Title Page

Title:

Cumulus cell acetyl-CoA metabolism from acetate is associated with maternal age but only partially with oocyte maturity

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Abstract

Objective: To use metabolism of cumulus cells (CCs) to predict oocyte competency.

Design: CC clumps that associate with oocytes are thought to provide the oocytes with growth and signaling factors. Thus, the metabolism of the CCs may influence oocyte function. This was a prospective and blinded cohort study that analyzed 403 individual sets of CC clumps from 36 participants. Thirty-one of the participants had paired oocyte maturity data. CCs were removed from oocytes after oocyte retrieval procedure, transported individually in vials to the research laboratory, incubated with stable isotope labeled substrates for 60 minutes, and analyzed using liquid chromatography-high resolution mass spectrometry (LC-HRMS) for isotopologue enrichment of major metabolic intermediates, including acetyl-CoA derived from the stable isotope labeled substrates.

Results: Mean enrichment of M+2 acetyl-CoA (mean, standard deviation), where M+0 is the unlabeled acetyl-CoA, M+1 contains 1 ^{13}C, M+2 contains 2 ^{13}C atoms, was for glucose (3.6, 7.7), for glutamine (9.4, 6.2), and for acetate (20.7, 13.9). Mean % enrichment of acetyl-CoA from acetate in CCs from women ≤34 (49.06, 12.73) decreased with age compared to CCs from women >34 (43.48, 16.20) (p=0.0004, t test). The CCs associated with the immature prophase I oocytes had significantly lower enrichment in M+2 acetyl CoA compared to the CCs associated with the metaphase I and metaphase II oocytes (difference: -6.02, CI: -1.74,-13.79, p=0.013). Limitations of this preliminary study include the difficulty in recovery of consistent numbers of CCs across oocytes, and the inability in this study to track oocyte function to the primary endpoint of successful birth.

Conclusion: Acetate metabolism in individual CC clumps was positively correlated with oocyte maturity and decreased with maternal age. These findings indicate that CC metabolism of short chain fatty acids like acetate should be investigated relative to oocyte function and age-related fertility.

Introduction

A major barrier in *in vitro* fertilization (IVF) remains that approximately 5% of aspirated human oocytes have the competence after fertilization to implant and develop into a child (1). Oocyte developmental competence decreases as a woman ages, and currently maternal age is the single best predictor of reproductive outcomes in women, highlighting an acute need for non-invasive biomarkers of oocyte competency to increase efficiency, lower costs, and reduce the chances of riskier multiple births (2). This has led to attempts to understand the aging and maturation process of oocytes for both etiologic understanding of fertility and predictive assays for oocyte function.

Notably, the metabolism of the oocyte *in vivo* is intimately linked to surrounding cells, such as the oocyte-associated somatic cumulus cells (CCs), which are critical to oocyte function in the ovarian follicle. CCs support oocyte development through the provision of essential nutrients and signaling molecules (3). CCs possess specialized cytoplasmic projections that penetrate through the zona pellucida [shell] and form gap junctions at their tips with the oocyte, generating an elaborate structure called the cumulus-oocyte complex (COC) (4, 5). Within the COC, CCs are thought to metabolize the bulk of the glucose to supply metabolic intermediates to the oocyte, where the COC glucose metabolism is pivotal in determining oocyte developmental competence (6). Because of the metabolic and communication link between the cumulus and the oocyte, glucose availability and metabolism within the cumulus can have a significant impact on oocyte meiotic and developmental competence (7).

During the oocyte retrieval procedure after controlled ovarian stimulation for IVF, the oocytes within specialized ovarian follicles, that support the growth and development of oocytes, are harvested. As only the oocyte is used in fertilization, the remainder of the cells and fluid is remnant sample and has been used as a source for non-invasive investigation of follicle function, even by destructive methods. This easy access has led

to insightful investigations into the metabolomics of the follicular fluid (8), but relatively few studies have been conducted on the metabolism of the recovered human CCs.

