Dried Blood Spots as a Suitable Matrix for Phenotyping the Metabolome: A Proof of Concept Study

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Introduction

Since about 1998, the metabolome[1] has come to be defined as a compendium of small molecules (< 1,000 Da) called metabolites that are either encoded by the host genome (endogenous) or introduced to the host (exogenous) through the diet, medication, or environmental exposures. Endogenous metabolites are essential for key physiologic functions,[2] while exogenous metabolites may benefit or harm the host, depending on the compound and/or level of exposure.[3] In this article, we discuss applications of metabolomics as a molecular profiling technology that provides a real-time snapshot of the metabolome and, hence, an individual's physiologic status. The underlying technology combines analytical chemistry techniques – typically high-performance chromatography and mass spectrometry – and advanced statistical methods to study the metabolomic profile.

The metabolome is highly sensitive to internal and external variables, including age, gender, diet, geographical location, and genetics,[4,5] making metabolomic profiling a powerful tool for assessing an individual's phenotype. The metabolic readout of the phenotype, "the metabotype," is a vital tool for biomedical research, drug development, and precision medicine.[6-9]

To date, metabolomics has been used to diagnose and prognosticate disease,[10-14] guide clinical decision making,[15-17] help shape preventative treatment strategies,[18,19] and monitor overall health and wellness.[20] Despite these contributions to precision medicine, adapting metabolomics-based diagnostic testing and follow-up monitoring into clinical practice is challenging due to the need for high performance liquid chromatography and mass spectrometry (HPLC/MS), which do not lend themselves to point-of-care. This highlights a critical need to establish and benchmark methods that enable cost effective access to metabolomics

for diagnosing and monitoring health within the boundaries of centralized technology. In this paper, we introduce a simple collection technology that can be used to generate results consistent with current collection-analysis approaches. We show that this simple collection technology establishes a robust baseline for subject sample evaluation.

The Value of Dried Blood Spots (DBS) as a Matrix for Metabolomics and Precision Diagnostic Applications

Protocols have been developed to profile metabolomes in serum, plasma, urine, and tissue.[21-23] However, most of these matrices require cold chain storage to keep metabolites stable and are not amenable to self-collection. Dried blood spots (DBS) are routinely used as a simple, inexpensive, and non-invasive method to analyze blood components for medical diagnoses. DBS require only a fingerstick-sized volume that patients can usually collect themselves and can be shipped and stored at ambient temperature with reasonable assurance of stability.

Using DBS to profile the metabolome has gained traction in recent years, with several studies reporting satisfactory analytic performance and pathway coverage.[24-27] However, the utility of DBS to capture the global, longitudinal, biological, and pathological metabolic changes within each individual – a metric vital to precision medicine – has not been extensively investigated. The objective of this study was to evaluate the performance and characteristics of metabolomic profiles from a collection of DBS. Our findings provide the foundation for future studies aimed at establishing untargeted metabolomic profiling of DBS as a mainstream diagnostic testing modality for wellness and precision medicine initiatives.

Methods

Study Participant Characteristics

This IRB-approved study was open to all non-pregnant individuals 18-75 years old. Subjects were internally recruited at Metabolon by the study team and provided informed consent before participating. All health information was self-reported.

This study included 2 cohorts: 1) 49 self-reported healthy donors; 25 males aged 27-69 and 24 females aged 20-62, and 2) 22 donors; 10 males aged 38-61, 10 females aged 34-69, and 2 donors who did not report their age or gender.

Sample Collection

For matched comparisons between DBS and plasma, a phlebotomist collected one venous whole blood sample per individual into an EDTA tube according to Metabolon's IRB-approved protocol. 50µl of each blood sample were spotted onto Whatman 903 Protein Saver Cards (Sigma Aldrich #WHA10534612). Plasma was isolated from the remaining sample then stored at -80°C. For longitudinal analyses each volunteer was asked to self-collect one DBS sample via fingerstick each weekday at their home for 30 days and return them to Metabolon at the end of each week. All blood spots were dried at room temperature for 4 to 24 hours, sealed in gas-impermeable bags with desiccant, and stored at -20°C.

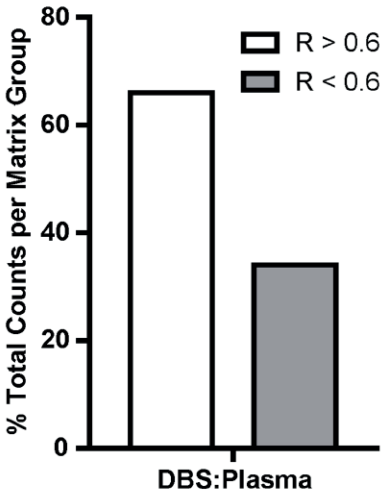
Sample Processing

Samples were processed according to validated

A

Super Pathway	Plasma	DBS
Lipid	450	304
Amino Acid	174	135
Xenobiotics	95	44
Nucleotide	31	32
Peptide	21	13
Cofactors and Vitamins	29	18
Carbohydrate	22	30
Energy	9	8
Partially Characterized	10	2
Total	**841**	**586**

B



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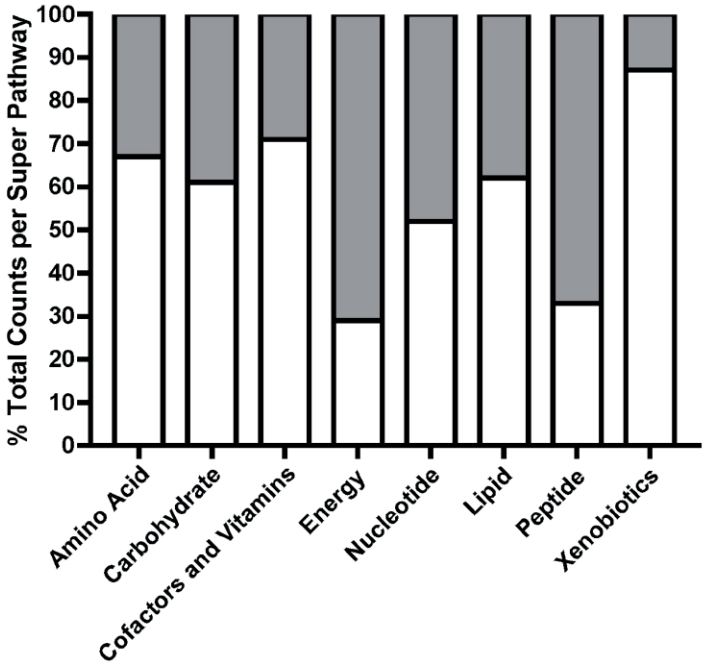


Figure 1: **Metabolite composition of DBS and plasma. (A)** A breakdown of super pathways covered by metabolites that were recovered from < 70% (35/49) of plasma and DBS samples. **(B)** Agreement between the relative abundances of each biochemical detected in > 70% of matched DBS and plasma samples. **(C)** Correlation comparison between recovered metabolites by super pathway.

