Quantitative Time-Dependent Variability and Independent Correlation of Eicosanoids in Human Plasma

Scholarly report submitted in partial fulfillment of the MD Degree at Harvard Medical School

Date: 30 January 2020

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Scholarly Report Title: Quantitative Time-Dependent Variability and Independent Correlation of Eicosanoids in Human Plasma

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Abstract

TITLE: Quantitative Time-Dependent Variability and Independent Correlation of Eicosanoids in Human Plasma

Gavin Ovsak, Mohit Jain, Susan Cheng

Purpose: Eicosanoids are small molecule lipid mediators of inflammation that, until recently, could not be measured in a high-throughput fashion or with high-resolution coverage. Eicosanoid-centric inflammation and related pathways have been implicated in the development of both acute and chronic major morbid conditions. Thus, eicosanoids hold promise as valuable biomarkers of disease risk. However, eicosanoid variability within and between individuals is not well understood. This knowledge gap limits the interpretability of detectable eicosanoid levels.

Methods: We performed peripheral blood eicosanoid sampling repeatedly in a cohort of 27 healthy individuals over the course of a year. Using these data, we conducted a comprehensive longitudinal analysis of the inter- and intra-individual variability of eicosanoids over time.

Results: We found that 79.2% of peripherally circulating eicosanoids were relatively stable over time. The vast majority of the over 700 eicosanoids that were repeatedly measurable demonstrated variability coefficients of < 0.5 (standard deviation / mean). There was less variation within individuals than between individuals. Cluster analyses revealed distinct groups of intercorrelated eicosanoids that demonstrated stable connectivity across individuals. Among these stable groups of eicosanoids, certain sentinel eicosanoids were identified as independently highly correlated with other eicosanoids with a group in a manner that was also consistently found across individuals.

Conclusions: Our results indicate that detectable and quantifiable eicosanoids in the human peripheral circulation are largely stable over time. Importantly, patterns of eicosanoid stability include persistent intercorrelated clusters that include sentinel eicosanoid species that demonstrate a consistently high independent correlation with many other eicosanoids. These sentinel eicosanoids may represent particularly important candidate markers or targets for further investigation of inflammation-related human disease.

Glossary of Abbreviations

UPGMA - Unweighted Pair Group Method with Arithmetic Mean
CANTOS - Canakinumab Anti-Inflammatory Thrombosis Outcomes Study

Scholarly Project Question

The aim of my exploratory basic research has been to characterize intra-individual eicosanoid variance and inter-eicosanoid relationships for the purpose of supporting the identification of potential biomarkers of disease and therapeutic targets.

Inflammation and inflammation-related pathways have been implicated in the development of both acute and chronic major morbid conditions[1-3]. Eicosanoids are small molecule lipid mediators of inflammation and have been relatively understudied. Eicosanoids have been historically challenging to measure in humans, with most prior studies having reported the measure of up to dozens of only the most highly abundant species at a time[6-7]. Recently developed technology now enables measurement of hundreds of eicosanoids in human plasma, including low abundance as well as higher abundance species. The diversity and range of the levels of these eicosanoids in human plasma have not previously been reported. Furthermore, the degree to which eicosanoids measurable in human plasma may vary within or between individuals over time is not known.

Previous work has been done to characterize the sources of variability between individuals by measuring eicosanoid levels with mass spectrometry at a single time point[8]. In this work, procedural and analytical factors regarding the collection, storage, and processing of samples was documented to be of significance for standardizing measurement of eicosanoids between labs. This study concluded by emphasizing that the natural variance of each circulating eicosanoid has not been established so far. They remark that knowledge of the natural intra-individual variance “will be crucial to appropriately power experimental designs and to enhance the identification of reliable and relevant biomarkers of disease”. This has been the impetus behind my scholarly project question.

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Contribution to Work

My role has been primarily to conduct independent, remote data analysis and visualization of an existing dataset of repeated samples over 700 eicosanoids for 27 healthy individuals over time. This anonymous data was previously collected for a separate study by a lab affiliated with my mentor, designed to look for consistent periodic changes in eicosanoids over extended time periods. I independently calculated the intra-individual coefficients of variance for each eicosanoid marker, as well as the inter-individual variance, and finally used network analysis methods to identify the spearman rank correlation coefficient between each eicosanoid pair to rank eicosanoids by their level of independent correlation with other eicosanoids in the set. Secondarily, subgroup analysis was repeated within individuals by sex.