In this study we utilized stable isotope tracing coupled with liquid chromatography-mass spectrometry (LC-MS) to quantify the substrate preferences for CC metabolism. This approach for *ex vivo* metabolic studies uses incubation with a stable (non-radioactive) isotope labeled metabolic substrate (e.g. $^{13}C_6$-glucose) and tracking the incorporation of that isotopic label into downstream metabolites (**Figure 1**). With detection by LC-MS, the isotopologues (molecules differing only in their number of isotopes), co-elute on the LC, but are resolved by the MS, allowing separate quantitation of each isotopologue. After performing an adjustment for natural isotope abundance, the resulting isotopologue enrichment gives the relative molar % of the product detected derived from the labeled substrate in the given incubation conditions. Importantly, this isotopologue enrichment is self-normalizing and is not as influenced by the number of CCs in each sample which is highly variable.

Based on previous literature that CCs are highly glycolytic, we expected to see utilization of glucose to generate anabolic intermediates, including the central metabolic intermediate acetyl-Coenzyme A (acetyl-CoA). However, initial experiments demonstrated that acetyl-CoA was poorly labeled by glucose, and that acetate, not glucose or glutamine, was the preferred substrate to generate the central metabolic intermediate acetyl-CoA in the recovered CCs. Little is known about the metabolism of short chain fatty acids like acetate in the CCs. Thus, based on the finding of relatively high acetate usage to generate acetyl-CoA, we examined the association between cumulus cell acetate metabolism to acetyl-CoA, maternal age, and oocyte maturity.

Materials and Methods

Chemicals

Water, methanol, acetonitrile, and ammonium acetate were Optima LC-MS grade solvents from Fisher Scientific (Pittsburgh, PA). Trichloroacetic acid and salts used in Tyrode's buffer were from Sigma-Aldrich (St. Louis, MO). Stable isotope-labeled substrates were from Cambridge Isotope Laboratories (Tewksbury, MA).

Patient Selection

Enrolled patients were scheduled for IVF at the Main Line Fertility Center (Bryn Mawr, Pennsylvania, USA). Women undergoing IVF were screened for inclusion in this study and informed consent was obtained from all individual participants before enrollment. Inclusion criteria included IVF patients between the ages of 28 and 42 years, with an anti-mullerian hormone (AMH) between 1.0 and 10 ng/ml, and follicle stimulating hormone $\leq$ 10 IU/ml, luteinizing hormone (LH) < 12 IU/ml, and estradiol < 50 pg/ml on day 2-4 of the menstrual cycle. A body weight $\geq$ 50 kg and a body mass index between 18 and 32 kg/m^2 were required. Patients were excluded for smoking, polycystic ovarian disease, endometriosis greater than Stage I, utilizing testicular sperm for IVF, and preimplantation genetic testing. This prospective study was approved by Western Institutional Review Board (WIRB).

Controlled Ovarian Stimulation Protocol

Patients underwent the standardized controlled ovarian stimulation for IVF using Gonal-F RFF Redi-ject pen (EMD Serono, Inc., Darmstadt, Germany). All patients received a fixed protocol of 300 IU recombinant FSH (rFSH) daily for the first four days of stimulation. After, a flexible protocol was used to optimize ovarian response. rFSH was adjusted by the patient's physician to 225 to 450 IU daily up to and including day of human chorionic gonadotropin (hCG) trigger administration. Cycles were monitored with follicular ultrasound measurements and serum estradiol concentrations throughout controlled ovarian stimulation. An antagonist was used to suppress endogenous pituitary LH for the prevention of premature LH surges by administering 0.25 mg/day of Cetroelix Acetate (EMD Serono) when follicle size reached 12 mm and continued up to and including

day of hCG trigger. Subcutaneous injection of 250 µg of hCG (Ovidrel, EMD Serono) was administered when at least three leading follicle sizes reached a diameter of ≥ 17 mm. The oocyte retrieval procedure was performed 36 h after hCG injection.

Biosample Collection

CCs were removed from cumulus-oocyte complexes immediately after the oocyte retrieval procedure as part of routine IVF lab protocol. Instead of being discarded, CCs were placed into individually labeled micro-centrifuge tubes containing one mL of transport medium, then delivered by medical courier with a 37°C heat pack to a university research laboratory where metabolic tracing and analysis were conducted. A total of 403 individual CC clumps and their associated oocytes from 36 patients were included in this study, and 31 patients and their CC clumps were paired with oocyte maturity data.

