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The follicular fluid adipo-cytokine milieu could serve as a prediction tool for fertility treatment outcomes

*Brandon A. Wyse<sup>a,†,\*</sup>, Noga Fuchs Weizman<sup>a,†</sup>, Miranda Defer<sup>a</sup>, Janice Montbriand<sup>b</sup>, Peter Szaraz<sup>a</sup>, Clifford Librach<sup>a,c,d</sup>*

<sup>a</sup> CReATe Fertility Centre, Toronto, ON, Canada; <sup>b</sup> Department of Anaesthesia, Sunnybrook Health Sciences Centre, Toronto, ON, Canada; <sup>c</sup> Department of Obstetrics and Gynecology University of Toronto, Toronto, ON, Canada; <sup>d</sup> Department of Physiology and Institute of Medical Sciences, University of Toronto, Toronto, ON, Canada

\* Corresponding Author

Brandon A. Wyse  
790 Bay St. Suite 420  
Toronto, ON  
Canada  
M5G 1N8  
[brandon@createivf.com](mailto:brandon@createivf.com) or [brandonwyse@gmail.com](mailto:brandonwyse@gmail.com)  
1-416-323-7727

<sup>†</sup>The authors consider that the first two authors should be regarded as joint First Authors

## ABSTRACT

Research question: Can the adipo-cytokine milieu of the follicular niche improve our ability to predict treatment outcomes in infertile patients?

Design: Follicular fluid samples from overweight patients were analyzed and compared with samples from matched normal-weight patients. Concentrations of Adiponectin, Chemerin, CRP, IL6, IL10, IL18, Insulin, Leptin, Prolactin, Resistin, TNF $\alpha$ , and BMP-15 were assessed by multiple magnetic bead immunoassay (MMBI) and ELISA and correlated with fertility treatment outcomes.

Results: Analysis of samples from 22 overweight and 22 normal-weight patients demonstrated that TNF $\alpha$  can predict oocyte maturation rate. When stratified by BMI, IL10 emerges as a better predictor of oocyte maturation in normal weight patients. Prolactin was a negative predictor for fertilization rate in the full cohort, and this prediction power was lost upon stratification. No adipo-cytokines were predictive of blastulation rate, and only age remained predictive. BMP-15 was a strong predictor of high quality blastulation in the full cohort, more so in the normal weight population.

Conclusions: The adipo-cytokine milieu of the follicular fluid provides a snapshot of the growing oocyte's environment and can help predict fertility treatment outcomes, fine-tuning our understanding of the dysregulation caused by increasing BMI. Inflammatory cytokines can

45 predict oocyte maturation; prolactin, oocyte competence; and BMP-15, high quality blastulation.  
46 Further analysis of these findings with a larger sample size and assessing individual oocytes,  
47 will help shed more light on the clinical significance of these findings.  
48

49 Keywords: Proinflammatory cytokines, Obesity, Adipo-cytokines, Assisted Reproduction  
50 Treatment, BMI and ART outcomes  
51

## 52 INTRODUCTION

53 Obesity has been termed a global pandemic and is on the rise among women of reproductive age  
 54 (Swinburn *et al.*, 2011; Ng *et al.*, 2014). Obesity negatively influences assisted reproductive technology  
 55 (ART) outcomes; the ability to achieve a pregnancy is lower when using oocytes of obese patients, and  
 56 once pregnant, obese patients are less likely to achieve live birth (Luke *et al.*, 2011b; Talmor and Dunphy,  
 57 2015). While some obese patients are more at risk than others, Loy *et al.* failed to correlate between  
 58 adiposity dispersion and ART outcomes (Loy *et al.*, 2018).

59 Adipose tissue, the largest organ in the body, secretes bioactive molecules, also known as adipo-  
 60 cytokines, into the systemic circulation. Obesity disrupts this secretion and induces a unique inflammatory  
 61 state (Funahashi, Shimomura and Matsuzawa, 2004; Ohashi, Ouchi and Matsuzawa, 2011; Gonzalez *et al.*,  
 62 2018). Pro-inflammatory cytokines such as interleukin-6 (IL6), tumor necrosis factor alpha (TNF $\alpha$ ),  
 63 and c-reactive protein (CRP) promote insulin resistance and cause a shift in the immune profile (Gonzalez  
 64 *et al.*, 2018). The adipokines leptin, resistin, and adiponectin modulate lipid and glucose metabolism, alter  
 65 insulin sensitivity, and have been shown to be involved in regulation of reproductive functions (Dupont *et al.*,  
 66 2012; Lin *et al.*, 2017).