> Our findings provide the foundation for future studies aimed at establishing untargeted metabolomic profiling of DBS as a mainstream diagnostic testing modality for wellness and precision medicine initiatives.

methods with certain modifications made for DBS.[28-30] Briefly, industry standard 2 x 6 mm punches were taken from each DBS and rehydrated by shaking with a small aliquot of water. Protein was precipitated by shaking with methanol on a SPEXC 2000 Geno/Grinder and centrifuging. For quality control (QC) purposes, several recovery standards were added to each sample before extraction. The extracted supernatants were divided into 4 aliquots then placed on a sample evaporator (SPE-Dry 96) to remove organic solvent. Dried extracts were stored overnight under nitrogen. Dry Whatman card punches (DBS blanks) were extracted using an identical method in every set to ensure curated biochemicals met a 3:1 signal-to-noise ratio.

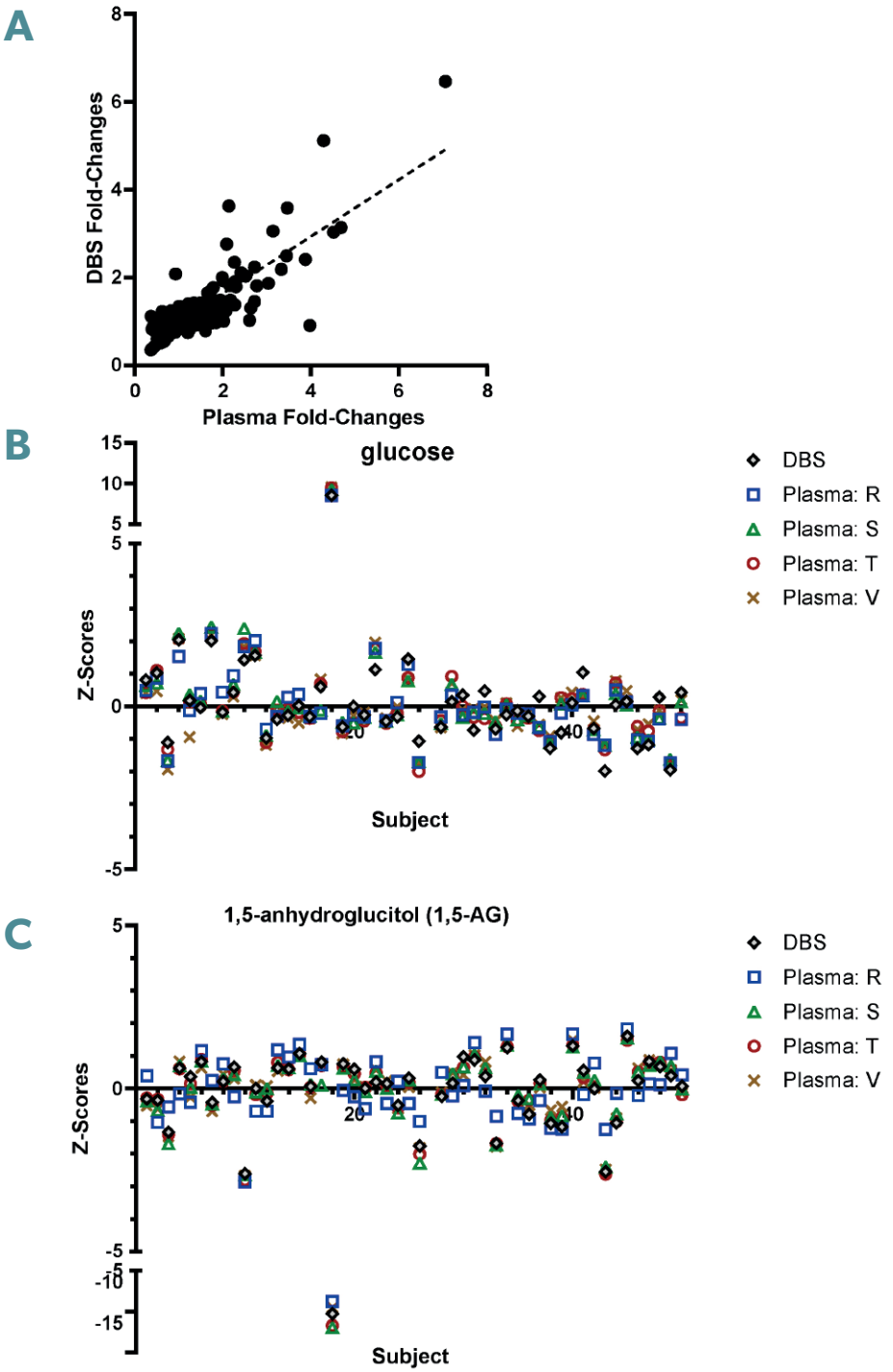


Figure 2: Agreement between fed/fasted signatures in DBS and plasma. (A) A graph plotting the fold changes for each metabolite shown in Table 2. **(B,C)** Plots of z-scores showing the analytic agreement between individual donors, sample matrices, and mass spectrometers. Letters R, S, T, and V indicate four different mass-spectrometry platforms used to analyze plasma samples. DBS samples were run on platform V.

A DBS QC sample was extracted with 4 technical replicates in every set to monitor reproducibility.

Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC/MS-MS)

Untargeted UPLC-MS/MS of known biochemicals was performed on samples extracted from DBS as described.[28-30] All samples were subjected to four different chromatography methods. Each of the 4 aliquots of dried extracts were reconstituted in a solvent optimized for each method. Aliquot #1 was analyzed using acidic positive ion conditions optimized for hydrophilic compounds. The extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1 x 100mm, 1.7um) using water and methanol containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Aliquot #2 was analyzed using acidic positive ion conditions optimized for hydrophobic compounds. The extract was gradient eluted from the same C18 column using methanol, acetonitrile, water, 0.5% PFPA and 0.01% FA. Aliquot #3 was analyzed using basic negative ion-optimized conditions on a dedicated C18 column. The extract was eluted from the column with methanol, water, and 6.5 mM ammonium bicarbonate (pH 8.0). Aliquot #4 was analyzed using negative ionization after eluting from an HILIC column (Waters UPLC BEH Amide 2.1 x 150 mm, 1.7 um) using a gradient consisting of water and acetonitrile with 10mM ammonium formate (pH 10.8).

Compound Identification and Data Analysis

Compounds were identified by comparing the mass-to-charge (m/z), retention time, and associated fragmentation spectra in each sample to a library of standard chemical entities as described.[28-31] Technical replicates of DBS QC samples were extracted in each 48 well plate and interspersed throughout the run to monitor the analytical variability of biochemicals. All sample sets met our acceptance criteria of < 10% relative standard deviation (RSD) for recovery standard variability and < 15% RSD for instrument variability. Raw "area under the curve" (AUC) values from samples were used to calculate the number of compounds quantified in each sample type. All values were log-transformed, then converted to z-scores using rankit regression to estimate the mean and standard deviation as described.[32] This analysis determined how many standard deviations the raw intensity of a given metabolite rose above or fell below the mean intensity of that metabolite in a dataset. Analyses were conducted in R[33] and Omicsoft Array Studio version 7.2.[34]

Table 1: Overview of the 49-donor cohort. This cohort consisted of 25 males aged 27-69 (average age 42) and 24 females aged 20-62 (average age 35). The fed group contained 12 males, aged 27-59 (average age 43) and 14 females, aged 20-62 (average age 35). The fasted group contained 13 males aged 23-69 (average age 41) and 10 females aged 27-48 (average age 35).

