Correlation-based network analysis for biomarkers in obesity

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***Abstract*—Background: Obesity is associated with chronic activation of the immune system and an altered gut microbiome, leading to increased risk of chronic disease development. As yet, no biomarkers have been found to distinguish individuals at greater risk of obesity-related disease. The aim of this study was to explore a correlation-based network approach to find existing patterns of immune-microbiome interactions in obesity.**

Results: The current study performed correlation-based network analysis on five different datasets obtained from 11 obese and 12 healthy weight men: anthropometric measures, metabolic measures, immune cell abundance, serum cytokine concentration, and gut microbial composition. The obese cohort had a denser network (total number of edges, n = 237) compared to the healthy network (n = 190). Within the obese network, immune cell abundance was found to be correlated to biomarkers from all four other datasets while in the healthy network, immune cell abundance was only correlated with serum cytokine concentration and gut microbial composition. Neutrophils within the obese immune cell abundance group were correlated with the most number of other biomarkers. Two different types of neutrophil measurements were taken, an abundance measure from immune cell gene expression and a whole blood count, with correlations to 10 and 6 other biomarkers, respectively. From the combined number of 16 biomarkers, 4 biomarkers were correlated between the two measurements: *Anaerostipes* abundance, *Blautia* abundance, *Escherichia*/*Shigella* abundance and *Flavonifractor* abundance.

Conclusion: The obesity-related dysregulation of immune biomarkers was suggested by the high connectivity of immune cells in the obese network compared to the healthy network. Our study also revealed the importance of integrated analysis to uncover immune-microbiome interactions in obesity that are likely to have been missed in univariate analysis.

Keywords—obesity, inflammation, network analysis, gut microbiome

I. Introduction

Obesity is a multifactorial disease that dysregulates many different body systems, including the immune system [1] and the gut microbiota [2], leading to increased risk of chronic diseases, including type 2 diabetes mellitus (T2DM), some cancers, and increased mental health problems [1]. Despite extensive research, no specific biomarker profile is clinically recognised to characterise individuals with a greater risk of developing obesity related disease [3]. A key reason is due to a failure in considering the interconnected nature of the immune system, host microbiota and metabolic interactions. Many functional studies have now recognised the need for integrated analysis to overcome the issue of redundancy [4, 5], whereby many biomarkers have similar roles, rendering univariate analysis ineffective. Recent technological advances that allow for multiple biomarker analysis are overcoming the limitations associated with biological complexity to understand the basis of diseases. However, the interpretation and visualisation of the significant amount of data generated from these methods still poses a challenge [6].

Correlation-based network analysis (CNA) has recently become a popular data-mining method as it allows complexity reduction of multidimensional data while still retaining the majority of information needed for interpretation [6]. CNA provides the means to visualise disease-related perturbations of molecular interactions to provide insight into key underlying mechanisms that drive disease development [7]. In biological network analysis, biomarkers are represented as nodes and the links between them as edges. A major component of CNA is betweenness centrality, the measure of shortest paths between any two nodes that pass through the node in question [6]. The node with the highest betweenness centrality score has the biggest impact on a system and should therefore be considered in treatment strategies. CNA has been used in many different studies, including a study by Nishihara et al., where it was used to reveal key molecules mutated in colorectal cancer. Nishihara et al. compared proximal and distal colorectal cancer networks and found two out of the 54 analysed biomarkers, microsatellite instability and *BRAF* mutation status, to play important roles in proximal colon carcinogenesis [7]. Understanding correlations between biomarkers provides insight into the pathogenesis of diseases which may advance research in prevention or treatment.

Studies examining immune profiles [1] and gut microbial composition [8] in obese individuals have found alterations in favour of pro-inflammatory biomarkers when compared to their lean counterparts. A study by Winer et al. has also found high pro-inflammatory to anti-inflammatory biomarker ratios in obese individuals that exacerbate chronic

disease development [9]. However, studies have still struggled to find biomarkers that may be targeted for therapeutic purposes. Due to the multitude of molecular interactions affected by obesity, a holistic approach is required to identify key biomarkers involved. This study aims to use CNA to compare anthropometric measures, metabolic measures, immune cell abundance, serum cytokine concentrations, and gut microbial compositions to identify biomarker profiles that distinguish obese from healthy weight individuals.