I am the primary author and wrote the manuscript below which summarized the results of this study. This manuscript has not been submitted yet. However, it is currently under review by the other authors.

Appendix

Quantitative Time-Dependent Variability and Independent Correlation of Eicosanoids in Human Plasma

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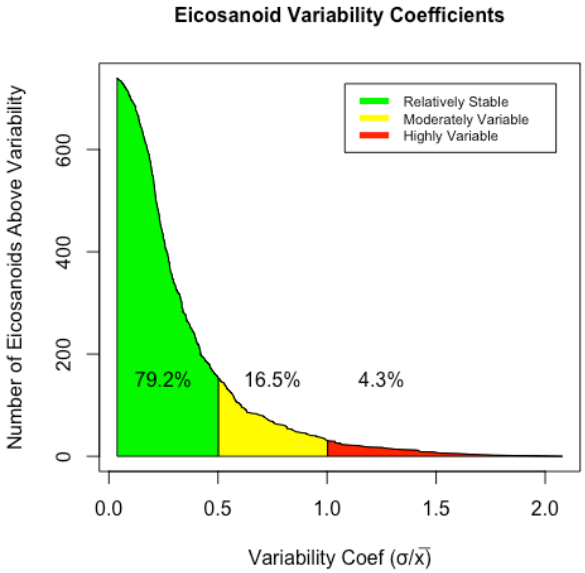
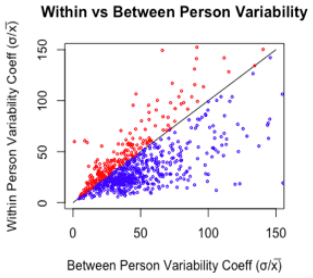
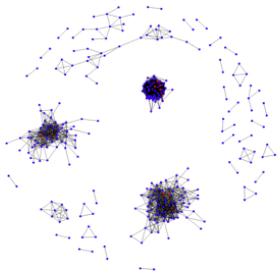
ABSTRACT

Eicosanoids are small molecule lipid mediators of inflammation that, until recently, could not be measured in a high-throughput fashion or with high-resolution coverage. Eicosanoid-centric inflammation and related pathways have been implicated in the development of both acute and chronic major morbid conditions. Thus, eicosanoids hold promise as valuable biomarkers of disease risk. However, eicosanoid variability within and between individuals is not well understood. This knowledge gap limits the interpretability of detectable eicosanoid levels. We performed peripheral blood eicosanoid sampling repeatedly in a cohort of 27 healthy individuals over the course of a year. Using these data, we conducted a comprehensive longitudinal analysis of the inter- and intra-individual variability of eicosanoids over time. We found that 79.2% of peripherally circulating eicosanoids were relatively stable over time. The vast majority of the over 700 eicosanoids that were repeatedly measurable demonstrated variability coefficients of < 0.5 (standard deviation / mean). There was less variation within individuals than between individuals. Cluster analyses revealed distinct groups of intercorrelated eicosanoids that demonstrated stable connectivity across individuals. Among these stable groups of eicosanoids, certain sentinel eicosanoids were identified as independently highly correlated with other eicosanoids with a group in a manner that was also consistently found across individuals. Our results indicate that detectable and quantifiable eicosanoids in the human peripheral circulation are largely stable over time. Importantly, patterns of eicosanoid stability include persistent intercorrelated clusters that include sentinel eicosanoid species that demonstrate a consistently high independent correlation with many other eicosanoids. These sentinel eicosanoids may represent particularly important candidate markers or targets for further investigation of inflammation-related human disease.





Eicosanoid Cluster Analysis



BACKGROUND

Inflammation and inflammation-related pathways have been implicated in the development of both acute and chronic major morbid conditions[1-3]. These pathways have served as primary or secondary targets of standard of care medical therapies including aspirin and statins[4]. The inflammatory hypothesis is still being evaluated however with mixed results on outcomes for drugs with isolated anti-inflammatory effect. This research has seen renewed or augmented interest in light of the CANTOS trial of 10,061 patients who received a monoclonal antibody targeting interleukin-1β which found a significant reduction in cardiovascular events over placebo with dose-dependence[5]. Eicosanoids are small molecule lipid mediators of inflammation and have been relatively understudied. Eicosanoids have been historically challenging to measure in humans, with most prior studies having reported the measure of up to dozens of only the most highly abundant species at a time[6-7]. Recently developed technology now enables measurement of hundreds of eicosanoids in human plasma, including low abundance and higher abundance species. The diversity and range of the levels of these eicosanoids in human plasma have not previously been reported. Furthermore, the degree to which eicosanoids measurable in human plasma may vary within or between individuals over time is not known. Intra-person variability, in particular, is important to understand when considering potential markers of acute or chronic physiological or pathophysiological processes that may underlie the