Fed				Fasted			
Male Subject	Age	Female Subject	Age	Male Subject	Age	Female Subject	Age
1	23	1	20	1	27	1	23
2	31	2	23	2	28	2	27
3	31	3	25	3	32	3	27
4	32	4	27	4	36	4	33
5	33	5	28	5	38	5	33
6	36	6	29	6	42	6	36
7	38	7	30	7	42	7	36
8	42	8	33	8	44	8	41
9	46	9	39	9	48	9	48
10	47	10	40	10	49	10	62
11	57	11	41	11	52		
12	59	12	43	12	62		
		13	46	13	69		
		14	53				

Results

Overview of Cohorts

For DBS verses plasma comparisons 49 self-reported healthy volunteers were divided into "fed" and "fasted" groups that were distributed by age and sex (**Table 1**). The fed group ate *ad libitum* while the fasted group abstained from all food and liquid intake other than water for 8 hours before sample collection.

DBS used in longitudinal analyses were collected from 22 subjects. Of these 22 subjects, 6 subjects reported no health issues, 14 subjects self-reported having at least one medical diagnosis, and 2 participants did not disclose their health status (**Table 2**). A general description of diet and log of medications taken on each study day by each study participant are shown in **Supplemental Figures 1 and 2**.

Metabolomic Profiles of Plasma and DBS

We characterized the global metabolomic profile captured in DBS punches and compared it to the profile in matched samples of plasma, a matrix routinely used for diagnostic testing for which the metabolome is well characterized.[35] Plasma samples were analyzed on four identical mass spectrometry (MS) platforms, referred to as R, S, T and V; DBS was analyzed once, on platform V. To confirm acceptable precision and accuracy of the data we analyzed 40 technical replicates of plasma (10 per platform) and 8 replicates of DBS. Of the 984 total metabolites detected in plasma, 854, 856, 859, and 842 were detected in all 10 replicates for platforms R, S, T, and V, respectively. The median RSDs of these sample pools were 9.6%, 7.6%, 8.7%, and 7.2% on platforms R, S, T, and V, respectively. A total of 861 metabolites were recovered from the DBS technical replicates. Of those, 604 were detected in all 8 replicates and had a median RSD of 8.8%. Observing a smaller number of metabolites in DBS than in plasma is not unusual or unexpected given the lower sample volume.

> There was high correlation between the fed/fasted ratios of the log transformed data in each sample type (r=0.84), demonstrating that the majority of the metabolome in plasma was retained in DBS despite the differences in the testing matrices and DBS having a 5-fold lower extraction volume.

Having demonstrated acceptable RSDs in the technical replicates we analyzed the donor samples. As noted above, 984 and 861 metabolites were recovered from plasma (across 4 platforms) and DBS samples, respectively. Of the 861 metabolites detected in total, 841 metabolites were detected in > 70% of plasma samples and 586 metabolites were detected in > 70% of DBS samples. All (100%) of super pathways and 94% of sub-pathways represented in plasma were also represented in DBS (**Figure 1A**).

Across all four platforms, 482 metabolites were found in > 70% (35) of the 49 matched donor samples. For this group of 482 metabolites, we calculated the correlation coefficients (r values) between each plasma dataset and the DBS dataset. The correlations of the plasma to DBS were 0.76, 0.74, 0.76, and 0.75 for platforms R,S,T, and V, respectively. The percentage of correlations that were at least 0.6 were 65%, 65%, 67%, and 66% for platforms R, S,T, and V, respectively (**Figure 1B**).

When averaged across all 4 platforms, 209 (44%) metabolites showed strong correlation (> 0.8), 107 (22%) showed moderate correlation (0.8-0.6, 0.6 inclusive) and 166 metabolites had correlation below 0.6. When DBS and plasma were compared relative to pathway coverage (**Figure 1C**), xenobiotics and cofactors/vitamins correlated strongest, with 87% and 71% of metabolites showing r > 0.6, respectively. By contrast, energy and peptide pathways had the lowest correlation, with only 29% of energy and 33% of peptide biochemicals achieving an r > 0.6. The weaker correlation between these molecules is not surprising given that energy and peptide metabolites tend to be enriched in erythrocytes and absent in plasma. Overall, metabolites generally found in plasma had the highest correlation between sample types, while metabolites found exclusively in erythrocytes had the lowest correlation. Altogether, these data show that plasma and DBS have similar pathway coverage but each with distinct characteristics.

Metabolomic Profiles of Fed and Fasted Individuals

Metabolomic profiles captured in DBS and plasma under "fed" and "fasted" conditions were compared. We used fasting as a point of comparison because it represents a clinical industry standard of collection with significant evidence showing stable, reproducible performance of small molecule diagnostics.[36] For statistical analysis of fed versus fasted, each metabolite was first re-scaled to have median=1, then imputed with its observed minimum, and finally the natural log-transformation was applied. For the plasma sets these computations were applied separately to each platform. Because of some of the imbalances of age and sex, an analysis of covariance (ANCOVA) was run with sex and log(age) as covariate. The fold changes for fasting status are based on the least-square means (LS MEANS) which are the means for the fed and fasted groups adjusted for the covariates. These analyses were performed in R version 4.2.2,[33] the R packages "car"[37] and "emmeans" version 1.8.3.[38]

To account for multiple comparisons, the false discovery rates were computed for each comparison using the q-value method of Storey and Tibshirani[39] and implemented with the R package "qvalue" version 2.30.0.[40] After analyzing DBS and plasma from fed and fasted groups we further evalutated the metabolites that demonstrated a significant fold change ($p<0.05$) in response to fasting. The false discovery rates corresponding to the $p<0.05$ threshold were 9.5%, 7.7%, 8.6%, 9.5%, and 24% for plasma, platforms, R, S, T, V, and DBS, respectively. We then focused on the plasma samples that were analyzed on the same platform as the DBS. To characterize the congruency of the metabolomes of DBS and plasma we plotted the fed/fasted fold-change ratios of the metabolites found in both sample matrices (**Figure 2A**). There was high correlation between the fed/fasted ratios of the log transformed data in each sample type ($r=0.84$), demonstrating that the majority of the metabolome in plasma was retained in DBS despite the differences in the testing matrices and DBS having a 5-fold lower extraction volume.

We also used glucose and 1,5-anhydroglucitol (1,5-AG) markers associated with both fasting and fed states, to measure variance in metabolite performance between individuals. We estimated the distribution range for glucose and 1,5-AG by calculating the deviation from the mean by computing the z-scores on the log-transformed data *without* imputes, using rankit regression to estimate the mean and standard deviation. Z scores for glucose and 1,5-AG for the 49 subjects were computed independently for each matrix and then plotted (**Figure 2 B,C**). While Z scores varied between individuals – sometimes by as much as 10 standard deviations – the difference in Z scores between plasma and DBS for each individual was less than 1 standard deviation. Altogether, these data show that DBS captures global metabolomic changes associated with fasting that are fairly consistent with those observed in plasma, while also capturing variability among individuals within a testing group.

Longitudinal Precision and Biological Variability Captured in DBS

We next evaluated the metabolic variability captured by DBS for individuals over time. We profiled DBS samples from 22 volunteers. Of these volunteers, six subjects did not report any health issues.; the rest (16) self-reported diagnoses shown in **Table 2**. Each donor was asked to self-collect at least one DBS sample every weekday for up to 30 days. Some donors did not complete the full study, and some provided more than one sample on some of the study days. Our analysis included all data generated from metabolic profiling of all DBS submitted for the study.