II. Methods

A. Study design and ethics

A correlation-based network analysis was performed on anthropometric measures, metabolic measures, immune gene expression, serum cytokine concentrations and gut microbial composition collected from 11 obese individuals with metabolic syndrome (MetS), as per the Adult Treatment Panel III criteria [10], and 12 healthy weight men. All participants were aged between 18 and 65 years without a history of medical conditions known to affect the immune system, including: cancer, Crohn's disease, liver disease, and irritable bowel syndrome. Additionally, participants were excluded if they use any immune-modulating medications or supplements, such as: non-steroidal anti-inflammatory drugs (NSAIDs), fish oil and probiotics. Ethics for this study was approved by the Griffith University Human Research Ethics Committee (MED 18.15.HREC) and all participants provided written informed consent prior to their involvement in the study.

B. Sample collection and analysis

Fasting blood samples were collected for analysis of metabolic (lipids, glucose, glycated haemoglobin [HbA1c]) and inflammatory (C-reactive protein [CRP], erythrocyte sedimentation rate [ESR], circulating cytokines) measures. In addition, RNA was isolated and analysed using an immune profiling panel of 770 genes (nCounter® PanCancer Immune Profiling Panel, NanoString Technologies, Washington, USA) to estimate the abundance of different immune cells, including mast cells, neutrophils and different T cell subsets. Faecal samples were also collected and microbial compositional sequencing was undertaken via 16S rRNA sequencing and taxonomic classification.

C. Correlation-based network analysis

To compare key demographic measures of obese and healthy weight participants, an unpaired t-test was used and measures were expressed as mean ± standard deviation. Differences in measures were considered significant if the p-value was less than 0.05. The dataset was split into five different variable groups: anthropometric measures, metabolic measures, immune cell abundance, serum cytokine concentrations, and gut microbial composition.

Correlation networks were constructed by calculating the Pearson correlation coefficient for each biomarker with all other biomarkers in all five variable groups. Each biomarker that has a strong correlation with another biomarker will appear as a node in the CNA. The existence of strong correlations between biomarkers of different variable groups was denoted by a single line connecting the variable groups involved, regardless of the number of correlations found. Nodes of biomarkers without strong correlations with any other biomarker were not included in the CNA. Strong correlations between biomarkers were defined by a Pearson correlation coefficient greater than $|\pm 0.7|$ and visually represented by a link between the two nodes. Due to the small sample size, the Pearson correlation coefficient threshold required for a correlation to be considered significant was set very high rather than using a significance level. A complete case correlation analysis was conducted, meaning that biomarkers with missing data were excluded from the network analysis.

All the variables involved in the correlation analysis were continuous variables. The node degree is the number of strong correlations a particular node has with other biomarkers. Different node sizes visually demonstrate the degree of each node, with a bigger sized node representing a greater node degree. The betweenness centrality score was also computed for each node, describing the number of shortest paths between any two biomarkers that passes through the node in question. A node with a high betweenness centrality score is more well-connected within the network and therefore considered to be the driver of the network. As each variable group has different numbers of nodes, it is difficult to compare betweenness centrality scores across variable groups. Instead, the essentiality of nodes in a network was compared by node degree and the ranking of betweenness centrality within their respective variable groups.

All the statistical analyses and network analyses were carried out with custom R (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) scripts.

III. Results

The molecular interactions altered by obesity were analysed by comparing networks within obese and healthy weight individuals through CNA. The characteristics of participants from the two distinct cohorts are described in Table I. Significant differences were observed in all the key demographic measures, except for age, between the two groups. By design, the obese cohort had a higher measure of variables that constitute the criteria for MetS [10]. Two major markers of inflammation, CRP and ESR, were also compared between the two groups and while there was no significant difference in ESR, the obese group had significantly higher CRP concentration ($p = 0.01$). As obesity has been described as a state of chronic low-grade inflammation [3], the obese group having a higher CRP level was expected. Based on this finding, the use of other analytical methods to find possible underlying molecular interactions between inflammatory biomarkers is warranted.