development of inflammatory disease states. Intra-person variability is also important to understand when evaluating potential targets for anti-inflammatory therapeutics. The baseline variability of eicosanoid levels is critical when evaluating the statistical significance of changes in eicosanoid levels with observed disease or controlled medication activity. Intra-person variability may therefore be an important factor in deciding which eicosanoid candidates to observe for significant changes. Studies of repeated measurements within individuals allow not only for analyzing time variability of eicosanoids but also for analyzing persistence of inter-eicosanoid level correlations, which may be of biological importance. Eicosanoid pairs that show high levels of relative intercorrelation not only during single random samples but also upon multiple repeated measurements over time may be connected through biologically mediated signaling pathways. The connectivity networks of eicosanoids are important to consider when determining candidates for pharmaceutical targeting of inflammation pathways, as certain molecules may be more important than others for causing large scale physiologic impact.

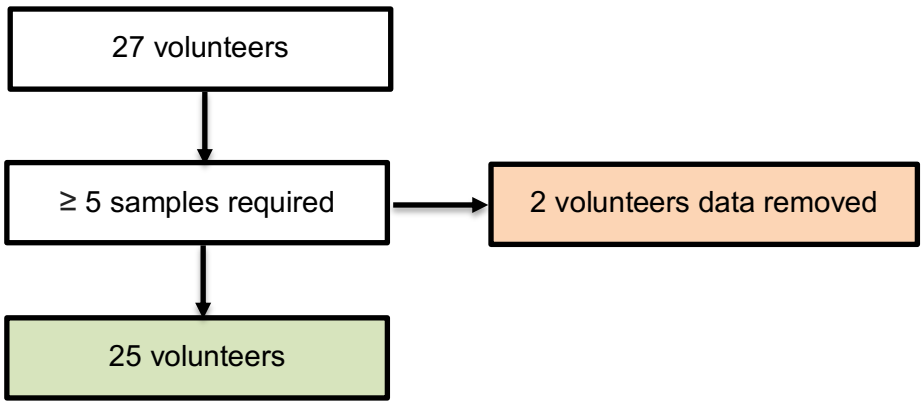
METHODS

Study design

In this study, plasma samples were collected from 27 volunteers without significant acute or chronic diseases who were not taking any medications (mean age 29 years, 60% women). Samples were collected over the course of an 89-day period. Plasma samples were sent to Jain Laboratory, Institute for Metabolomics Medicine, UCSD, for mass spectrometry analysis of composite eicosanoid species.

Data management

Two volunteers had less than 5 samples collected and were removed from the data set. Between 9-19 plasma samples were collected each from the remaining 25 volunteers with an average of 17 samples per individual.



Across all patients, a total of 771 distinct eicosanoid species were identified as being present. Not all eicosanoids were detected in each patient sample, and some were more widely detected than others. In order to compare eicosanoid levels across patients, a data-completeness criterion of over 2/3 of data being present across individuals was applied. 649 eicosanoids (84%) met these data-completeness criteria and were included in further analysis (Figure 1).