67 The wide array of adipo-cytokines in the follicular fluid (FF) can be grouped into several major  
 68 families including the adipokines (i.e. leptin, resistin, and adiponectin), the inflammatory cytokines [i.e.  
 69 TNF $\alpha$ , CRP, IL6, interleukin-18 (IL18), and interleukin-10 (IL10)], and the metabolic cytokines (prolactin,  
 70 chemerin, and insulin). Gonzalez *et al.* found that adipo-cytokines correlated with lipid levels in the FF  
 71 and hypothesized that local dyslipidemia was responsible for inferior ART outcomes (Gonzalez *et al.*,  
 72 2018). Others have yielded conflicting findings (Lee *et al.*, 1987; Rosenbusch, Djalali and Sterzik, 1992;  
 73 Mounzih, Lu and Chehab, 1997; Mendoza *et al.*, 1999; Takikawa *et al.*, 2010; Várnagy *et al.*, 2013; Singh,  
 74 Suragani and Krishna, 2014; Hajiaghayi *et al.*, 2019). These discrepancies stem, in part, from  
 75 heterogeneous study designs, small sample sizes, and not accounting for co-effects of different adipo-  
 76 cytokines from the same family or from different families. In this study, we aimed to determine: 1) which  
 77 adipo-cytokines predict fertility treatment outcomes, and specifically 2) the effect of increasing BMI and  
 78 the resultant disruption of the FF milieu on these predictive adipo-cytokines.

## MATERIALS AND METHODS

### Ethics approval

This study was approved by the University of Toronto Research Ethics Board (approval #29237).

### Study design

Retrospective analysis of FF samples collected through the CReATe Biobank (Toronto, ON, Canada).

Overweight and normal weight patients were matched, based on baseline characteristics (Table 1).

#### *Inclusion criteria*

- Patients consenting for biobanking their waste material
- Patients with a BMI of 25 or more were matched with patients with BMI of 18.5-24.9

#### *Exclusion criteria*

- Underweight patients (BMI<18.5)
- Patients with polycystic ovarian syndrome diagnosis (Rotterdam Criteria, 2004)
- Patients with endometriosis diagnosed by laparoscopy

### Sample and data collection

Samples were collected during oocyte retrieval at CReATe Fertility Centre (Toronto, ON, Canada) between January 2018 - May 2019. Patients were treated with standard antagonist in vitro fertilization (IVF) protocols. Extracted clinical data included demographics (age, BMI), fertility-related characteristics [anti-Müllerian hormone (AMH)], sperm parameters, and estradiol plasma levels on day of triggering oocyte maturation (peak E2), as well as treatment outcomes (maturation, fertilization and blastulation rates, and the proportion of high-quality blastocysts).

### Sample preparation

Neat FF was processed to remove follicular cells (centrifuged at 700xg for 10 min at 4°C) and snap frozen on dry ice. FF from one mature sized follicle (>18mm) was obtained from the BioBank, thawed on ice, and spun at 16,000xg for 4 min at 4°C to pellet precipitates. Cleared FF was aliquoted for both multiple magnetic bead immunoassay (MMBI) and Enzyme-linked immunosorbent assay (ELISA).

### Adipo-cytokine Measurement in FF by Multiplex Magnetic Bead Immunoassay (MMBI)

Cleared samples were diluted with Calibrator Diluent and distributed into a 96 well plate. A custom immunoassay (Luminex Assay, R&D Systems, Oakville, ON) was utilized for assessing the following

analytes: Adiponectin, Chemerin, CRP, IL6, IL10, IL18, Insulin, Leptin, Prolactin, Resistin, and TNF $\alpha$ . Analytes were captured using specific immunoglobulin-coated magnetic beads, a biotin-conjugated antibody was added to provide analyte-specific quantitative readout, and a fluorophore-conjugated (R-phycoerythrin - PE) detection antibody was added to amplify the signal to detection range. Unbound molecules were removed by repeat washes. Finally, the beads were resuspended and analyzed using the MACSQuant Analyzer flow cytometer (Miltenyi Biotec, Cologne, Germany).

#### Flow Cytometry and Quantification by MACSQuant Analyzer

Events corresponding to beads were selected based on forward scatter (FSC) and side scatter (SSC) (Supplemental Figure 1A). Specific single analyte bead populations were identified and selected using allophycocyanin (APC) and R-phycoerythrin-Vio770 (PE-Vio770) filters (Supplemental Figure 1B). Each individual population was then assessed using the PE filter to determine the median fluorescence intensity (MFI) of the population (Supplemental Figure 1C). The MFI of the standards were plotted against their known concentrations and a standard curve was constructed. The concentrations were determined for each analyte by linear regression.