A multi-level correlation network was built for the two groups (Fig. 1). In the CNA, each node represented a biomarker that had a strong correlation with another biomarker, denoted by a link between two nodes based on a Pearson correlation analysis. The total number of edges was higher in the obese network ($n = 237$) compared to the healthy network ($n = 190$). In addition, the obese network was more highly interconnected, with correlations between biomarkers in all variable groups (Fig. 1). Interestingly, immune cells within the healthy network were not found to be correlated with two of the four other biomarker groups: anthropometric

TABLE I. DEMONGRAPHIC CHARACTERISTICS AND METABOLIC MEASURES IN OBESE (N = 11) AND HEALTHY WEIGHT (N = 12) MALES.

	Obese with Mets (n = 11)	**Healthy (n = 12)**	***P-value***
Age (Years)	47.74 ± 8.52	40.98 ± 12.36	0.1*
BMI (kg/m2)	35.25 ± 3.57	23.05 ± 1.30	<0.001*
Waist (cm)	177.82 ± 10.31	82.71 ± 5.03	<0.001*
Fat Mass (%)	34.2 ± 2.30	20.48 ± 2.52	<0.001*
Muscle Mass (%)	26.3 ± 2.09	36.27 ± 2.87	<0.001*
Visceral Fat	16.64 ± 3.53	5.75 ± 1.45	<0.001*
Metabolic variables			
MetS	3.55 ± 0.69	0.17 ± 0.39	<0.001*
SBP (mmHg)	144.55 ± 13.37	122 ± 4.78	<0.001*
DBP (mmHg)	96.91 ± 9.98	76.58 ± 6.49	<0.001*
Triglycerides (mmol/L)	2.18 ± 0.50	1.10 ± 0.62	<0.001*
Cholesterol (mmol/L)	5.58 ± 1.01	5.08 ± 1.15	0.24*
HDL (mmol/L)	1.13 ± 0.18	1.54 ± 0.34	<0.001*
LDL (mmol/L)	3.46 ± 0.87	3.03 ± 0.88	0.22*
HbA1c (%)	5.36 ± 0.43	5.23 ± 0.26	0.42*
Glucose (mmol/L)	5.74 ± 0.71	5.20 ± 0.33	0.04*
CRP (mg/L)	1.77 ± 0.86	0.95 ± 1.04	0.01*
ESR (mm/hr)	6.18 ± 4.62	3.58 ± 0.90	0.27*
AUSDRISK	16.91 ± 4.64	6.25 ± 2.93	<0.001*

* *P* value is based on an unpaired t-test using log-transformed data.
BMI: body mass index; BP: blood pressure; MetS: metabolic syndrome; AUSDRISK: Australian type 2 diabetes risk; HDL: high-density lipoprotein; LDL: low-density lipoprotein; HbA1c: haemoglobin A1c, CRP: C-reactive protein; ESR: erythrocyte sedimentation rate
MetS: scored out of a maximum of five based on presence of five defined metabolic syndrome features

measures and metabolic measures. The high interconnectivity of immune cells in the obese network compared to the healthy network suggests that immune cells are heavily affected in obesity.

Further analysis into the dysregulation of immune cells in obesity found that neutrophils were the biomarkers with the highest node degree. Additionally, within the immune cell abundance group, neutrophils had the second highest betweenness centrality score (0.20) with the first being Treg cells (0.22). The high involvement of neutrophils in the obese network suggests it as a potential mediator in obesity.

Two different types of neutrophil measurements were analysed in this study: neutrophil abundance estimated from gene expression analysis and absolute neutrophil count in whole blood. In the obese network, neutrophil abundance was positively correlated with macrophage abundance (correlation coefficient [ρ] = 0.80), *Blautia* abundance (ρ =0.78), *Escherichia*/*Shigella* abundance (ρ = 0.74), natural killer (NK) cell abundance (ρ = 0.74), *Flavonifractor* abundance (ρ = 0.73), Treg cell abundance (ρ = 0.73), *Anaerostipes* abundance (ρ = 0.72), *Akkermansia* abundance (ρ = 0.71), and *Holdemania* abundance (ρ = 0.70) Neutrophil abundance was negatively correlated with lymphocytes (ρ = -0.73), as expected with neutrophil-to-lymphocytes ratio (NLR) being a measure of inflammation [11]. As with neutrophil abundance, absolute neutrophil count in whole blood was also found to be positively correlated with *Escherichia*/*Shigella* abundance (ρ = 0.91), *Blautia* abundance (ρ = 0.88), *Anaerostipes* abundance (ρ = 0.81), and *Flavonifractor* abundance (ρ = 0.79). Other correlations involving absolute neutrophil counts were: *Butyricicoccus* abundance (ρ = 0.81) and white blood cell count (ρ = 0.75). The discrepancy between correlations in neutrophil measures in gene expression and whole blood emphasise the importance of utilising integrated analysis to unveil results that would have otherwise been missed.