Cumulus Cell Metabolic Tracing

All tracing was conducted by laboratory analysts blinded to any sample characteristics. CC metabolism was traced by centrifugation of the CC clump at 200 x g for 5 min at 25°C, removal of the supernatant, and then addition of the labeling media. For labeling media we used Tyrode's buffer (139 mmol/L NaCl, 3 mmol/L KCl, 17 mmol/L $NaHCO_3$, 3 mmol/L $CaCl_2$, and 1 mmol/L $MgCl_2$) pH= 7.4 pre-incubated at 20% O_2 and 5% CO_2 containing as a carbon source either 5 mmol/L [$^{13}C_6$]-glucose or 5 mmol/L glucose in combination with either 1 mmol/L [$^{13}C_2$]-acetate or 100 µmol/L [$^{13}C_5$ $^{15}N_2$]-glutamine. Labeling was conducted by addition of labeling media, a 3 sec vortex, and then incubation in a 37°C water bath for 1 hour in 1.5 mL plastic Eppendorf tubes. After completion of labeling incubation, cumulus cells were centrifuged at 1000 x g for 5 min at 4°C, supernatant was removed, and either 1 mL of 80:20 methanol:water pre-chilled to -80°C (for lactate and pyruvate analysis) or 1 mL of 10% trichloroacetic acid (w/v) in water (for acyl-CoA analysis) was added. Samples were frozen at -80°C until extraction and analysis. Samples were extracted by thawing at 4°C, probe tip sonication for 15 sec, and

then centrifugation at 16,000 x g for 10 min at 4°C. Supernatant was extracted with an Oasis HLB solid phase extraction (SPE) cartridge washed with 1 mL methanol and then equilibrated with 1 mL water. After loading of the supernatant, the SPE column was washed with 1 mL water, then eluted into a 10 mL glass tube with 1 mL methanol with 25mM ammonium acetate. The eluent was evaporated to dryness under nitrogen, resuspended in 50 µL 5% (w/v) 5-sulfosalicylic acid and 10 µL was injected for LC-HRMS analysis.

Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS)

Acyl-CoAs were analyzed as previously described in a quantitatively validated method (9) on an Ultimate 3000 Quaternary UHPLC coupled to a Q Exactive Plus mass spectrometer operating in the positive ion mode with a heated electrospray ionization probe (mark II) in an IonMax Source housing. Samples were kept in a temperature controlled autosampler at 6°C and LC separation was performed as previously described on a Waters HSS T3 2.7 µm particle size 2.1 x 150 mm column. LC conditions were as follows; column oven temperature 30°C, solvent A water with 5 mM ammonium acetate, solvent B 95:5 acetonitrile: water with 5 mM ammonium acetate, solvent C (wash solvent) 80:20 acetonitrile: water with 0.1% formic acid. The gradient was as follows: 0.2 mL/min flow at 98% A and 2% B for 1.5 min, 80% A 20% B at 5 min, 100% B at 12 min, 0.3 mL/min 100% B at 16 min, 0.2 mL/min 100% C at 17 min, held to 21 min, then re-equilibrated at 0.2 mL/min flow at 98% A and 2% B from 22 to 28 min. Flow from 4-18 minutes was diverted to the instrument. Operating conditions on the mass spectrometer were as follows; auxiliary gas 10 arbitrary units (arb), sheath gas 35 arb, sweep gas 2 arb, spray voltage 4.5 kV, capillary temperature 425°C, S-lens RF-level 50, aux gas heater temperature 400°C, in-source CID 5 eV. Scan parameters were optimized during the experiments, but final conditions were alternating full scan from 760-1800 *m/z* at 140,000 resolution and data independent acquisition (DIA) looped 3 times with all fragment ions multiplexed at a normalized collision energy (NCE) of 20 at a resolution of 280,000. An isolation width of 7 *m/z* with an offset of 3 *m/z* was used to capture all relevant isotopologues for targeted acyl-CoAs. Data was processed in Xcalibur and or TraceFinder (Thermo).