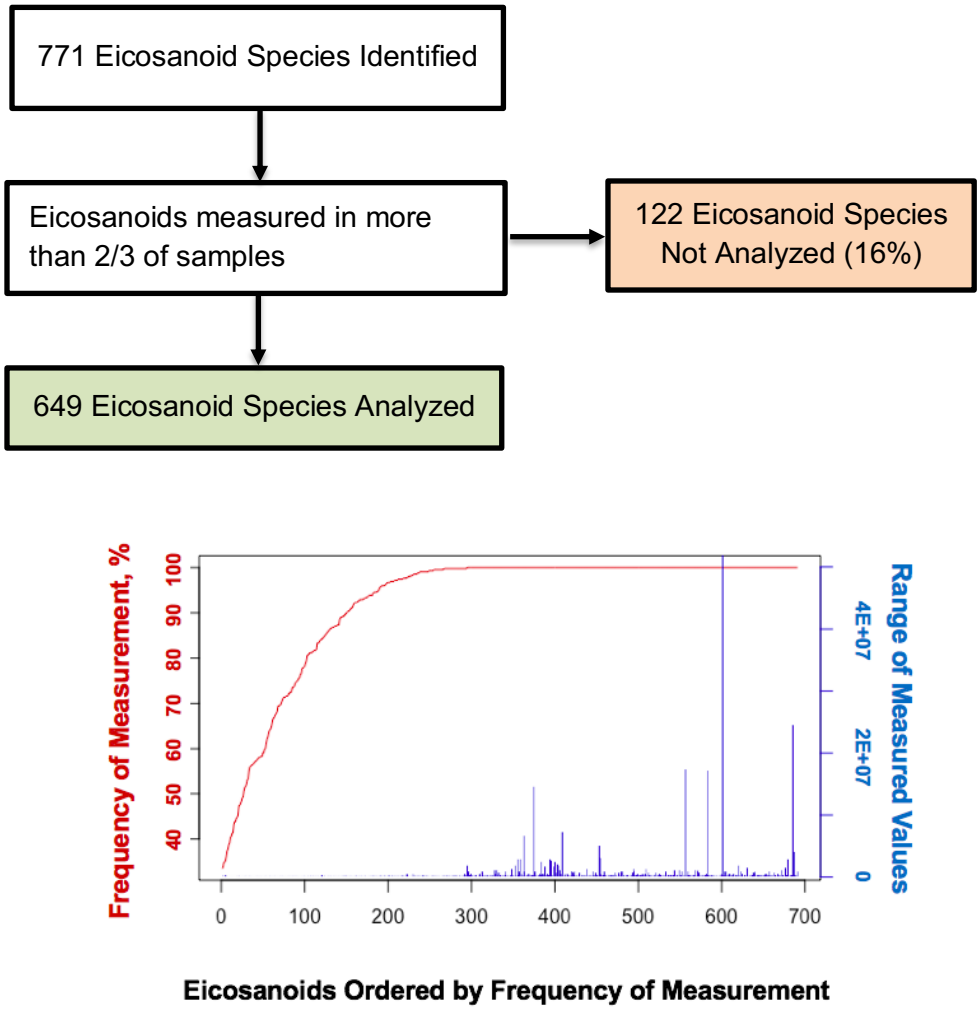


Figure 1. Of the over 771 human plasma eicosanoids analyzed, 649 eicosanoids (84%) were detectable in over 2/3 of samples. A majority (57.45%) were measurable in every plasma specimen (i.e. 100% frequency of measurement).

DATA ANALYSIS

First, the frequency and range of all eicosanoids were measured (Figure 1). Next, in order to understand the extent to which circulating plasma eicosanoids vary in their measurable levels over time within people, a coefficient of variation was calculated for each eicosanoid species. This measure of variation is the standard deviation divided by the mean and is useful for comparing relative variability across data with significant differences in individual means. Using the coefficient of variation, we then categorized eicosanoids as relatively stable (<50 var. coeff.), moderately variable (50-100% var. coeff.), and highly variable (>100% var. coeff.) (Figure 2, Panel A).

The extent to which eicosanoid variation is related to differences between versus within individuals was then analyzed. This was accomplished by comparing the average variability coefficient for each eicosanoid within people to the variability coefficient of individual eicosanoid means between people (Figure 2, Panels B and C). To illustrate between-person variability, a randomly selected eicosanoid was plotted with subject ID on an x-axis with each sample data point displayed along the y-axis in a strip plot overlaid with a boxplot (Figure 2, Panel D).

Inter-eicosanoid correlation was then analyzed over time across all subjects to identify clusters of correlated eicosanoids. Correlation was measured with ranked spearman R^2 correlation coefficients between every pair of individual eicosanoids. Cluster analysis was performed with multiple techniques for visualization of key network properties. First, correlation matrices were visualized as heatmaps clustered by UPGMA, with an additional column showing the variability class for each eicosanoid from Figure 2 (green: stable, yellow: moderate, red: highly variable). Next, significant correlations between eicosanoids were represented as connections between nodes in a connected network diagram. In the network diagram, connections between eicosanoid nodes with spearman coefficients above 0.8 were included as links between nodes in a 2D physics-simulation using d3.js [8].