While both measurements of neutrophils were correlated with a total of 16 other biomarkers in the obese network, the healthy network only found 4 correlations between both neutrophil measures. Neutrophil abundance was correlated with the abundance of a gut microbe from the *Lachnospiraceae* family (ρ = 0.71) and absolute neutrophil counts was correlated with *Holdemania* abundance (ρ = 0.76), *Turicibacter* abundance (ρ = 0.70), and white blood cell count (ρ = 0.77). The difference in node degree of neutrophils in the obese network compared to the healthy network implies that neutrophils are key immune factors essential in studies looking to understand mechanisms that underpin obesity-associated disease risk.

IV. DISCUSSION

Correlation-based network analysis was constructed using datasets that have been reported by current literature as being dysregulated in obesity, leading to an increased risk of chronic disease development. The datasets were built by analysing samples from 11 obese and 12 healthy weight men and included: anthropometric measures, metabolic measures, immune cell abundance, serum cytokine concentration, and gut microbial composition Functional studies in obesity have only recently taken the overlapping roles of biomarkers into account in analytical approaches, overcoming issues of redundancy. A multidimensional CNA was therefore used in this study to overcome this limitation and challenge the theory that metabolic disease development is due to changes in only a few individual biomarkers. The aim of this study was to compare the networks constructed for the two studied