#### Enzyme-Linked Immunosorbent Assay

Bone morphogenetic protein 15 (BMP-15) was quantified using ELISA (Novus Biologicals, Toronto, ON). Sample dilutions were determined by performing serial dilutions (neat, 1:2, 1:4, 1:10, 1:50) of a subset of four samples (data not shown). All samples were then assayed at the optimal dilution (1:50), in a 96 well plate. Analytes were captured on the well surface and the biotin-detection antibody was added. Unbound antibody was washed away, and an HRP-conjugated secondary antibody was added. Colorimetric substrate solution was added and incubated in the dark to facilitate colour change. The reaction was stopped, and optical density (OD) was quantified using FilterMax F5 Plate Reader (Molecular Devices, San Jose, USA) at 495nm. The analytes were quantified by linear regression.

#### Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software version 25. Participants were matched between normal and high BMI groups using propensity scores created by logistic regression with the covariates age, AMH, total motile sperm count, and estradiol. Logistic regression using the propensity score failed to reach significance ( $p>0.05$ ). Continuous

135 outcomes, average, standard error of the mean (SEM) and relative risks were created within each group  
136 and sub-strata, and linear regression was conducted while accounting for important potential  
137 confounders. Categorical variables were represented by n and %, and logistic regression was performed.  
138 Given the small sample size we chose to perform backwards selection within groups of variables, which  
139 allows the identification of the most parsimonious model while preserving power. All adipo-cytokines and  
140 covariates were first assessed at the univariate level for relationships with outcomes using appropriate  
141 parametric and non-parametric tests. Significant predictors were selected as potential covariates in a final  
142 model. Normality of variables was examined through skew/kurtosis analysis, as well as visually. Extreme  
143 outliers were captured using standardized scores and graphs and were removed from the final analysis.  
144 Multicollinearity was assessed with variance inflation factor and tolerance.  
145 To determine if the loss of prediction ability of some of the adipo-cytokines observed in the stratified  
146 groups was due to a loss of correlation between the adipo-cytokine and the outcome, we performed  
147 correlation and locally estimated scatterplot smoothing (LOESS) analysis. LOESS curves were used to  
148 visually examine non-linear relationships and changes across BMI. ROC curves were calculated for the  
149 final logistic regression models predicting each of the measured outcomes, with a Youden index used for  
150 calculating the sensitivity and specificity for each model.  
151 For the stratification analysis, to control for multiple comparisons and reduce the risk for false positives,  $p$   
152  $< 0.02$  was considered significant, and  $0.021 < p < 0.06$  was considered a trend. For the correlation  
153 analysis a power calculation showed that for an expected moderate correlation, a sample size of 36  
154 would suffice. In this analysis  $R < 0.3$  was considered a small effect size,  $R = 0.3-0.5$  a moderate effect  
155 size, and  $R > 0.5$  a large effect size (Cohen, 1988).

## RESULTS

### Study population and matching process

The study cohort consisted of 22 overweight patients matched with 22 normal-weight patients. Matching was based on baseline characteristics that could affect the measured outcomes (Table 1).

### Multiplex Magnetic Bead Immunoassay and ELISA quantification of the adipo-cytokine milieu in FF

All assayed adipo-cytokines were detected in FF samples (mean  $\pm$  SEM): adiponectin (261697.6  $\pm$  31441.1 pg/ml), BMP-15 (67.5  $\pm$  8.9 pg/ml), chemerin (956.1  $\pm$  84.9 pg/ml), CRP (344761.9  $\pm$  36392.6 pg/ml), IL6 (1.1  $\pm$  0.2 pg/ml), IL10 (0.5  $\pm$  0.04 pg/ml), IL18 (29.1  $\pm$  3.3 pg/ml), insulin (29.5  $\pm$  5.7 pg/ml), leptin (5412.2  $\pm$  598.7 pg/ml), prolactin (1950.9  $\pm$  181.1 pg/ml), resistin (980.4  $\pm$  70.5 pg/ml), and TNF $\alpha$  (0.7  $\pm$  0.07 pg/ml).

### Adipo-cytokines prediction of ART treatment outcomes

TNF $\alpha$  and leptin were both significant positive predictors of oocyte maturation. However, TNF $\alpha$  was the strongest predictor and the only one in the final model following backwards selection (OR = 10.2). An ROC curve based on the prediction model for oocyte maturation showed an area under the curve (AUC) of 0.8 (with a sensitivity of 0.79, and specificity of 0.7) (Figure 1A). When stratifying by BMI, the anti-inflammatory cytokine IL10 and the proinflammatory cytokine IL18 were predictive of oocyte maturation amongst women with a normal BMI (OR = 1.25), with IL10 remaining predictive in the final model after controlling for covariates. This model had robust predictive ability as shown by its ROC curve (AUC = 0.98; sensitivity of 1, specificity of 0.91) (Figure 1B). There were no significant predictors for oocyte maturation in the overweight cohort. Table 2 depicts regression analysis for all significant adipo-cytokines predicting oocyte maturation.