Due to tightly connected clustering of highly correlated eicosanoids, it was hypothesized that within triplets of highly correlated eicosanoids, connections which had the lowest correlation were most likely to represent the cumulative relationship through the third eicosanoid with accumulated noise from each of the other two stronger connections. Based on this framework, a pruned network diagram was produced which only included connections which met the previous strength criteria of 0.8 as well as met the criteria that no stronger connections existed connecting each eicosanoid through a common third eicosanoid. Connections with previously

connected intermediates were assigned a color to visualize groups from highly connected bundles.

To identify eicosanoid targets for investigation which are central to each correlation cluster, eicosanoids were ranked by the sum of their spearman correlation coefficients with other eicosanoids across pruned connections. The top 15 most connected eicosanoids were labeled “Sentinel Eicosanoids” to highlight their strong relationships to many other eicosanoids, not better explained through intermediate eicosanoid correlations. The top 15 sentinel eicosanoids were identified and highlighted as red points in unpruned and pruned network diagrams.

Systematic cluster analysis (heat maps, pruned and unpruned correlation maps, and sentinel eicosanoid ranking) was repeated within data subsets from each sex to identify potential sex-differences in physiologic clusters. Cluster analysis was also done on single time points across people to illustrate the added value of time series analysis for cluster discrimination. Finally, cluster analysis was performed on data from four representative subjects to compare inter-subject differences.

RESULTS

A summary of basic characteristics of the study sample is shown in Table 1. It is apparent from this plot that the ranges for each individual eicosanoid varied over many orders of magnitude. When analyzing repeated measures, over half (79%) of eicosanoids were observed to be relatively stable (exhibiting variability coefficients <50% deviation from mean levels), while 17% were observed to be moderately variable (exhibiting variability coefficients between 50% to 100%), and only 4% were observed to be highly variable (exhibiting > 100% variability coefficients) (Figure 2, Panel A).

Of eicosanoids that were highly variable, the majority of variability was due to between person rather than within person variability, as shown in Figure 2, Panel B. On a fine-grained level, data from one eicosanoid is shown in Figure 2, Panel C which illustrates significant between person variation.

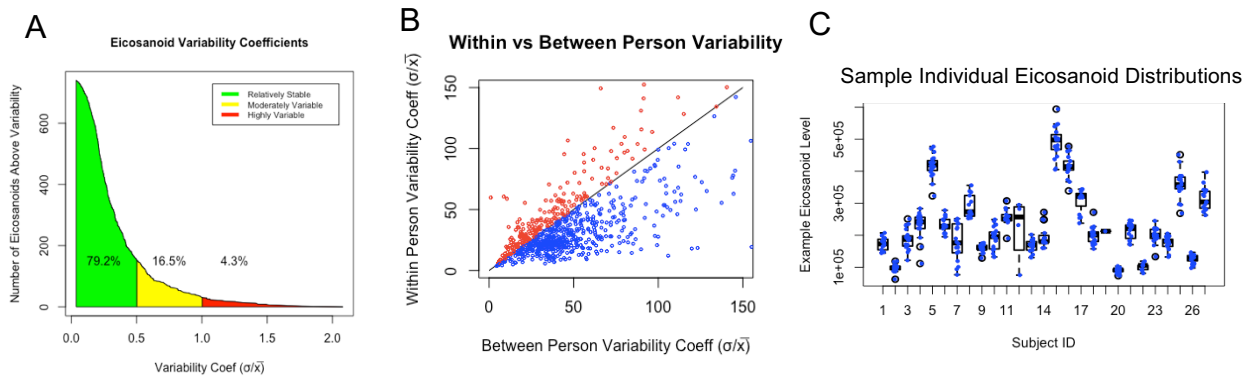


Figure 2. Eicosanoids Variability Over Time.

The results of cluster analysis on longitudinal samples across patients revealed three primary clusters that tended to remain highly correlated over time (Figure 3), with the majority of analytes within these large clusters also tending to be relatively stable over time; (Figure 3, Panel A).

While these clusters are tightly correlated internally, by pruning connections based on the previously described method, there appear to be a handful of central eicosanoids which are directly correlated with a large number of others in sunburst patterns (Figure 3, Panel B). No stronger connections exist connecting the central eicosanoids to each of their pruned connections through any third eicosanoid. These central eicosanoids are specifically identified as the top sentinel eicosanoids in Figure 3, Panel D and are identified using their rt/min followed by their m/z properties from mass spectrometry.

