Linoleic acid (%)
Omega−3 fatty acids (%)
Omega−6 fatty acids (%)
PUFA (%)
MUFA (%)
Saturated fatty acids (%)
−0.2 −0.1 0.0 0.1 0.2
Change in sperm parameter (SD) per SD increase in metabolite
Motility
Concentration

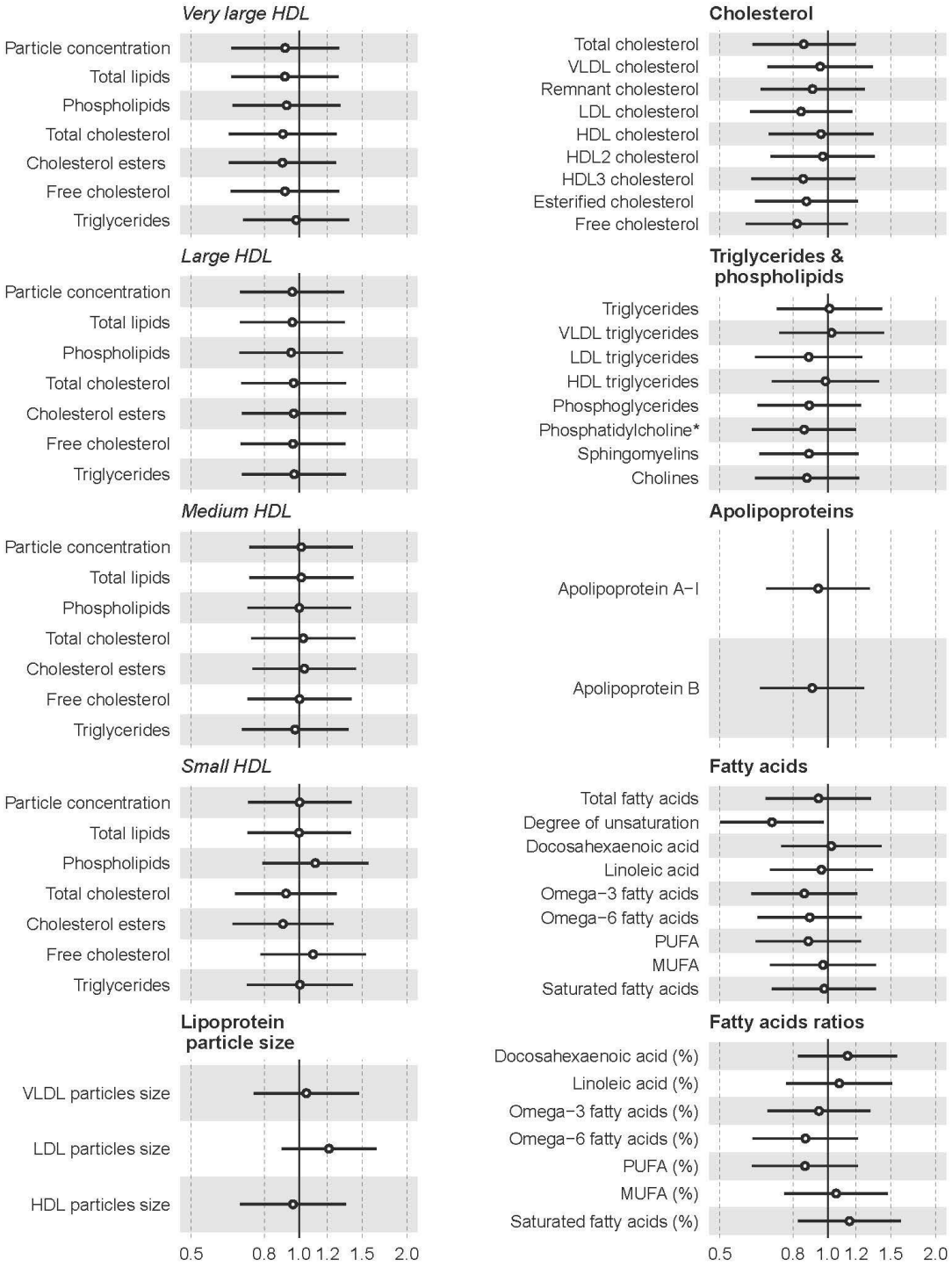

Glycolosis related metabolites
Glucose
Lactate
Pyruvate
Citrate
Glycerol
Amino acids
Alanine
Glutamine
Glycine
Histidine
Branched−chain amino acids
Isoleucine
Leucine
Valine
Aromatic amino acids
Phenylalanine
Tyrosine
Ketone bodies
Acetate
Beta−hydroxybutyrate
Fluid balance
Creatinine
Albumin
Inflammation
Glycoprotein acetyls
−0.2
−0.1
0.0
0.1
0.2
Change in sperm parameter (SD) per SD increase in metabolite
Motility
Concentration