192

193 **Data analysis**

194 Normalization of isotopologue distribution by natural isotopic abundance was conducted via an open
195 source resource, FluxFix (10), using experimentally derived normal isotopic distributions from cumulus cell
196 clumps labeled with non-isotopically enriched glucose. Due to this adjustment, any sample that had zero signal
197 intensity for M+0 or M+2 was excluded from analysis, since these values would not be interpretable. For
198 isotopologue analysis, summary statistics were calculated in Graph Pad Prism (v7).

199 Metabolic analysis was conducted by investigators blinded to status and identity of all samples/patients,
200 and analysis of correlation with oocyte maturity and age was conducted by an independent biostatistician, who
201 had no role in the design or collection of data, using JMP Pro 13.0 software. Unit of analysis was on each cumulus
202 cell clump sample, since we were primarily interested in the unique metabolism of the cumulus cells associated
203 with individual oocytes.

204

205 **Results**

206

207 *Cumulus cells have a high capacity to derive acetyl-CoA from acetate relative to glucose and glutamine.*

208 Cumulus cells incubated with either [$^{13}C_6$]-glucose, [$^{13}C_5$]-glutamine, [$^{13}C_2$]-acetate for one hour were
209 analyzed for isotopologue enrichment of the central carbon intermediate acetyl-CoA. Isotopologue enrichment,
210 calculated with FluxFix (10), gives the relative molar % of the product detected derived from the labeled
211 substrate in the given incubation conditions. Thus, in this context, the isotopologue notation describes how
212 many labeled carbons are in the analyte of interest corresponding to M+0 as the unlabeled acetyl-CoA, M+1
213 containing one ^{13}C, M+2 containing two ^{13}C atoms. M+2 represents the maximum labeling of the acyl-group (the
214 acetyl group) in acetyl-CoA. Mean isotopologue enrichment of M+2 acetyl-CoA (mean, standard deviation) was
215 for glucose (3.6, 7.7), for glutamine (9.4, 6.2), and for acetate (20.7, 13.9) (**Figure 2**).

Enrichment of acetyl-CoA from acetate is decreased in cumulus cells from older women.

Both measures of metabolism and fertility are known to decline with age. To examine if this was true in CCs, we tested if acetyl-CoA enrichment from acetate was different from women ≤34 than >34. This cut-off is based on the distribution of ages within our sample, the age categories reported by the Society for Assisted Reproduction (SART), and the American College of Obstetricians and Gynecologists (ACOG) recognition of age 35 as a guideline for increased fertility counseling. Mean % enrichment of acetyl-CoA in CCs from women ≤34 (49.06, 12.73) was higher (p=0.0004, t test) than in CCs from women >34 (43.48, 16.20) (**Figure 3**).

Although we had pre-specified 34 as a cutoff before statistical analysis, we conducted an exploratory analysis to examine the effect of treating age as a continuous variable. The equation of the regression was estimated at % acetyl-CoA = 54.46 – 0.215(age), with a p-value for the slope coefficient of 0.314. The high variance in acetyl-CoA labeling and the restriction of our sample population to ages 26-42 limit the utility of this analysis.

Generation of acetyl-CoA from acetate is associated with mature oocytes.

Based on the discovery that cumulus cells have a high capacity to utilize acetate to generate acetyl-CoA, we were interested if the enrichment in acetyl-CoA from acetate correlated with properties of the associated oocyte, especially oocyte maturity. For 490 individual samples of cumulus cells from 31 patients where oocyte maturity data was available, we tested if M+2 acetyl-CoA enrichment from acetate was different in cumulus cells associated with oocyte maturity. Three types of oocytes are obtained at retrieval – mature metaphase II oocytes, immature metaphase I oocytes (no polar body), and very immature prophase I oocytes (no polar body, contain a germinal vesicle) The cumulus cells associated with the immature prophase I group had significantly lower enrichment in M+2 acetyl CoA compared to the cumulus associated with the other oocytes (difference: -6.02, CI: -1.74,-13.79, p=0.013) (**Figure 4**).