Longitudinal tracking of selected metabolites

The relative abundance of most metabolites revealed global metabolic trends over time while also capturing the biological variability of each individual. Some metabolites that exemplify individual characteristics are shown in **Figure 3**. While many of these findings are from an n of 1 setting, the results from the study can be verified by the extensive personal information volunteers disclosed on their health history forms and daily logs. For example, Donor 2, who had been diagnosed with probable insulin resistance, had noticeably low levels of 1,5-AG, (**Figure 3A**) a marker of short-term glycemic control that decreases as blood glucose levels exceed the renal threshold for glucosuria.[41] This same donor demonstrated a relatively high level of ergothioneine (**Figure 3B**), a xenobiotic found mainly in mushrooms and beans, which aligned with the donor's reported vegetable-rich diet. Donor 9, who had a history of hypothyroidism, exhibited low levels of DHEA-S (**Figure 3C**), an androgenic steroid that is typically decreased in patients with thyroid dysfunction.[42,43] DHEA-S is also elevated in women with hyperandrogenism,[44] and Donor 2, who exhibited the highest levels of this metabolite in the cohort, had a history of polycystic ovary syndrome (PCOS). Donor 9 was taking corticosteroids to treat asthma and had the lowest level of androstenediol (3beta, 17beta) disulfate (**Figure 3D**), an androgenic steroid that can be potently downregulated in response to corticosteroid therapy Tryptophan betaine and N,N,N-trimethyl-5-aminovalerate

Table 2: Overview of the 22-donor cohort. This cohort consisted of 10 males aged 38-61 (average age 48), 10 females aged 34-69 (average age 46), and two adults of unknown age and sex. Each donor reported their medical diagnoses and the prescription medications they took during the study. While the number of DBS collected from each subject varied all data was included in the analyses. ADHD=attention deficit hyperactivity disorder, PCOS=polycystic ovary syndrome.

Subject Identifiter	Sex	Reported Medical History	Prescription Medications	Number of DBS Collected
DNR19	M	Asthma	Zyrtec	30
DNR25	M	Healthy	None	30
DNR05	M	Healthy	None	23
DNR15	M	Healthy	None	25
DNR18	M	History of Kidney Stones	None	30
DNR13	M	Hyperlipidemia, Hypercholesterolinemia	None	31
DNR21	M	Osteoarthritis, Hypercholesterolinemia	None	27
DNR04	M	ADHD, Hypothyroidism	Vyvanse, Adderall, Synthroid	28
DNR26	M	Insomnia, Bipolar Disorder, Type II Diabetes, Hypertension, Hypogonadism	Zestoretic, Synthroid, Depo-Testosterone, Lamictal, Lipotor, Cymbalta, Abilify Maintena	10
DNR22	M	Gastroesophigeal Reflux Disease	None	30
DNR28	F	Healthy	None	20
DNR02	F	ADHD, Reynauds Syndrome, Probable Insulin Resistance, PCOS, Migraines, Hypercholesterolinemia	Vyvanse, Adderall, Xanax	20
DNR16	F	Hypothyroidism	Synthroid	20
DNR27	F	Healthy	None	21
DNR01	F	Anemia, Migraines	Klonopin, Lexapro, Relpax	28
DNR06	F	Asthma, Sleep Apnea, Depression, ADHD, Osteoarthritis, Gastroesophageal Reflux Disease	Adderall, Xopenex HFA, Wellbutrin SR, Kelnor 1/35	12
DNR10	F	Migraines, PCOS	None	19
DNR09	F	Asthasma, ADHD, Hypothyroidism, Hypertension	Vyvanse, Strattera, Ritalin, Porair (Albuterol Inhaler), Zestoretic, Synthroid	23
DNR20	F	Healthy	None	26
DNR29	F	Healthy	None	5
DNR 11	Null	Null	Null	18
DNR 14	Null	Null	Null	7

(**Figure 3E and 3F**) are intermediate molecules associated with tryptophan metabolism.

No obvious dietary, medical, or medicinal conditions were noted by the subjects that might have caused variability in abundance between individuals; and while our findings revealed differences between individuals, they also showed that levels remained consistent *within* each subject for the duration of the study. Of important note, this between-within contrast shows that biological individual-to-individual differences are exceeding the analytical variability associated with DBS analysis and home collection, showing that our findings represent the metabolic fingerprints of the study participants rather than analytical noise.

We also note that the levels of some metabolites fluctuated considerably, both within an individual and across individuals over the study. Representative metabolites associated with amino acid metabolism (4-acetamidobutanoate), diet (dimethylglycine), and environmental factors (4-hydroxychlorothalonil and perfluorooctane sulfonic acid (PFOS)) are shown in **Figure 4**. The levels of the metabolite 4-acetaminobutanoate was highly consistent for some participants (e.g., Donors 2, 10, 15, and 29) but fluctuated dramatically for others, such as Donor 26. Some of the more extreme outliers could be explained by diet. For example, dimethylglycine is an amino acid found in many edible plants including beans and brown rice. Donor 20 reported eating these foods more often than other study participants.

The elevated PFOS level shown in Donor 5, a self-reported vegetarian, may indicate higher levels of exposure from food and/or lower clearance compared to the rest of the cohort since PFOS may be associated with pesticide metabolism to treat plants and vegetables. While conclusions about the causative factors for the observed biological variabilities cannot be drawn without further study, these data show that individual metabolic fingerprints can be observed at a singular time point from a DBS sample.

Altogether, these data show that DBS can capture biological variability between individuals to a high degree of precision and that the analytical variability with DBS is minor compared to the individual-to-individual differences associated with this cohort.