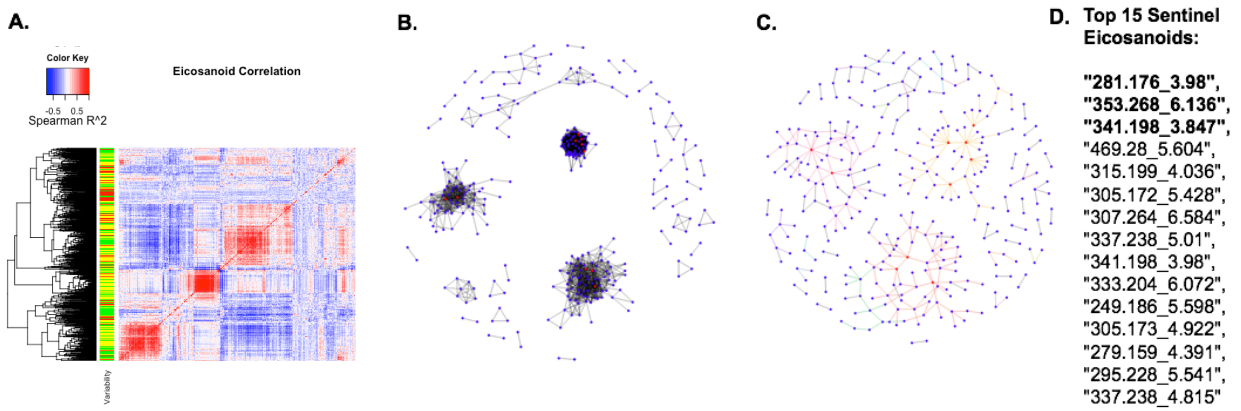


Figure 3. Eicosanoid Cluster Analysis.

Observing the results of cluster analysis in data subsets restricted to male and female individuals, eicosanoid clusters remained largely stable (Figure 4). Notably, the top three sentinel eicosanoids from cluster analysis in the total dataset are within the top 15 sentinel eicosanoids in sex-specific clusters.