Finally, we examined if there was a difference in acetyl-CoA isotopologue enrichment between CCs that were associated with embryos that were selected for transfer to the uterus versus those that were not. CCs that were associated with embryos that were not selected for transfer had no significant difference in isotopologue enrichment (-2.54, CI: 3.29, -8.38, p=0.38). Similarly, comparing SART scoring of embryo quality (Grade A vs other grades) on day 3 of embryo culture, there was no significant difference in isotopologue enrichment (1.46, CI: 4.9 to -1.97, p=0.40). Comparing differences by all embryo grades, there were no significant differences in acetyl-CoA isotopologue enrichment between groups (p=0.51).

Discussion

Our findings indicate that cumulus cells have a high capacity to derive acetyl-CoA from acetate. This also partially contrasts to previous literature, implicating cumulus cells as highly glycolytic, since labeling was conducted in the presence of 5mM unlabeled glucose for glutamine and acetate tracing. Importantly, this suggests that the role of glycolysis in CCs may not be to feed acetyl-CoA, via conversion to pyruvate, into the Krebs cycle for oxidative phosphorylation or export as citrate to generate cytoplasmic acetyl-CoA. This alternative use of glucose would be in-line with reports that CCs use glucose to generate extra-cellular matrix components or increased products of the hexosamine pathway (11).

Strengths of this study include the novelty of applying stable isotope assisted metabolomics to individual oocyte-associate cumulus cell complex metabolism. Studying the *ex vivo* metabolism of human cumulus cells is difficult because of the paucity of recovered cells associated with single oocytes, variability in the numbers of cells, and the effects of endogenous metabolism beyond the control of the experimentalist. With stable isotope tracing, it is possible to establish a substrate/product relationship, in comparison to the metabolomics experiments already conducted that only examined the contents of the follicular fluid with no regard as to their origin. Isotope tracing performed *ex vivo* reduces the major confounder of diet and resulting changes in

circulating metabolites. Finally, isotope tracing is "self-normalizing" such that variation in cell numbers are accounted for because each isotopologue is normalized to the metabolite pool within each sample (e.g. acetyl-CoA M+2 is a fraction of total acetyl-CoA in that sample) (10).

Although we found a difference in acetyl-CoA labeling by oocyte maturity, a limitation of this study is that we did not follow through data collection to oocyte competence to pregnancy or to baby. Oxygen consumption, a rough gauge of metabolic activity, is an existing quality marker for human oocyte competence conditioned by ovarian stimulation regimens (12). Furthermore, the oxygen consumption rates of embryos have been found to be associated with successful implantation and can be used to select the embryo with the best developmental potential (13). However, this quantitation of metabolic function was a relatively poor predictor, with an odds ratio of 1.037-1.732 (95% CI) for normal vs non-fertilized/abnormal oocytes (12). Future work may be able to improve upon this prediction incorporating more specific substrates in combination with oxygen consumption. The finding of high levels of acetate utilization, and significant glutamine labeling into acetyl-CoA suggests that exploration of other potential substrates may have utility.

Our finding of decreased acetyl-CoA generation from acetate in cumulus cells from women >35 is biochemically interesting. The aging process of the oocyte is complex and includes inter-related impaired mitochondrial dysfunction, oxidative stress, diminished metabolic activity, potential accumulation of DNA damage, and activity of several cell-signaling systems (14). Pathological perturbations of the coordinated somatic cell-oocyte interactions by metabolic disease and/or maternal aging can induce molecular damage to the oocyte can alter macromolecules, induce mitochondrial mutations, all of which can harm the oocyte (15). It is also thought that these pathological processes harm the related CCs. Endometriosis may be associated with mitochondrial dysfunction in pooled CCs (16), and subjects with endometriosis may have a defect in CC mitochondrial function, which likely contribute to decreased fertilization and implantation rates (17). Generation of acetyl-CoA is possible from a variety of substrates, but two major sources include glucose and acetate. A switch between glucose and acetate is alternatingly dependent on the enzymes ATP citrate lyase

(ACLY) that generates glucose-derived acetyl-CoA from citrate exported from the mitochondria and the Acyl-coenzyme A synthetase short-chain family members that form acetyl-CoA from acetate and CoASH (18). In this study, our exclusion criteria limits the extrapolation of our findings to wider age ranges and pathological settings. Future studies using stable isotope labeling of cumulus cells may be useful in non-invasive study of the metabolic consequences of endometriosis and other diseases, especially in the context of fertility.