When assessing fertilization rate, prolactin was the sole predictor for fertilization rate in the full cohort (OR = 0.36), with an AUC of 0.76 (sensitivity of 0.77, specificity of 0.78) (Table 2) (Figure 1C). There were no predictive cytokines for fertilization rate in the stratified cohorts (Table 2).

When assessing the adipo-cytokines for prediction of blastulation rate, none remained in the final model, leaving patient age as the only significant factor (B coefficient = -0.36, OR = 0.7) (Table 2) (AUC = 0.82, sensitivity of 1, specificity of 0.52) (Figure 1D). The same was true for the stratified high BMI group, with patient age being the only significant predictor of blastulation rate (B coefficient = -0.56, OR = 0.6) (Table 2).

BMP-15 was the main predictor for high quality blastocyst development among the full cohort (B coefficient = 0.26,  $R^2 = 0.2$ ) (Table 2). This predictive power was maintained among the normal weight population (B coefficient = 0.57,  $R^2 = 0.36$ ). However, the predictive power of BMP-15 was disrupted in the overweight population where it did not predict high quality blastulation. Table 2 depicts regression analysis for all significant adipo-cytokines predicting high quality blastulation.

#### Adipo-cytokines correlating with increasing BMI

When assessing the adipo-cytokines predictive of oocyte maturation, TNF $\alpha$  and leptin positively correlated with BMI in the full cohort ( $R = 0.55$  and  $R = 0.66$ , respectively) (Table 3). A LOESS graph demonstrated consistent correlation between TNF $\alpha$  and BMI, and a disruption of this correlation for leptin at BMI of 28 (Figures 2A and 2B). Both the pro-inflammatory cytokine IL18 and the anti-inflammatory cytokine IL10 correlated with BMI in the full cohort ( $R = 0.37$  and  $R = 0.50$ , respectively), and these were driven by correlations in the normal weight population ( $R = 0.55$  and  $R = 0.43$ , respectively) (Figures 2C and 2D) (Table 3). Prolactin was predictive of fertilization rate in the overall cohort however, it did not correlate with BMI (Figure 2E). Finally, BMP-15 was predictive of a high quality blastulation rate and displayed a strong positive correlation with BMI in the overall cohort ( $R = 0.59$ ) (Figure 2F) (Table 3).

## DISCUSSION

The causal relationship between obesity and ART outcomes has long been debated (Luke *et al.*, 2011a; Luke *et al.*, 2011b; Christofolini *et al.*, 2014; Einarsson *et al.*, 2017; Espinós *et al.*, 2017; Friedler *et al.*, 2017; Kluge *et al.*, 2019). Adipose tissue secretes bioactive substances into the systemic circulation (Gonzalez *et al.*, 2018), and from there they are transudated into the follicular environment (McRae *et al.*, 2012). Increased adiposity leads to dysregulation of glucose and lipid metabolism, as well as increased systemic inflammation, all of which could impact oocyte development and maturation (Snider and Wood, 2019).

Oocyte competence and embryo development are determined by multiple factors in the follicular environment. The analysis of FF components provides information on metabolic changes in this microenvironment and is easily available during oocyte pick-up (Revelli *et al.*, 2009). Because of this intimate connection between obesity, metabolism, and inflammation, markers of these pathways should be explored simultaneously. Here we aimed to build on the foundational work of Gonzalez *et al.* by exploring the unique inflammatory follicular environment in the overweight population (Gonzalez *et al.*, 2018). In the current study, we explored an enhanced FF adipo-cytokine milieu and their power to predict fertility treatment outcomes, and established how these predictive markers are affected by increasing BMI.

When investigating oocyte maturation and the predictive ability of adipo-cytokines, leptin and TNF $\alpha$  were both found to be predictors of oocyte maturation in the overall group at the univariate level. Following model building, TNF $\alpha$  remained the only adipo-cytokine predicting oocyte maturation by both regression analysis and ROC. We found that leptin had minimal predictive power for oocyte maturation, and this was not retained after controlling for covariates. TNF $\alpha$  has been previously shown to enhance folliculogenesis and ovulation by promoting vascularization and suppressing granulosa cell proliferation and spontaneous apoptosis, (Tilly *et al.*, 1992; Deguchi *et al.*, 1996; Bili *et al.*, 1998; Baka and Malamitsi-Puchner, 2006; Kollmann *et al.*, 2017). Furthermore, TNF $\alpha$  showed a strong positive correlation with BMI in the overall group, as previously reported (La Vignera *et al.*, 2011).