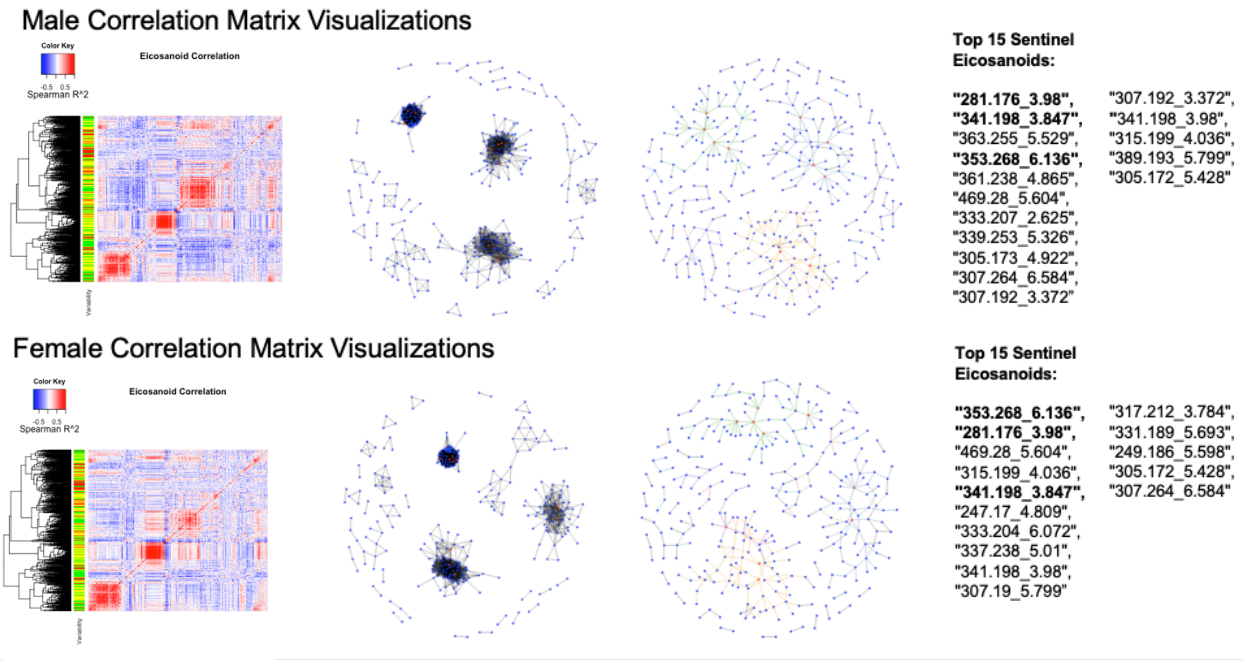


Figure 4. Cluster analysis repeated within data from each sex. Between sexes, eicosanoid clusters remained largely stable, likely due to common underlying essential physiology. The top three sentinel eicosanoids from **Figure 3** are highlighted in bold as they reappeared.

Restricting data from a single time point yielded notably less discrete inter-eicosanoid clusters (Figure 5).

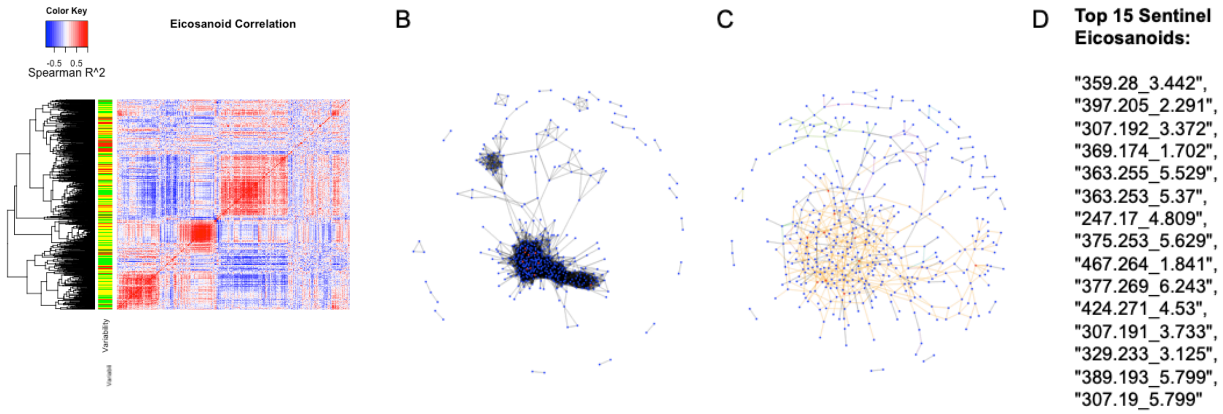


Figure 5. Correlation matrix visualizations from data restricted to the first time point across subjects. Restricting to data to a single time point yielded much less discrete inter-eicosanoid clusters in Panels B and C than the cluster analysis results of Figures 3, 4, and 6.

Isolating data to time series within individuals revealed similar primary three clusters across individuals (Figure 6). Individual cluster analysis had more variation in sentinel eicosanoids identified, however, all four individuals had sentinel eicosanoids within their top 15 eicosanoids which were in the top 3 sentinel eicosanoids overall from Figure 3.