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Figures

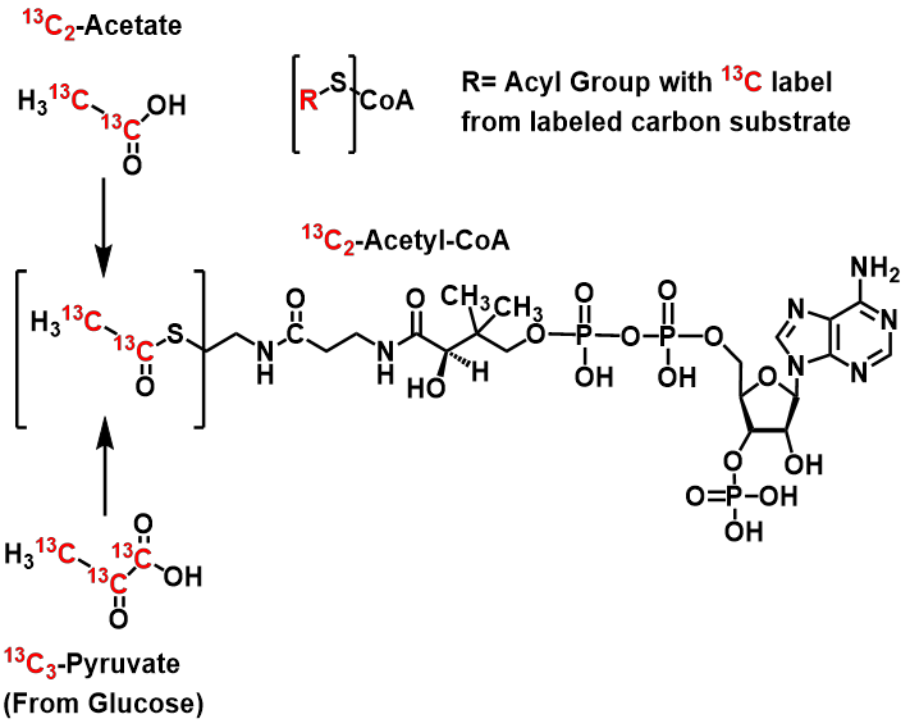

Figure 1. Stable Isotope Metabolic Tracing. Metabolic tracing from (**A**) Generally, acyl-CoA labeling can occur by any metabolite where the R=acyl group can be variably labeled by carbon derived from substrates. Stable isotope labeling into $^{13}C_2$-acetyl-CoA can be achieved through $^{13}C_2$-acetate or $^{13}C_3$-pyruvate derived from $^{13}C_6$-glucose.