Leptin, a satiety signal regulating food intake and energy expenditure (Mantzoros, 2000; Almog *et al.*, 2011), is also an angiogenic factor which regulates VEGF expression and has been shown to

increase the developmental competence of oocytes (De Placido *et al.*, 2006; Joo *et al.*, 2010). Some evidence suggests it enacts its role in controlling ovarian function via reduction of estradiol production by granulosa cells (Agarwal *et al.*, 1999). As previously described and recapitulated in this study, leptin levels correlated positively with TNF $\alpha$  levels, which could offer an explanation to its predictive power for oocyte maturation (Nikolettos *et al.*, 2004). Further studies are needed to assess whether the prognostic role for FF leptin in human reproduction is independent of other factors and to elucidate the underlying mechanisms (Mantzoros, 2000; Anifandis *et al.*, 2005). The LOESS graph for leptin (Figure 2B) is a testament to the dysregulation that occurs in the follicular environment of overweight patients, and may offer an explanation to the lower ART success rates obtained with oocytes of obese patients.

When assessing the adipo-cytokine predictive power for oocyte maturation in the normal and overweight groups, TNF $\alpha$  no longer predicted oocyte maturation, and only the normal weight group had significant predictors of oocyte maturation. Both IL10 and IL18 were predictive of oocyte maturation at the univariate level, and following model building only IL10 remained predictive. Both IL10 and IL18 have been previously reported to be positively correlated with follicular maturation and ovarian response to stimulation (Sarapik *et al.*, 2012; Nuñez-Calonge *et al.*, 2016; Alhilali *et al.*, 2019). In the current study, following ROC analysis, IL10 displayed robust sensitivity and specificity. To further explore these dynamics, we looked at the correlation of these inflammatory adipo-cytokines with BMI and demonstrated that while TNF $\alpha$  consistently correlated with BMI, the correlation for IL10 and IL18 was disrupted in the overweight range. This may suggest metabolic dysregulation that affects the follicular environment and oocyte development in the overweight population and should be explored further in larger-scale clinical studies.

Next, we assessed fertilization rate, with prolactin being the sole negative predictor for fertilization rate in the overall group, corroborating some, but not all previous literature (Lee *et al.*, 1987; Reinthaller *et al.*, 1987; Kamel *et al.*, 1994). There were no predictors identified in the stratified groups. Prolactin may act as a "co-gonadotropin" (Porter, Brumsted and Sites, 2000), and because small immature follicles are more sensitive to the inhibitory action of prolactin on steroidogenesis and folliculogenesis than large follicles, it has a modulatory role in follicular growth and maturation and, consequently, in the selection of

the ovulatory follicle (Kawagoe and Hiroi, 1990). Because of its complex relationship to ART outcomes, and the fact that FF prolactin levels are affected by many other fertility-related circumstances (Reinhaller *et al.*, 1987), prolactin is generally not considered a reliable marker of oocyte competence (Revelli *et al.*, 2009). The current study shows that even though prolactin does not correlate with BMI, it may potentially serve as a predicting marker for fertilization, an area in which further exploration is warranted.

No adipo-cytokines were predictive of blastulation rate, however BMP-15 was significantly predictive of high quality blastulation in the overall cohort, as previously reported (Wu *et al.*, 2007; Persani *et al.*, 2014; Riepsamen *et al.*, 2019). This finding further highlights the importance of increasing the developmental competence of the oocyte to produce high quality blastocysts (Hussein, Thompson and Gilchrist, 2006). In the stratified groups, BMP-15 remained predictive of high quality blastulation rate in the normal weight group and no adipo-cytokines were predictive in the overweight group. BMP-15 only correlated in patients whose BMI ranged between 23-30 kg/m<sup>2</sup>. This finding could be potentially attributed to the heterogeneity of this population in the study cohort and a larger sample size is needed to verify this finding.

The strength of our study design included the matching that was done: to minimize the biological variability between the groups, to increase the statistical power, and to tease out differences in the FF milieu that could be attributed to increasing BMI. We excluded cases of patients with polycystic ovarian syndrome (PCOS) and/or endometriosis to avoid the effects these conditions may have on the composite of the FF (Karaer *et al.*, 2019; Zhang *et al.*, 2020). The correlation analyses between predictive adipo-cytokines and BMI helped us determine whether differences in predictive abilities between different BMI strata and the complete cohorts stemmed from loss of power (i.e smaller sample sizes) or from disruption of the FF composite in the overweight population. Among the covariates we explored, peak E2 level was the most influential on oocyte maturation and fertilization, while age impacted later stages of development, namely blastulation. As these experiments were done on previously biobanked samples, we are only able to comment on cycle outcomes and unable to assess the fate of each individual oocyte. This study was not designed to explore differences in outcomes between patients in different BMI categories. Further analysis of the correlations identified in this study are needed with larger sample sizes to incorporate interaction analysis and assess individual oocytes. Correlating levels of the predictive

adipo-cytokines identified in the FF with levels of the same adipo-cytokine in the blood and validation of these findings in different clinical settings are a critical next step towards developing a clinically-applicable test.