Lipoprotein subclasses
Extremely large VLDL
Very Small VLDL
Very large VLDL
IDL
Large VLDL
Large LDL
Medium VLDL
Medium LDL
Small VLDL
Small LDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
0.5
0.8
1.0
1.2
1.5
2.0
OR for low vs normal TMSC per SD increase in metabolite


Very large HDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
Large HDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
Medium HDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
Small HDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
Lipoprotein particle size
VLDL particles size
LDL particles size
HDL particles size
0.5
0.8
1.0
1.2
1.5
2.0
Cholesterol
Total cholesterol
VLDL cholesterol
Remnant cholesterol
LDL cholesterol
HDL cholesterol
HDL2 cholesterol
HDL3 cholesterol
Esterified cholesterol
Free cholesterol
Triglycerides & phospholipids
Triglycerides
VLDL triglycerides
LDL triglycerides
HDL triglycerides
Phosphoglycerides
Phosphatidylcholine*
Sphingomyelins
Cholines
Apolipoproteins
Apolipoprotein A−I
Apolipoprotein B
Fatty acids
Total fatty acids
Degree of unsaturation
Docosahexaenoic acid
Linoleic acid
Omega−3 fatty acids
Omega−6 fatty acids
PUFA
MUFA
Saturated fatty acids
Fatty acids ratios
Docosahexaenoic acid (%)
Linoleic acid (%)
Omega−3 fatty acids (%)
Omega−6 fatty acids (%)
PUFA (%)
MUFA (%)
Saturated fatty acids (%)
0.5
0.8
1.0
1.2
1.5
2.0
OR for low vs normal TMSC per SD increase in metabolite


Glycolysis related metabolites
Glucose
Lactate
Pyruvate
Citrate
Glycerol
Amino acids
Alanine
Glutamine
Glycine
Histidine
Branched−chain amino acids
Isoleucine
Leucine
Valine
Aromatic amino acids
Phenylalanine
Tyrosine
Ketone bodies
Acetate
Beta−hydroxybutyrate
Fluid balance
Creatinine
Albumin
Inflammation
Glycoprotein acetyls
0.5
0.8
1.0
1.2
1.5
2.0
OR for low vs normal TMSC per SD increase in metabolite


Lipoprotein subclasses
Extremely large VLDL
Very Small VLDL
Very large VLDL
IDL
Large VLDL
Large LDL
Medium VLDL
Medium LDL
Small VLDL
Small LDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
0.5
0.8
1.0
1.2
1.5
2.0
OR for TMSC < 5 million vs TMSC > 5 million per SD increase in metabolite


Very large HDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
Large HDL
Particle concentration
Total lipids
Phospholipids
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Cholesterol esters
Free cholesterol
Triglycerides
Medium HDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
Small HDL
Particle concentration
Total lipids
Phospholipids
Total cholesterol
Cholesterol esters
Free cholesterol
Triglycerides
Lipoprotein particle size
VLDL particles size
LDL particles size
HDL particles size
0.5
0.8
1.0
1.2
1.5
2.0
Cholesterol
Total cholesterol
VLDL cholesterol
Remnant cholesterol
LDL cholesterol
HDL cholesterol
HDL2 cholesterol
HDL3 cholesterol
Esterified cholesterol
Free cholesterol
Triglycerides & phospholipids
Triglycerides
VLDL triglycerides
LDL triglycerides
HDL triglycerides
Phosphoglycerides
Phosphatidylcholine*
Sphingomyelins
Cholines
Apolipoproteins
Apolipoprotein A−I
Apolipoprotein B
Fatty acids
Total fatty acids
Degree of unsaturation
Docosahexaenoic acid
Linoleic acid
Omega−3 fatty acids
Omega−6 fatty acids
PUFA
MUFA
Saturated fatty acids
Fatty acids ratios
Docosahexaenoic acid (%)
Linoleic acid (%)
Omega−3 fatty acids (%)
Omega−6 fatty acids (%)
PUFA (%)
MUFA (%)
Saturated fatty acids (%)
0.5
0.8
1.0
1.2
1.5
2.0
OR for TMSC < 5 million vs TMSC > 5 million per SD increase in metabolite