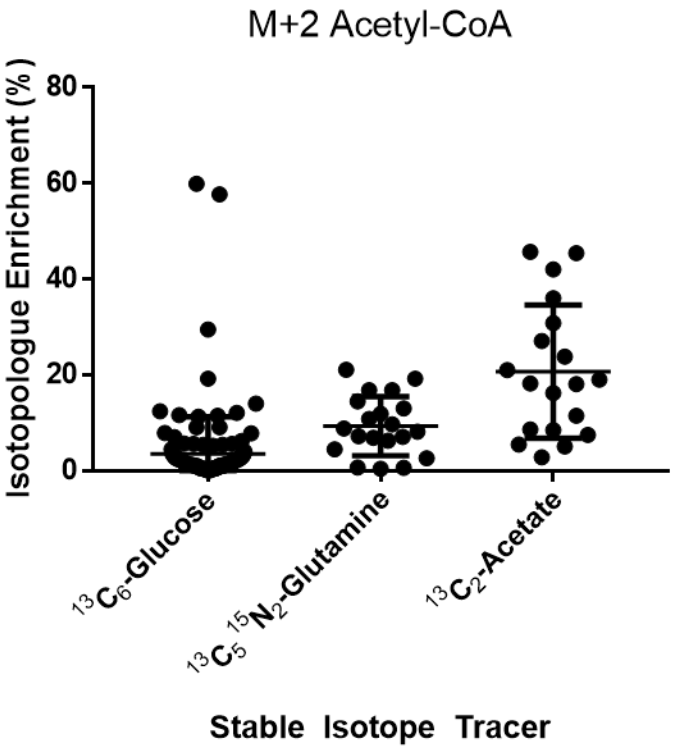

Figure 2. Isotopologue enrichment in acetyl-CoA M+2 from 3 major carbon tracers in cumulus cells (CCs). One hour incubations with CC clumps from individually retrieved oocytes in stable isotope tracer media containing the indicated labeled substrate. CCs have a higher capacity for generating acetyl-CoA from acetate than glucose, despite the literature on the glycolytic nature of glucose.

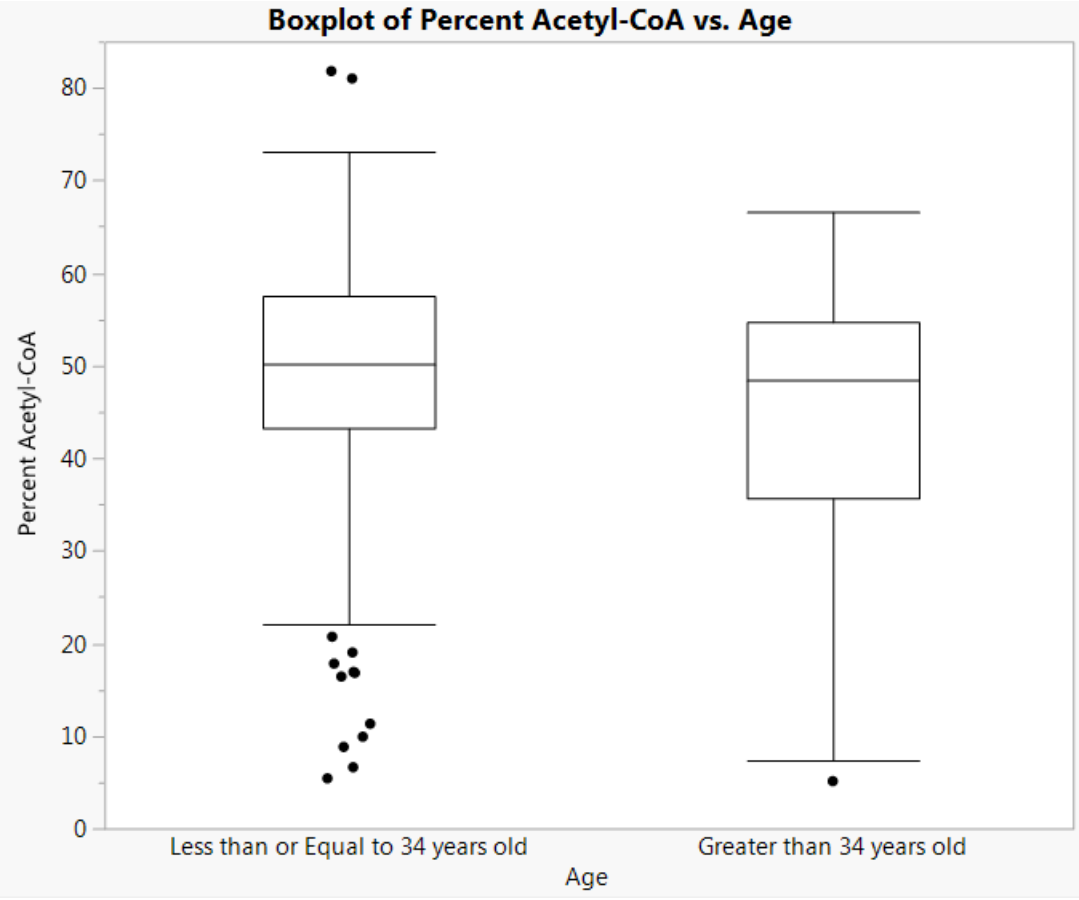

Figure 3. Isotopologue enrichment in acetyl-CoA M+2 in cumulus cells (CCs) from participants ≤34 or >34.