In conclusion, we identified inflammatory cytokines that can serve as surrogate markers in predicting oocyte maturation, we added to the evidence pertaining to the importance of prolactin in oocyte fertilization, and we highlighted the importance of BMP-15 in promoting high-quality blastulation. These findings further contribute to the understanding of the dysregulation that occurs in the microenvironment surrounding the developing oocyte in cases of obesity and have also identified further areas required for study.

#### **AUTHOR CONTRIBUTIONS**

B.A.W, N.F.W, M.D., and C.L developed the concept and designed the study. B.A.W, N.F.W, and M.D. analyzed the data and interpreted the results. B.A.W and M.D. performed experiments, with essential guidance from P.S. J.M. performed statistical analyses, with biological interpretation by N.F.W and B.A.W. B.A.W. and N.F.W drafted the manuscript. All authors critically reviewed the manuscript and approved the final version of the manuscript.

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#### **CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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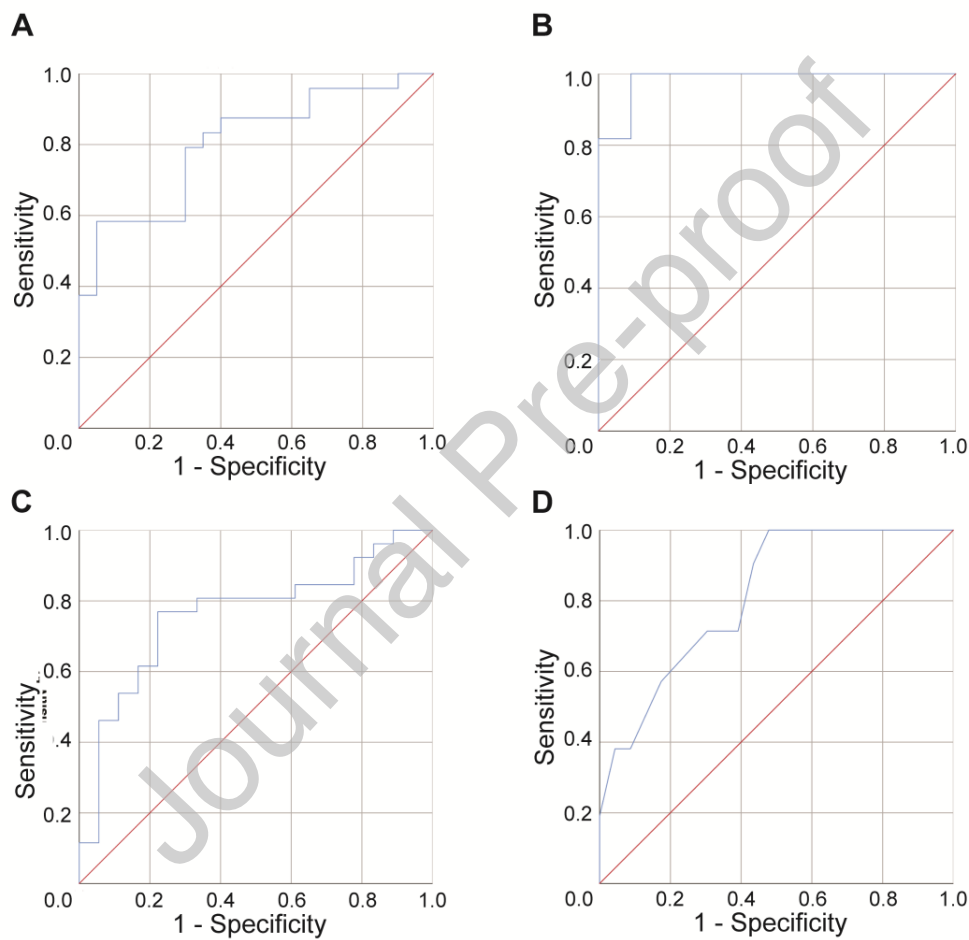
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496 Brandon leads a team investigating biomarkers of oocyte quality, maturity, and female infertility.  
497 He maintains several cross-institutional collaborative projects investigating oocyte maturation,  
498 communication, and growth. He completed his MSc at the University of Guelph, where he  
499 studied epigenetics, establishing a model of epigenetic regulation in the human malaria causing  
500 agent, *Plasmodium falciparum*.  
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#### 502 **Key Message**