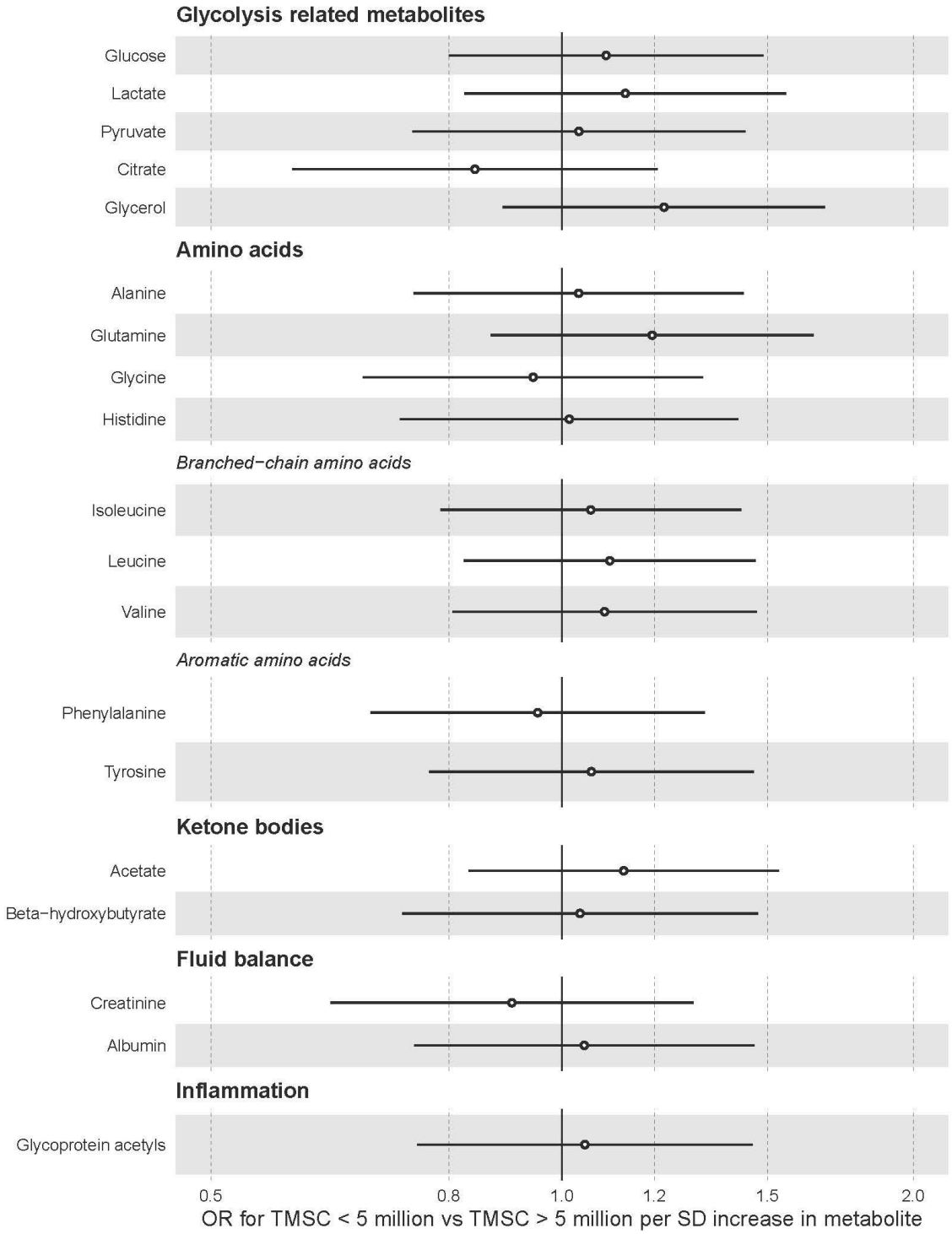
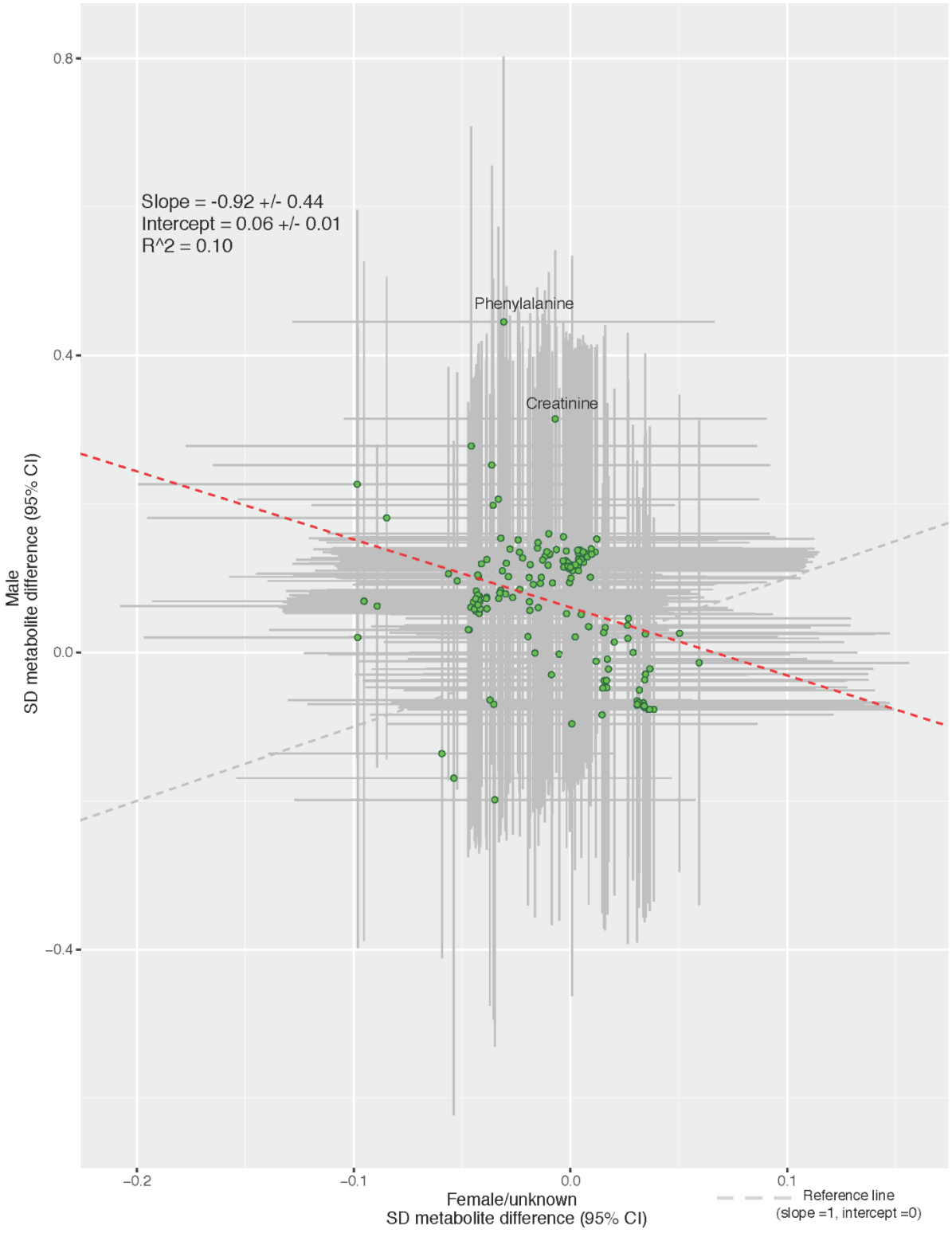