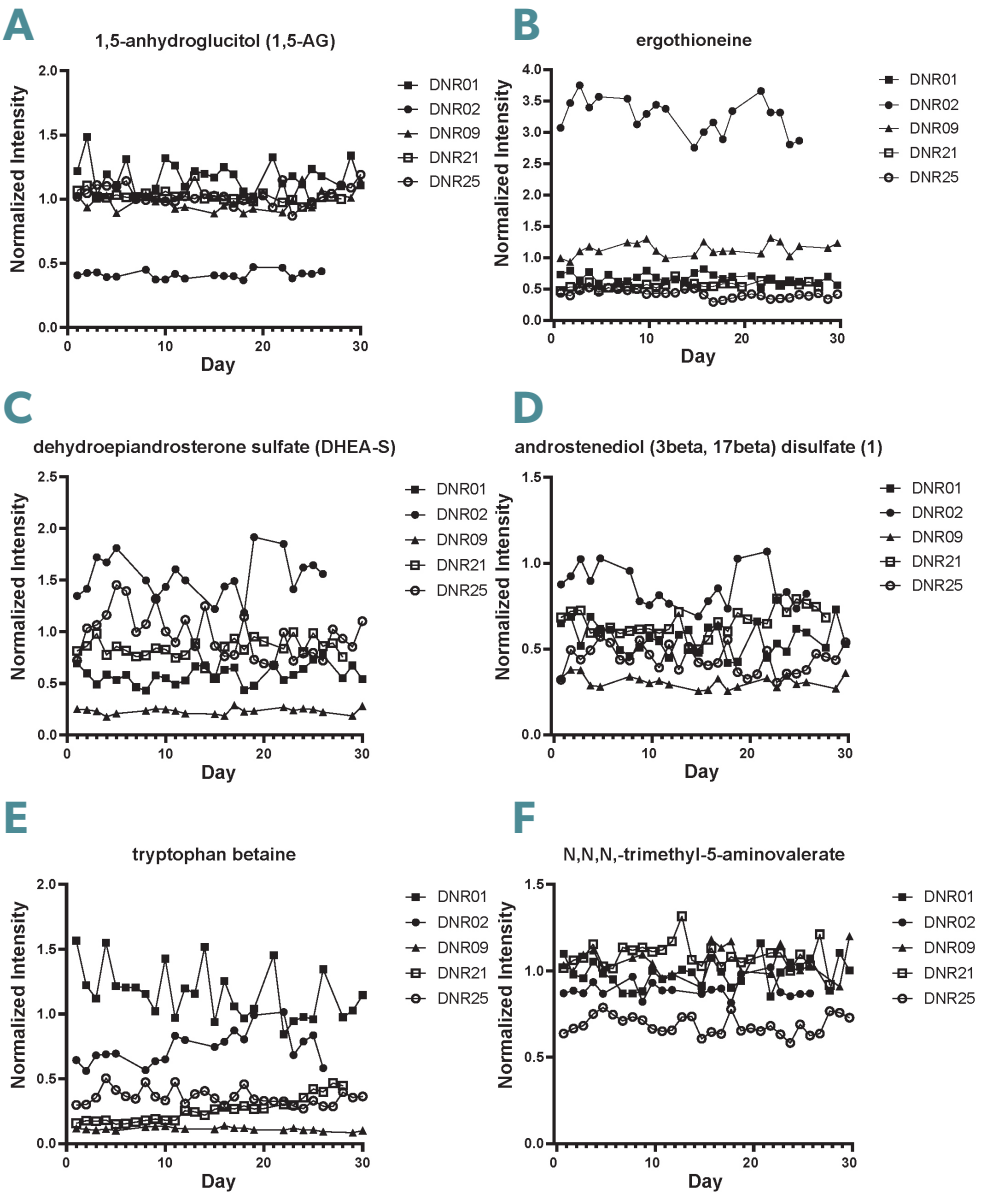


Figure 3: Individual biological variability and longitudinal precision captured by DBS. (A-F) The relative abundances of representative metabolites were plotted over the 30-day study period. Subject DNR25 was healthy, while subjects DNR01, 02, 09, and 21 had been diagnosed with various conditions and were being treated with prescription medications listed in Table 2.

Longitudinal tracking of selected hormones

To further evaluate the ability of DBS as a modality for "metabolic fingerprinting" based on the global metabolome, we focused the analysis on hormone profiles of the 22-donor cohort to test whether we could identify the two individuals in the population that had a reported history of PCOS. Analysis of the biochemical profiles for each of the subjects revealed a specific steroid hormone profile in multiple subjects (**Figure 5**). The lowest levels of the steroid hormones across the cohort corresponded to male and post-menopausal female participants. Among pre-menopausal females who were not taking estrogen-based birth control we identified two with significantly lower levels of the progestin steroid 5alpha-pregnan-3beta,20alpha-diol disulfate, a steroid sulfate that increases during ovulation and is known to be downregulated in PCOS.[45] Given the small number of cases in this study, it is impossible to make any claims regarding the strength of this particular finding. Rather the preliminary evidence highlights the proof of concept for the ability of DBS samples to capture metabolic variability associated with specific biological function, including the female reproductive cycle. Based on these observations, we propose that DBS can be extended to disease diagnosis for related conditions.

Discussion

As noted throughout this article, significant advancement in the performance of analytical platforms and informatics tools enable the measurement of thousands of metabolites in a variety of biofluids and tissues.[46,47] These analytical tools have provided insights

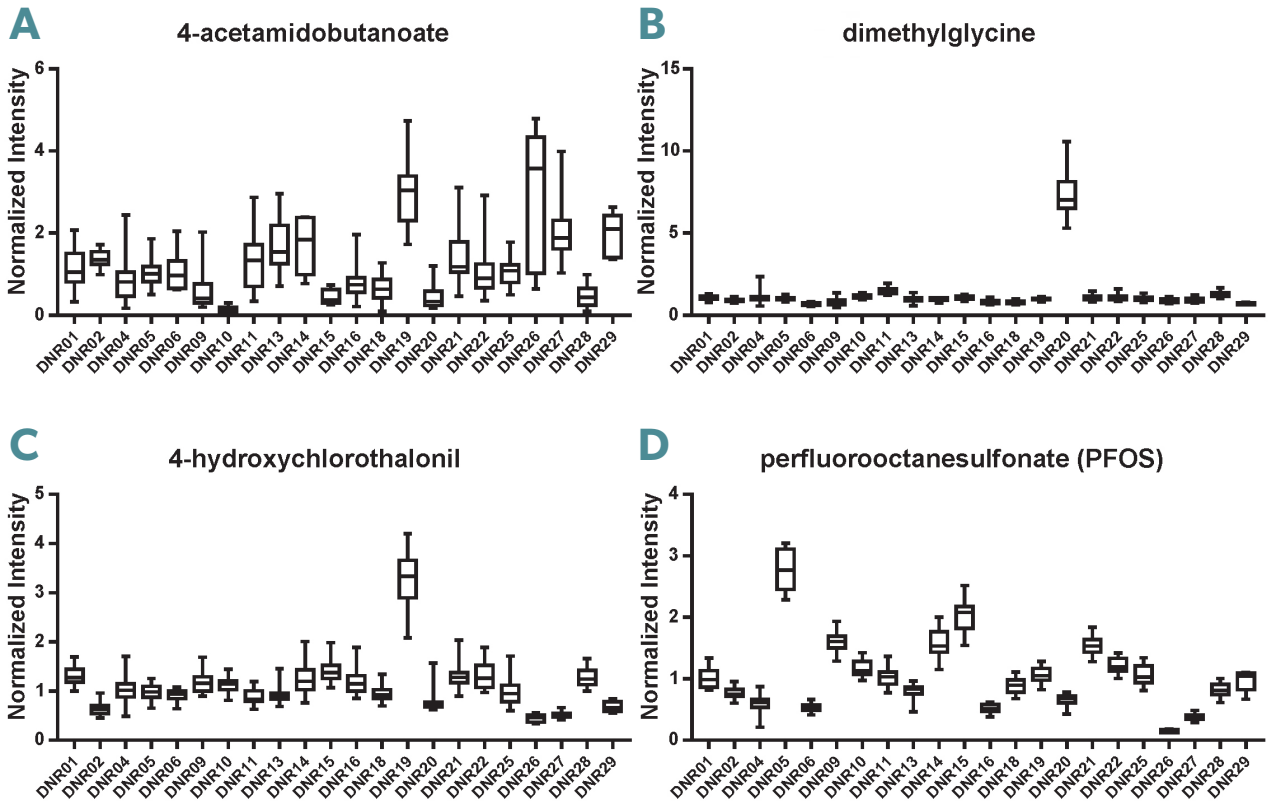


Figure 4: Representative metabolites showing individual metabolic fingerprints across the cohort. The box plots represent all data for each biochemical and each donor across the entire data set. The box represents 80% of the data and the whickers represent the top and bottom 10% of the data. The line in the middle of the boxes represents the median level for that donor. **(A)** Levels of a metabolite associated with amino acid metabolism that was shown to have a wide range of individual variability. **(B)** Levels of a metabolite derivative of the amino acid glycine that is found mainly in beans and liver. **(C-D)** Levels of metabolites associated with pesticide metabolism.

on mechanisms that underly numerous diseases including neuropsychiatric disorders,[48,49] cardiovascular disease,[50,51] cancer,[52,53] and diabetes.[54] Metabolomics may also inform a patient's prognosis and response to treatment.[55-59] Continued use of metabolomics-based testing will define deviations in global metabolic pathways from healthy reference populations in clinically stratified cohorts. Information from these reference studies will become the baseline for discovery and validation of prognostic and diagnostic biomarkers of wellness or disease activity, enable sub-classification of disease, and serve as a tool for monitoring disease activity, recurrence, and/or response to treatment.