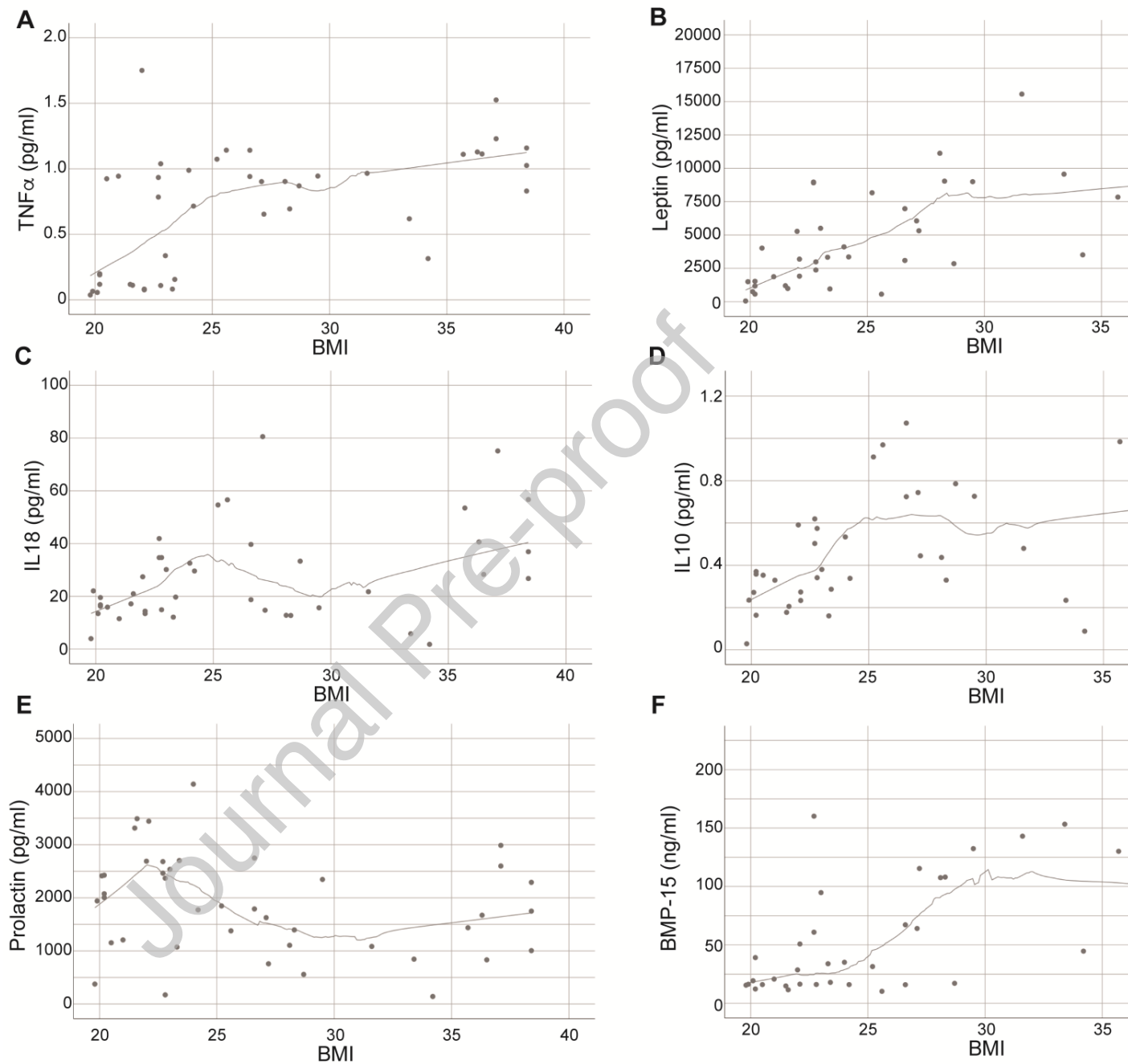
503 Herein we provide a novel methodology for assessing adipo-cytokines in the human follicular  
504 fluid and evaluating the interplay between them, obesity and ART outcomes. Based on our  
505 findings, follicular adipo-cytokine levels can become the basis for multifactor prediction models  
506 for ART outcomes in patients of differing BMIs.

## FIGURE LEGENDS



**Figure 1:** Receiver operating characteristic curves (ROC) of individual adipo-cytokines and outcomes. A) For TNF $\alpha$  predicting oocyte maturation in the full cohort; B) For IL10 predicting oocyte maturation in the normal BMI cohort; C) For prolactin predicting fertilization rate in the full cohort; D) For age predicting blastulation rate in the full cohort.

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520 **Figure 2:** Locally estimated scatterplot smoothing (LOESS) correlations for the corresponding adipo-  
 521 cytokines and BMI in the full cohort. A) TNF $\alpha$ ; B) Leptin; C) IL18; D) IL10; E) Prolactin; F) BMP-15.