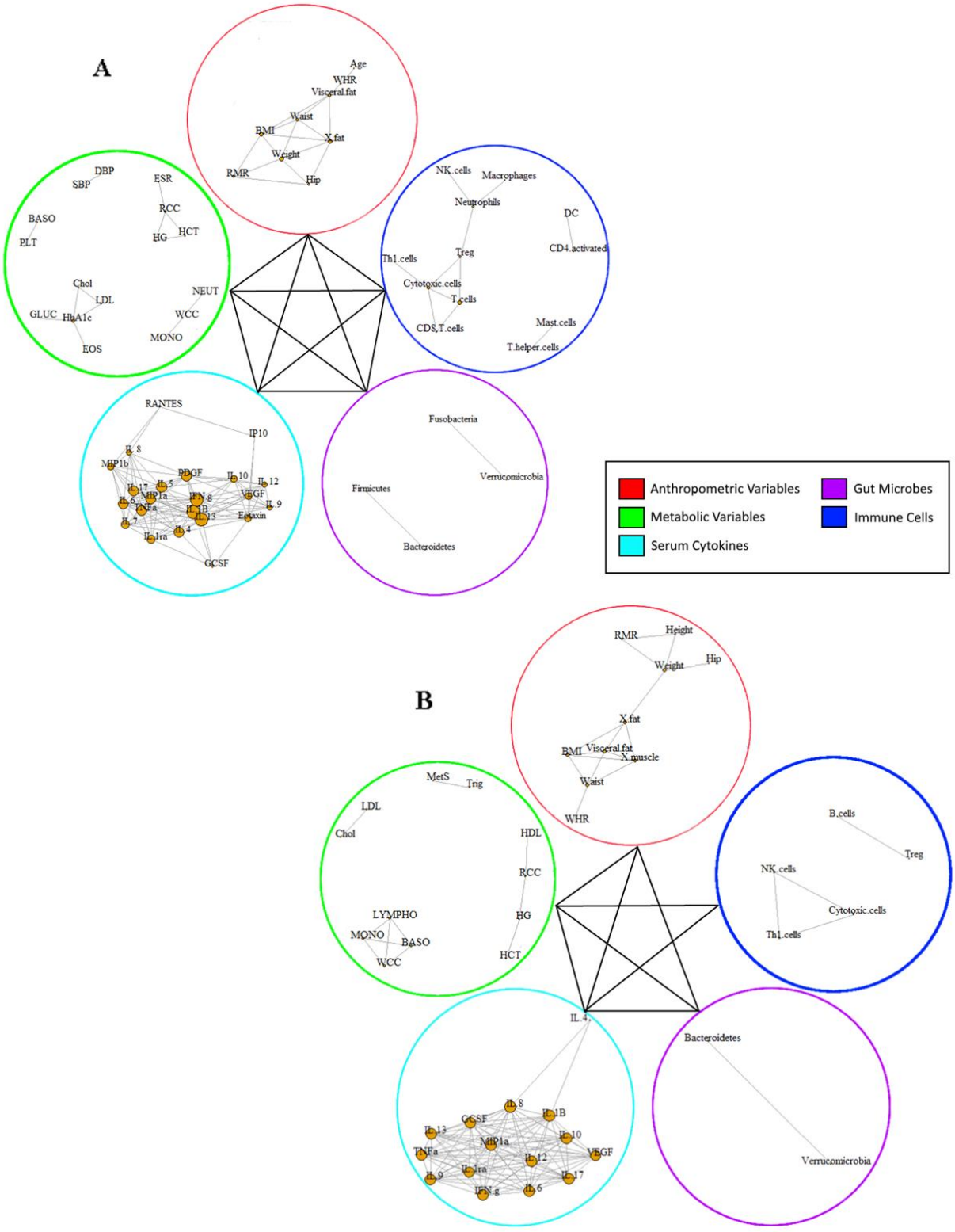

Fig. 1. Multi-level CNA constructed for obese (**a**) and healthy weight (**b**) participants. AUSDRISK: Australian type 2 diabetes risk; WHR: waist-hip ratio; BMI: body mass index; X.fat: percentage fat mass; X.musc: percentage muscle mass; RMR: resting metabolic rate; SBP: systolic blood pressure; DBP: diastolic blood pressure; MetS: metabolic syndrome; Chol: cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; HbA1c: haemoglobin A1C; GLUC: glucose; EOS: eosinophils; MONO: monocytes; WCC: white cell count; NEUT: neutrophils; HG: haemoglobin; HCT: haematocrit; RCC: red cell count; ESR: erythrocyte sedimentation rate; PLT: platelet; BASO: basophils; CRP: C-reactive protein; LYMPHO: lymphocyte; NK cells: natural killer cells; DC: dendritic cells; Treg: T-regulatory cells; Th1: T-helper 1 cells; T cells: T lymphocytes; B cells: B lymphocytes; VEGF: vascular endothelial growth factor; IL-: interleukin-; IP10: interferon gamma-induced protein 10; PDGF: platelet-derived growth factor; IFN.g: interferon gamma; TNFa: tumour necrosis factor alpha; GCSF: granulocyte-colony stimulating factor; MIP1a: macrophage inflammatory protein 1 alpha; MIP1b: macrophage inflammatory protein 1 beta

cohorts to identify key biomarkers that may be targeted for treatment strategies.

When comparing the network constructed for each cohort, the obese cohort produced a denser network than the healthy weight cohort. The differences in the number of correlations suggests that the obese network is more complex and developed than the control network. The concept of a more complex network confirms the paradigm that obesity alters multiple parameters across a broad range of biological systems. This supports the need for integrated analytical approaches to deconstruct the complexity of the biological dysregulation in obesity and to determine which biomarkers may be central for investigation in future studies. The correlation analysis used in this study supports the use of cluster-based analysis to better understand obesity-related disease.

Biomarkers in the obese network were correlated with other biomarkers within their own variable group as well as those from other variable groups. On the other hand, immune cell biomarkers in the healthy network were only seen to be correlated with two other variable groups: serum cytokines and gut microbiome. The contrast between correlations in the obese network and the healthy network suggest that obesity strongly dysregulates the immune system. Studies in both animals and humans have also found obesity-related changes in immune cell numbers and activity that were linked with chronic diseases [12-15]. A closer look into the results of the current study found that within immune cell markers of the obese network, neutrophils were strongly correlated with a number of variables. Neutrophils were found to have correlations with a total of 16 other biomarkers, both within and beyond its own variable group, and had the second highest betweenness centrality score within the immune cell abundance group. The involvement of neutrophils in a high number of molecular interactions suggests the possibility of neutrophils being a regulatory cell in obesity. The correlations that involved neutrophils support current literature describing neutrophils as pro-inflammatory immune cells, with higher frequencies found in obese subjects compared to their lean counterparts [16]. The pro-inflammatory nature of neutrophils is demonstrated in this study through positive correlations found with pro-inflammatory immune cells, including macrophages and NK cells. Surprisingly, neutrophils were also seen to be positively correlated with anti-inflammatory Tregs. A possible explanation for this is found in a study by Mishalian et al. which found neutrophils to be capable of recruiting Tregs, exacerbating the impairment of the immune system in disease [17].