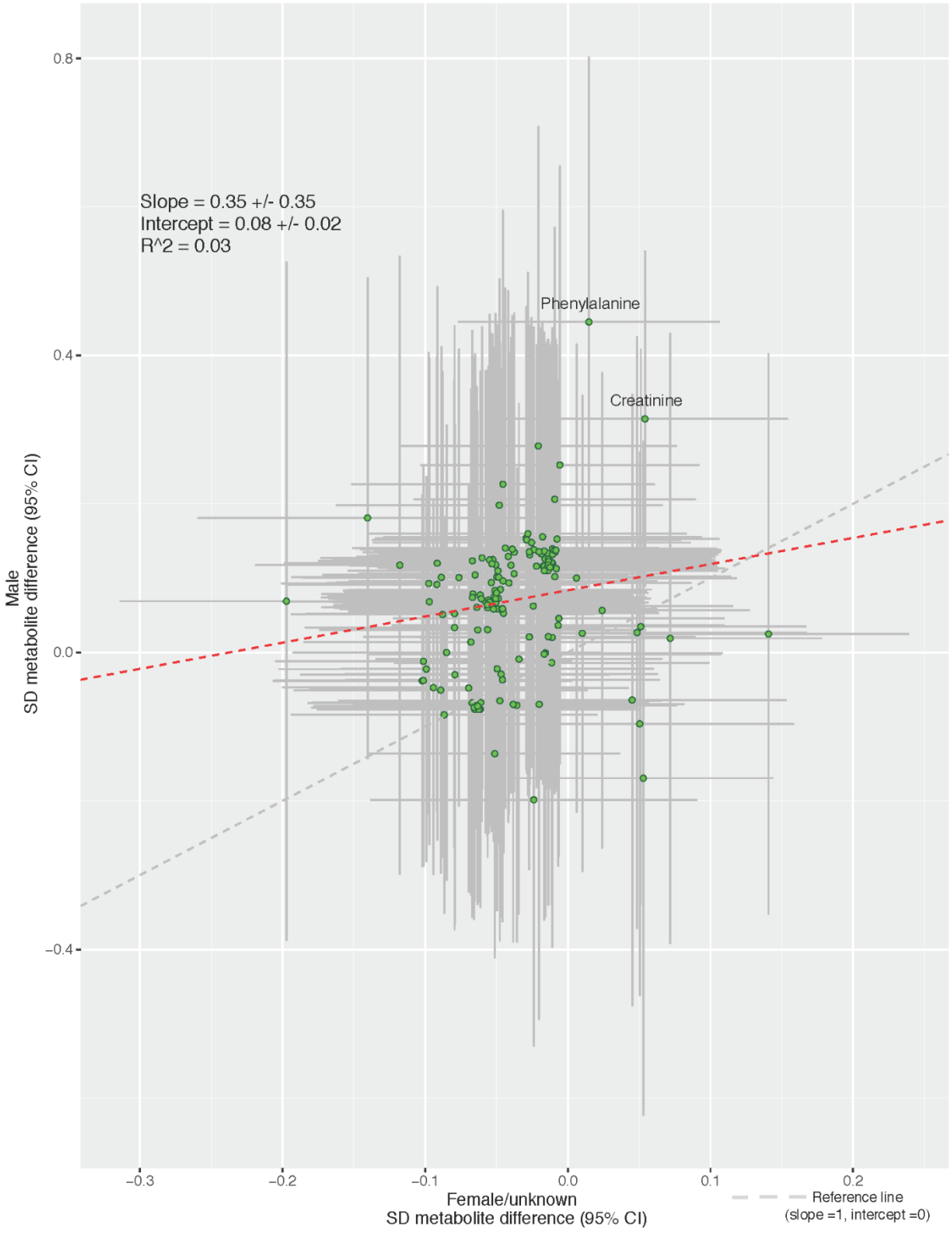