DBS represents an optimal solution for expanding access to metabolomics for monitoring wellness owing to their easy collection, low-cost shipping and storage, and lack of reliance on cold-chain and phlebotomy services. Several studies have demonstrated the utility of DBS in metabolomics-based diagnostic testing,[60-62] however the performance of DBS samples for broad metabolic fingerprinting and assessing overall wellness has not been rigorously tested. Here, we aimed to establish a protocol for DBS collection and to compare the metabolomic profiles from plasma samples (which are routinely used for clinical testing) to profiles obtained from DBS. The overarching goal was to demonstrate the utility and value of DBS for evaluating the global metabotype of individual patients.

Using Metabolon's untargeted discovery mass spectrometry platform, we captured a comprehensive metabolomic profile from DBS samples and assessed the metabolite variability

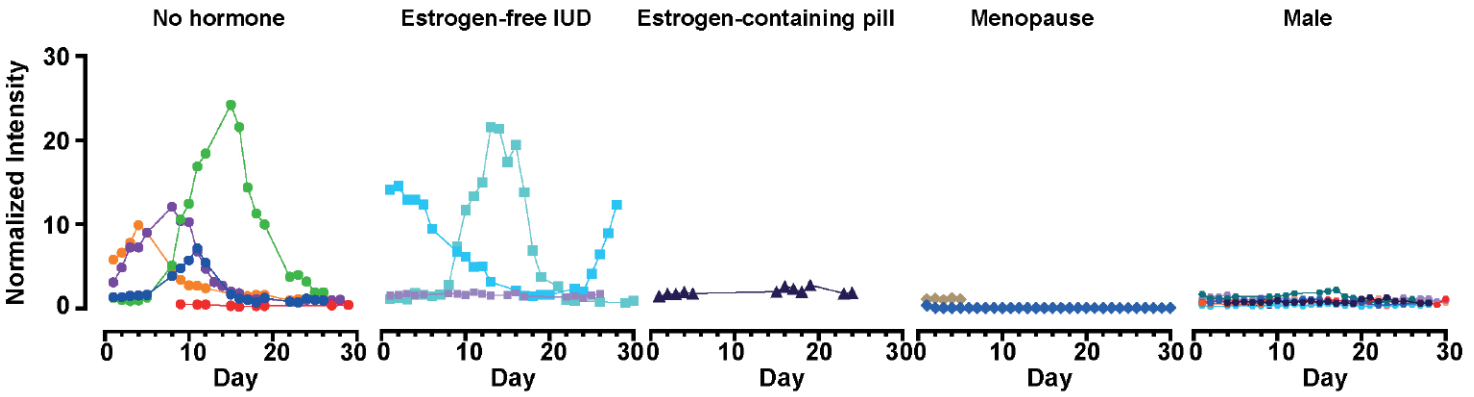


Figure 5: **Analysis of steroid hormone levels for each donor over the 30-day study.** Decreased levels of a steroid hormone revealed two study participants who had a history of PCOS: blue circles shown in "No Hormone" and purple squares shown in "Estrogen-free IUD".

across individuals under conditions of feeding and fasting. We showed that DBS captured a metabolic profile similar to that of plasma with the exception of metabolites that are enriched in erythrocytes (**Figure 1**). DBS also captured physiologic signature changes associated with fasting (**Figure 2A**). While these changes varied significantly between individuals, there was a high agreement between plasma and DBS signatures within individuals (**Figure 2B,C**), indicating that DBS captured biological variability with high fidelity.

We assessed longitudinal precision and biological variability by analyzing DBS collected at home over 30 days. DBS captured stable and broad metabolic variability between study participants and metabolites per individual for the study period (**Figure 3**). This data agrees with established concepts regarding tight control of levels of certain metabolites in the blood, while also revealing that the DBS methodology is capable of monitoring this level of biological precision. There were also examples of metabolic signature differences that could be explained by dietary, medicinal, or environmental factors unique to those individuals, where metabolites had both greater individual-to-individual differences but also greater variability in how tightly maintained those metabolites were per individual (**Figure 4**). Finally, we demonstrated that the individual variability captured in the DBS metabolome can potentially inform disease activity (**Figure 5**).

Altogether, these data demonstrate that samples generated from DBS can provide accurate and precise monitoring of metabolomic profiles and valuable insights on the alterations in the underlying biology of the individual. These findings support the assertion that DBS is a suitable matrix for profiling the metabotype. We recognize the limits of a small population study as a proof of concept and recognize that additional testing must be done with larger cohorts to confirm and validate our findings. We also note that plasma remains the gold standard for clinical diagnostics, and at present, should be used when a clinical diagnosis needs to be validated. Given that point, our findings show that in situations when cold storage or phlebotomy is not feasible, DBS represent a viable alternative matrix for profiling the metabotype and should therefore be further evaluated for metabolomics-based wellness testing as a potential testing modality within clinical standard of care. Further, we propose that metabolomic profiling with DBS will increase the affordability, diversity, and access for precision medicine applications. PMQ

Kelli D. Goodman

Kelli is a Principal Scientist in Research and Development at Metabolon with over 15 years of experience in method development, Good Laboratory Practice, liquid chromatography/mass spectrometry (LC/MS), and analytical chemistry. She manages the development of new metabolomics products from feasibility testing to launch. She played a pivotal role in Metabolon's ISO-9001 New Product Launch for metabolomics in dried blood spot card samples and now leads the ongoing evaluations of alternate at-home capillary blood collection devices for metabolomics.

Matthew W. Mitchell

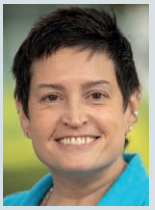
Matthew has a PhD in Statistics from North Carolina State University and serves as the Director of Statistics at Metabolon. He has extensive experience applying a wide variety of statistical applications to large metabolomics datasets. He also works closely with Metabolon's mass spectrometry (MS) research team to build biochemical reference libraries for MS analysis. His contributions include assessing library matches, software peak integration, structure elucidation, and designing experiments to further refine library matching decisions.

Laura J. Sommerville

Laura has a PhD in Cellular and Molecular Physiology with expertise in vascular proliferative diseases, smooth muscle cell biology, and clotting disorders. After completing her postdoctoral fellowship in 2019 she transitioned to medical writing and is now a Senior Medical Writer at Metabolon. She serves as the primary author of Metabolon's NIH grant applications and manuscripts and supports the writing of product development reports, regulatory documents, and marketing materials.