Isotopologue enrichment in acetyl-CoA CCs from $^{13}C_2$-acetate was 4.3% higher in women ≤34 versus >34 (p= 0.0004, CI: 2.3, 8.8).

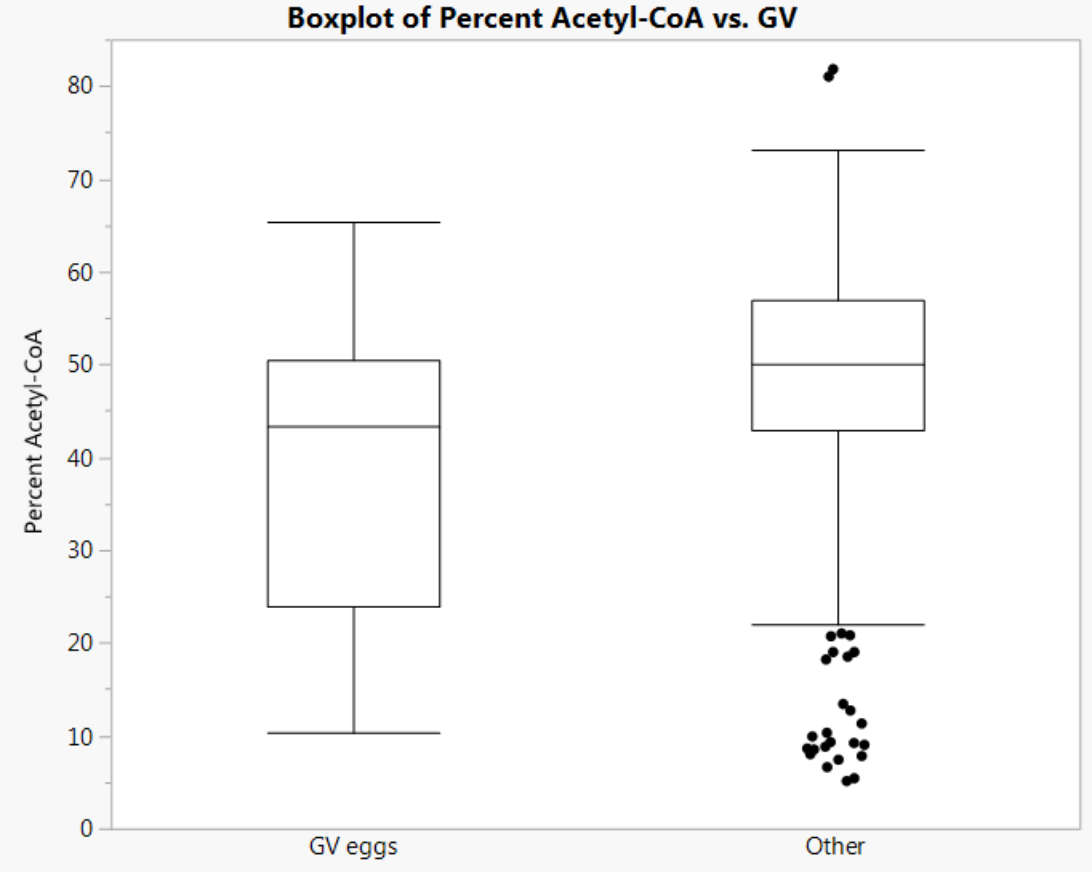

Figure 4. Isotopologue enrichment from CCs associated with prophase I oocytes containing germinal vesicles (GV eggs) compared to metaphase I and II oocytes. Isotopologue enrichment in acetyl-CoA CCs associated with GV eggs was 6.02% lower than in CCs associated with metaphase I and II oocytes (p=0.013, CI: -1.74,-13.79).