Glycolysis related metabolites
Glucose
Lactate
Pyruvate
Citrate
Glycerol
Amino acids
Alanine
Glutamine
Glycine
Histidine
Branched−chain amino acids
Isoleucine
Leucine
Valine
Aromatic amino acids
Phenylalanine
Tyrosine
Ketone bodies
Acetate
Beta−hydroxybutyrate
Fluid balance
Creatinine
Albumin
Inflammation
Glycoprotein acetyls
0.5
0.8
1.0
1.2
1.5
2.0
OR for TMSC < 5 million vs TMSC > 5 million per SD increase in metabolite

Figure 4: Scatterplot of associations between sperm concentration and motility and metabolites in those males with female or unknown cause of infertility (N = 398) and restricted to males with a reported male partner cause of infertility (N = 66).

Figure 4a shows associations with concentration by cause of infertility, Figure 4b shows associations with motility by cause of infertility. The green dots highlight the individual metabolites, with phenylalanine and creatinine highlighted. The grey dashed line reflects the reference line (slope 1, intercept 0) and the red dashed line is the best line of fit.


Slope = -0.92 +/- 0.44
Intercept = 0.06 +/- 0.01
R^2 = 0.10
Phenylalanine
Creatinine
0.8
0.4
0.0
−0.4
−0.2
−0.1
0.0
0.1
Male
SD metabolite difference (95% CI)
Female/unknown
SD metabolite difference (95% CI)
Reference line
(slope =1, intercept =0)


Slope = 0.35 +/- 0.35
Intercept = 0.08 +/- 0.02
R^2 = 0.03
Phenylalanine
Creatinine
0.8
0.4
0.0
−0.4
−0.3
−0.2
−0.1
0.0
0.1
0.2
Male
SD metabolite difference (95% CI)
Female/unknown
SD metabolite difference (95% CI)
Reference line
(slope =1, intercept =0)