Rangaprasad Sarangarajan

Rangaprasad serves as the Chief Scientific Officer at Metabolon. He has over a decade of experience leading multi-functional teams to integrate biology, technology, and artificial intelligence analytics to develop multi-omic platforms for the discovery of disease-specific and novel therapeutic targets. He supports ongoing company initiatives by identifying unique differentiated commercial opportunities then building teams and plans to achieve corporate goals.



Karen L. DeBalsi

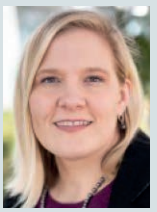
Karen has a PhD in Pharmacology and Cell Biology with expertise in mitochondrial metabolism and DNA mutagenesis as they relate to aging and cancer. Early in her 6 year tenure at Metabolon she served as a Senior Study Director in the division of Discovery and Translational Sciences. Under that role she led numerous projects aimed at identifying metabolic signatures of kidney disease, liver disease, and inborn errors of metabolism. In her current role of Principal Scientist in Diagnostic Metabolomics she manages biomarker discovery and validation initiatives for the development of diagnostic metabolomics assays for various diseases.

Adam D. Kennedy

Adam is an accomplished biochemist and immunologist with over 40 peer-reviewed publications and 5 patents, awarded and pending. During his 11 year tenure at Metabolon he has led over 200 projects aimed at biomarker discovery, validation, and product development for kidney diseases, liver diseases, type 2 diabetes, and inborn errors of metabolism. He currently serves as the Associate Director of Clinical Metabolomics where he played a critical role in the development of CLIA/CAP validated tests for insulin resistance and impaired glucose tolerance. He is currently leading the development of a screening test for rare diseases.

P. Ross Gunst

Ross is a Principal Statistician with over 12 years of experience at Metabolon. He provides statistical expertise to a wide range of areas including analyses for commercial and academic clients, large cohort studies, CLIA validated precision medicine studies, and research and development work. He has extensive experience working with cross functional teams of commercial and academic researchers and Metabolon biochemists to develop analysis plans that allow for optimal discovery of scientific insights from metabolomic data. Ross has performed analyses on metabolomic data which has provided the basis for over 100 peer reviewed papers.



Anne M. Evans

Annie is the Head of Research and Development for the discovery metabolomics and lipidomics profiling technologies at Metabolon. The metabolomics and lipidomics methodologies developed under Dr. Evans have been the analytical basis for many thousands of commercial and academic metabolomics and lipidomics studies from over 800 institutions worldwide. Currently, her research is focused on further developing Metabolon's technologies as well as investigating new technologies to be able to support further initiatives into large human cohort studies to study human health while also making metabolomics technologies precise and robust enough to study individual patient health (precision medicine).

Summary Points

1. Metabolomics has become a valuable tool for precision medicine initiatives but remains inaccessible for routine diagnostic testing because metabolomics technologies do not lend themselves to ease of access or affordability.
2. Dried blood spot (DBS) collection is an ideal method for expanding access to metabolomics owing to easy collection and low-cost shipping and storage.
3. The metabolomics profile captured by DBS is consistently similar to that of plasma.
4. DBS captures universal metabolomic changes associated with fasting while also capturing the metabolic fingerprint of individuals within a cohort.
5. DBS samples have the ability to capture metabolic variability associated with specific biological functions, including the female reproductive cycle, that can be extended to disease diagnosis.
6. Our proof-of-concept findings show that DBS is a suitable sample matrix for untargeted, global metabolomic profiling of individuals.

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Supplemental Figure 1: Diet Summary

Donor ID	Animal Protein	Plant Protein	Refined Carbohydrates	Vegetables	Fruit	Dairy	Added Fats	Sweets
DNR 1	Chicken, beef, fish			All vegetables	All fruits	Soft cheeses		Chocolate
DNR 2	Salmon, chicken, pork	Vega protein shake, almonds, edamame		Cruciferous, spinach, peppers		None	Coconut oil. Flax milk	Dark chocolate
DNR 4	Chicken, eggs, beef	Protein bars	Low carb tortilla, noodles, sweet potatoes	Green beans, salad, tomato sauce, sprouts	All fruits	Whey protein, yogurt, cheese	Mayonnaise	Nutella, m&ms, soda
DNR 5	None	Beans, almonds, hummus	Pasta. rice. Sandwich bread	Salad, tomato sauce, green beans, peas, pepper, carrot, radish	Apples	Yogurt, cheddar cheese		Pop-tarts
DNR 6	Did not report							
DNR 9	Chicken, steak, eggs		Pasta, granola bar, potato chips	Salad, green vegetables, corn, salsa	Strawberries and raspberries			Ice cream
DNR 10	Poultry, beef, eggs		Pasta, wheat crackers	All vegetables	All fuits	Yogurt, cheese		Diet soda
DNR 11	Did not report							
DNR 13	Chicken		Cereal (Special K), sandwich bread, pizza, chips	All vegetables	Bananas	Yogurt, cheese		Ice cream
DNR 14	Did not report							
DNR 15	Did not report							
DNR 16	Chicken	Lentils, chickpeas	Curry rice, bread, cheeze its	All vegetables	All fruits	Cheese, yogurt		
DNR 18	Chicken, beef (ground and jerkey)	Peanut butter, beans	Pasta, bread, crackers, rice, taco shell	All vegetables	All fruits	Cheese, yogurt	Butter. EVOOm pesto	
DNR 19	Chicken, eggs, grass-fed beef	Peanuts, almonds, sunflower seeds, protein noodles, whey protein (power, bar)	Brown rice	All vegetables	All fruits (raw and (dried), Lara bar	Cheese, whey protein		Dried fruit, honey
DNR 20	Did not report							
DNR 21	Salmon, chicken, pork, beef, eggs	Nuts	Popcorn	Green vegetables	Blueberries, apples, plums	Yogurt, cheese	MCT oil, butter	Dark chocolate
DNR 22	Eggs, all meat	Pumpkin seeds	Ramen	All vegetables	None	None		Candy, pop-tarts, soda
DNR 25	Chicken	Protein chake, Rx bar	Cereal (Heritage Grain)	Salad	None	Whey protein	Salad dressing	
DNR 26	Ham, beef	Peanut Butter	Potatoes, bread	Broccoli, peas	Bananas	Yogurt, cheese		
DNR 27	Chicken	Kefir	Pasta	Salad		Yogurt, kefir		
DR 28	Chicken		Pizza, whole wheat toast, chips	Salad	Bananas	Yogurt		