Previous studies have also reported on the link between neutrophils and the gut microbiome [18-21]. A study by Li et al. isolated peripheral neutrophils from participants with the aim of identifying and characterising neutrophil-associated microbiomes [22]. The study classified the isolated specimen into four phyla: Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria [22]. Consistent with the observations reported by Li et al. [22], the current study also found neutrophils to be positively correlated with gut microbes belonging to the Firmicutes (*Butyricicoccus*, *Blautia*, *Flavonifractor*, *Anaerostipes*, and *Holdemania*) and Proteobacteria (*Escherichia*/*Shigella*) phyla.

In this study, two different types of neutrophil measurements were obtained: neutrophil abundance determined by gene expression analysis and absolute neutrophil counts in whole blood. Comparisons of the two different neutrophil measurements found similarities and differences in their correlations. Neutrophil abundance was correlated with 10 other biomarkers while absolute neutrophil count was correlated with 6 other biomarkers. From the total number of 16 biomarkers correlated with neutrophils, only four biomarkers were found to be correlated with both neutrophil measures, *Anaerostipes* abundance, *Blautia* abundance, *Escherichia*/*Shigella* abundance and *Flavonifractor* abundance. The unique interactions between the two neutrophil measures indicate that if the study was to only include neutrophil abundance in the analysis, 6 other molecular interactions would have been missed. Similarly, if only absolute neutrophil count was included in the analysis, correlations with 2 other biomarkers would have been missed. The importance of multidimensional analysis in studies seeking to illuminate underlying molecular interactions has therefore been highlighted.

There are many advantages to using CNA in high-throughput biological studies. A t-test conducted on the same dataset found very little significant differences between the two cohorts in biomarkers. Both neutrophil abundance and absolute neutrophil count in whole blood were not found to be significantly different between obese and healthy weight individuals ($p = 0.962$ and $p = 0.322$, respectively). However, the construction of a correlation network was able to reveal many underlying molecular interactions involving neutrophils that led to the idea that neutrophils are key regulators in obesity. The findings of this study warrant further research targeting obese individuals with high neutrophil counts who may stand to benefit the most from chronic disease risk-reduction strategies.

Another advantage to using correlation networks in multidimensional data is the discovery of patterns that were not considered in the initial study design. The current study compared markers of inflammation between obese and healthy cohorts to find key biomarkers that distinguish the two. Naturally, ESR and CRP would be measured as two major markers of inflammation. However, while there were significant differences in CRP between the two studied cohorts, ESR measures were not significantly different. The difference in results between these two markers highlights the importance of finding negative correlations between neutrophils and lymphocytes. As neutrophil-to-lymphocyte ratio (NLR) is another marker of subclinical inflammation [11], this finding further demonstrates the pro-inflammatory profile of obese individuals in the study. Without CNA, this finding would have been missed by a study that only compared biomarkers between the two cohorts using a simple test statistic, such as a t-test.

The limitations of this study have also been recognised, in particular the small sample size that was used. As a pilot study, the current work was exploratory and utilised high correlation coefficient cut-offs rather than p-values to define significant results. Another limitation is the small number of molecular pathological markers included in the analysis. While many studies in obesity have looked at markers within adipose tissue, the current study performed analysis on

peripheral blood as biopsy studies are extremely invasive and difficult, particularly for a pilot study. Despite these limitations, the study was still able to gather a multitude of results that validates further research with larger sample sizes and datasets.

V. Conclusion

A network approach showed that biomarkers in the obese network had higher interconnection compared to biomarkers in the healthy network. Comparisons of the two networks revealed immune systems to be highly dysregulated in obesity and further analysis found the immune cell neutrophil to be involved in many molecular interactions in obesity. This study demonstrated the need for integrated analysis of different body systems to identify biomarkers that may be targeted for treatment strategies.

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