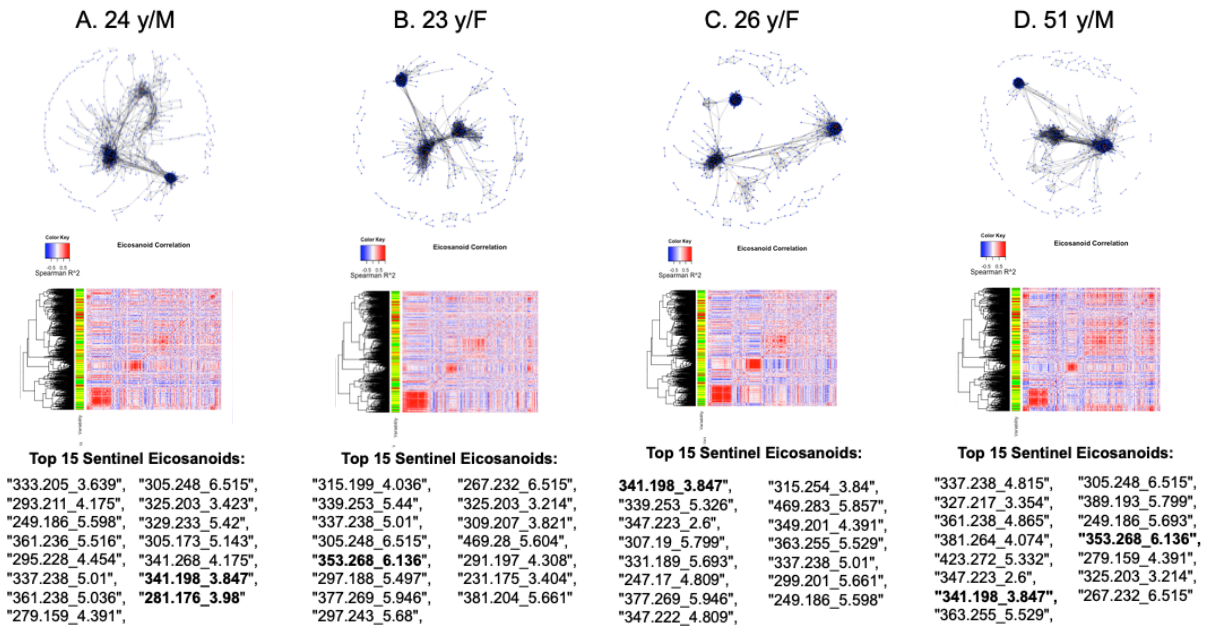


Figure 6. Correlation matrices from four individuals

DISCUSSION

It is now possible to reliably measure several hundred distinct eicosanoid species in human plasma, in a repeated fashion, allowing for analyses of within individual as well as between individual variability, even for eicosanoid species that tend to be relatively low in abundance in circulating plasma. In this analysis of a comprehensive panel of eicosanoids measured repeatedly in healthy humans, we observed 79% to be relatively stable (with <50% variability coefficient) while 16.5% were moderately variable (50% to 100% variability coefficients) and only 4% were highly variable (>100% variability coefficient). Overall, eicosanoids tend to vary much less within people than they vary between people.

Within every subject, certain large groups of eicosanoids tend to remain highly correlated with each other across repeated measures, predominantly in a very stable time-invariant relationship. These eicosanoids may reflect important time-invariant biological relationships between certain eicosanoid-related pathways. In particular, in the three largest clusters, one cluster was largely representative of eicosanoids in the putative class of prostaglandins, another was composed of unsaturated fatty acids, octadecanoids, and docosanoids, and the last cluster was composed of a mix of dydroxy/hydroperoxyeicosatrienoic acids and prostaglandins.

Eicosanoid measurements are currently rarely done in a repeated series within individuals. This study has shown the importance of within-individual time series in multiple ways. Between individual variation has been shown to be more significant than within person variation with large differences between individual means. Also, when data is restricted to initial time-points for cluster analysis (Figure 5), there is much less ability to discriminate inter-eicosanoid relationship networks which are consistently observed within each individual (Figure 6) and across groups of individuals over time series (Figure 4). Sentinel eicosanoids which are especially strongly connected to a network of stable eicosanoids have also been identified for future investigation (Figure 4).

CONCLUSION

In conclusion, it has become feasible to measure the levels of large numbers of eicosanoids in individuals across time. Inflammatory pathways which involve eicosanoid species have been implicated in the pathophysiology of significant acute and chronic morbidity, which suggests the potential for use of eicosanoid species as biomarkers of disease or targets of therapy. To

achieve these aims, it is important to understand the underlying baseline variability of these species over time within and between individuals. We analyzed a longitudinal time-series of a comprehensive panel of eicosanoid in healthy individuals. These analyses have shown that eicosanoids are predominantly stable (79% with a variability coefficient <50%), vary more between individuals than within individuals, and are intercorrelated in consistent networks across individuals. Significant variation in levels between individuals highlights the importance of time-series data for each individual studied to determine meaningful correlation with external inflammation perturbations. In addition, multiple candidates have been identified for further study which are sentinel eicosanoids with independently high correlation to many other eicosanoids and which are predominantly stable over time. These highly intercorrelated eicosanoids may represent fundamental eicosanoid pathways. Even minor persistent perturbations of these pathways could have important biological consequences.

APPENDIX

Supplemental Table 1: Subject Demographics

Sex	M (11), F (16)
Age	Mean: 29, St. Dev: 7
Weight	Mean: 68kg, Standard Deviation: 15kg
Height	Mean: 169cm, Standard Deviation: 9cm

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