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|  | <b>Overall BMI<br/>(n=44)</b> | <b>Normal BMI<br/>(n=22)</b>  | <b>High BMI<br/>(n=22)</b>    | <b>P Value</b> |
|--|-------------------------------|-------------------------------|-------------------------------|----------------|
| <i>Age (years)</i>                                 | 35.3±0.6<br>(23-42)           | 35.1±0.7<br>(31-40)           | 35.5±1.0<br>(23-42)           | 0.80           |
| <i>AMH (pmol/L)</i>                                | 16.6±1.1<br>(5.8-38.2)        | 15.8±1.4<br>(7.3-30.5)        | 17.3±1.6<br>(5.8-38.2)        | 0.52           |
| <i>Total Motile Sperm Count<br/>(TMSC) (10E+6)</i> | 19.4±2.6<br>(0-60)            | 22.5±4.2<br>(0.5-60)          | 16.3±3.1<br>(0-56)            | 0.26           |
| <i>Estradiol (E2) (pmol/L)</i>                     | 10744.4±666.1<br>(4056-26780) | 10453.1±880.9<br>(4056-23800) | 11035.8±995.5<br>(6279-26780) | 0.67           |

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|                                 |                                  | Overall BMI (n=44)     |                        |       | Normal BMI (n=22)  |   |                        |       |
|---------------------------------|----------------------------------|------------------------|------------------------|-------|--|---|------------------------|-------|
|                                 |                                  | OR (CI)                | B                      | p     | OR (CI)  | B   | p                      |       |
| Maturation Rate <sup>†</sup>    | <i>Leptin</i>                    | 1.2<br>(1.01 to 1.456) | 0.19                   | 0.04  | <i>IL18</i>  | 1.9 (0.98 to 1.26)  | 0.6                    | 0.06  |
|                                 | <i>TNFa</i>                      | 10.2<br>(1.7 to 60.9)  | 2.3                    | 0.01  | <i>IL10</i>  | 1.3x10 <sup>12</sup><br>(111.86 to 1.39 x10 <sup>22</sup> ) | 27.9                   | 0.02  |
|                                 | <i>Final Model (TNFa+PeakE2)</i> | 10.2<br>(1.7 to 60.9)  | 2.3                    | 0.01  | <i>Final Model (IL10+BMI)</i>  | 1.3x10 <sup>12</sup><br>(111.86 to 1.39 x10 <sup>22</sup> ) | 27.9                   | 0.02  |
| Fertilization Rate <sup>‡</sup> | <i>Prolactin</i>                 | 0.36<br>(0.16 to 0.81) | -1.02                  | 0.01  | No significant predictors at the univariate level available for model building |   |                        |       |
|                                 | <i>Final Model (Prolactin)</i>   | 0.36<br>(0.16 to 0.81) | -1.02                  | 0.01  |  |   |                        |       |
| Blastulation Rate <sup>§</sup>  | <i>Chemerin</i>                  | 1.0<br>(1.0 to 1.0)    | -0.002                 | 0.06  | No significant predictors at the univariate level available for model building |   |                        |       |
|                                 | <i>Final Model (Age)</i>         | 0.7<br>(0.56 to 0.87)  | -0.36                  | 0.002 |  |   |                        |       |
| High Quality Blastulation Rate  | <i>BMP-15</i>                    | 0.2*                   | 0.25                   | 0.006 | <i>BMP-15</i>  | 0.36*   | 0.57                   | 0.004 |
|                                 | <i>Final Model (BMP-15)</i>      | 0.2*                   | 0.26<br>(0.09 to 0.43) | 0.004 | <i>Final Model (BMP-15)</i>  | 0.36*   | 0.57<br>(0.21 to 0.93) | 0.004 |

†Controlled for Peak E2; ‡Controlled for Peak E2 and BMI; §Controlled for Age and AMH; \*R<sup>2</sup> from linear regression analysis

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|                   | Overall BMI (n=44) |                                    | Normal BMI (n=22) |                                    | High BMI (n=22) |                                    |
|-------------------|--------------------|------------------------------------|-------------------|------------------------------------|-----------------|------------------------------------|
| <b>Biomarkers</b> | <i>P value</i>     | <i>Correlation coefficient (R)</i> | <i>P value</i>    | <i>Correlation coefficient (R)</i> | <i>P value</i>  | <i>Correlation coefficient (R)</i> |
| <i>TNFi</i>       | <b>0.0001</b>      | <b>0.55</b>                        | 0.58              | —                                  | 0.29            | —                                  |
| <i>IL18</i>       | <b>0.016</b>       | <b>0.37</b>                        | <b>0.008</b>      | <b>0.55</b>                        | 0.81            | —                                  |
| <i>IL10</i>       | <b>0.001</b>       | <b>0.50</b>                        | <b>0.045</b>      | <b>0.43</b>                        | 0.75            | —                                  |
| <i>Leptin</i>     | <b>0.00001</b>     | <b>0.66</b>                        | <b>0.018</b>      | <b>0.50</b>                        | 0.15            | —                                  |
| <i>Prolactin</i>  | 0.054              | -0.29                              | 0.90              | —                                  | 0.81            | —                                  |
| <i>BMP-15</i>     | <b>0.00005</b>     | <b>0.59</b>                        | 0.33              | —                                  | 0.10            | —                                  |

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