Supplemental Figure 2: Daily Drug Log

Day	Donor ID						
	DNR 1	DNR 2	DNR 4	DNR 5	DNR 6	DNR 9	DNR 10
1	Lexapro, Hydrocortisone	Vyvanse, Adderall, Benadryl	Synthroid, Aspirin, Trazodone	None Reported	Adderall, Xopenex HFA, Zyrtec, Wellbutrin SR, Kelnor 1/35	Vyvanse, Strattera, Ritalin, Flonase, Zestoretic, Synthroid	
2	Lexapro	Vyvanse, Adderall, Benadryl	Synthroid, Aspirin, Trazodone		Adderall, Xopenex HFA, Zyrtec, Wellbutrin SR, Kelnor 1/35	Vyvanse, Strattera, Ritalin, Flonase, Zestoretic, Synthroid	Ibuprofen
3	Lexapro	Vyvanse, Adderall, Benadryl	Synthroid, Aspirin, Trazodone		Adderall, Xopenex HFA, Zyrtec, Wellbutrin SR, Kelnor 1/35	Vyvanse, Strattera, Proair, Zestoretic, Synthroid	
4	Lexapro, Ibuprofen	Vyvanse, Adderall, Benadryl	Synthroid, Aspirin, Trazodone		Adderall, Xopenex HFA, Zyrtec, Wellbutrin SR, Kelnor 1/35	Vyvanse, Strattera, Zestoretic, Synthroid	
5	Lexapro, Hydrocortisone	Vyvanse, Adderall	Synthroid, Aspirin, Trazodone		Adderall, Xopenex HFA, Zyrtec, Wellbutrin SR, Kelnor 1/35	Vyvanse, Strattera, Zestoretic, Synthroid	Ibuprofen
6	Lexapro		Synthroid, Aspirin, Trazodone				
7	Lexapro, Relpax, Ibuprofen		Synthroid, Aspirin, Trazodone				
8	Lexapro	Vyvanse, Adderall, Benadryl	Synthroid, Trazodone			Strattera, Ritalin, Zestoretic, Synthroid	Ibuprofen
9	Lexapro	Vyvanse, Adderall, Benadryl	Adderall, Synthoid, Econazole nitrate, Miconazole nitrate, Clotrimazole, Trazodone			Strattera, Ritalin, Zestoretic, Synthroid	Ibuprofen
10	Lexapro	Vyvanse, Adderall, Benadryl, Melatonin	Adderall, Synthoid, Econazole nitrate, Miconazole nitrate, Clotrimazole, Trazodone			Strattera, Ritalin, Zestoretic, Synthroid	Prilosec
11	Lexapro	Vyvanse, Adderall, Benadryl, Melatonin	Adderall, Synthoid, Econazole nitrate, Miconazole nitrate, Clotrimazole, Trazodone			Strattera, Ritalin, Zestoretic, Synthroid	Prilosec
12	Lexapro	Vyvanse, Adderall, Benadryl, Melatonin	Adderall, Synthoid, Econazole nitrate, Miconazole nitrate, Clotrimazole, Trazodone			Vyvanse, Strattera, Zestoretic, Synthroid	Prilosec, Claritin
13	Lexapro		Adderall, Synthoid, Econazole nitrate, Miconazole nitrate, Clotrimazole, Trazodone				Prilosec, Claritin
14	Lexapro		Adderall, Synthoid, Econazole nitrate, Miconazole nitrate, Clotrimazole, Trazodone				
15	Lexapro	Vyvanse, Adderall, Benadryl, Melatonin	Vyvanse, Synthoid, Clotrimazole, Trazodone		Adderall, Xopenex HFA, Zyrtec, Wellbutrin SR, Kelnor 1/35	Strattera, Ritalin, Zestoretic, Synthroid	Prilosec, Claritin
16	Lexapro	Vyvanse, Adderall, Benadryl	Vyvanse, Synthoid, Clotrimazole, Trazodone		Adderall, Xopenex HFA, Zyrtec, Wellbutrin SR, Kelnor 1/35	Strattera, Ritalin, Zestoretic, Synthroid	Prilosec, Claritin
17	Lexapro, Clonopin, Xanax	Vyvanse, Adderall, Xanax, Benadryl	Vyvanse, Synthoid, Trazodone		Adderall, Xopenex HFA, Zyrtec, Wellbutrin SR, Kelnor 1/35	Strattera, Ritalin, Zestoretic, Synthroid	Prilosec, Claritin
18	Lexapro	Vyvanse, Adderall, Xanax	Synthoid, Trazodone		Adderall, Xopenex HFA, Zyrtec, Wellbutrin SR, Kelnor 1/35	Strattera, Ritalin, Zestoretic, Synthroid	
19	Lexapro	Vyvanse, Adderall, Xanax, Benadryl	Synthoid, Trazodone		Adderall, Xopenex HFA, Zyrtec, Wellbutrin SR, Kelnor 1/35	Strattera, Ritalin, Zestoretic, Synthroid	
20	Lexapro		Synthoid, Trazodone				
21	Lexapro, Relpax, Ibuprofen		Synthoid, Trazodone				
22	Lexapro	Vyvanse, Adderall, Xanax, Benadryl	Vyvanse, Trazodone			Vyvase, Strattera, Zestoretic, Synthroid	Prilosec, Ibuprofen
23	Lexapro, Relpax, Ibuprofen	Vyvanse, Adderall, Benadryl, Melatonin	Vyvanse, Synthoid, Aspirin, Trazodone		Adderall, Xopenex HFA, Zyrtec, Wellbutrin SR, Kelnor 1/35	Vyvase, Strattera, Zestoretic, Synthroid	Prilosec
24	Lexapro	Vyvanse, Adderall, Benadryl, Melatonin	Vyvanse, Synthoid, Aspirin, Trazodone		Adderall, Xopenex HFA, Zyrtec, Wellbutrin SR, Kelnor 1/35	Vyvase, Strattera, Zestoretic, Synthroid	Prilosec
25	Lexapro	Vyvanse, Adderall, Benadryl, Melatonin	Vyvanse, Synthoid, Aspirin, Trazodone			Vyvase, Strattera, Zestoretic, Synthroid	Prilosec
26	Lexapro, Ibuprofen	Vyvanse, Adderall, Xanax, Benadryl, Melatonin	Vyvanse, Synthoid, Aspirin, Trazodone				Prilosec
27	Lexapro		Vyvanse, Synthoid, Aspirin, Trazodone				
28	Lexapro		Vyvanse, Synthoid, Aspirin, Trazodone			Vyvase, Strattera, Zestoretic, Synthroid	
29						Vyvase, Strattera, Zestoretic, Synthroid	
30						Vyvase, Strattera, Zestoretic, Synthroid	

continues...

Supplemental Figure 2: Daily Drug Log *(continued)*

Day	Donor ID						
	DNR 11	DNR 13	DNR 14	DNR 15	DNR 16	DNR 18	DNR 19
1	None reported	None reported	None reported		Synthroid	None reported	Zyrtec
2					Synthroid		Zyrtec
3					Synthroid		Zyrtec
4					Synthroid		Zyrtec
5				Ibuprofen	Synthroid		Zyrtec
6				Ibuprofen			Zyrtec
7							Zyrtec
8					Synthroid		Zyrtec
9					Synthroid		Zyrtec
10					Synthroid		Zyrtec
11					Synthroid		Zyrtec
12					Synthroid		Zyrtec
13							Zyrtec
14							Zyrtec
15					Synthroid		Zyrtec
16					Synthroid		Zyrtec
17					Synthroid		Zyrtec
18					Synthroid		Zyrtec
19					Synthroid		Zyrtec
20							Zyrtec
21							Zyrtec
22					Synthroid		Zyrtec
23					Synthroid		Zyrtec
24					Synthroid		Zyrtec
25					Synthroid		Zyrtec
26					Synthroid		Zyrtec
27							Zyrtec
28							Zyrtec
29							
30							

continues...

Supplemental Figure 2: Daily Drug Log *(continued)*

	Donor ID						
Day	**DNR 20**	**DNR 21**	**DNR 22**	**DNR 25**	**DNR 26**	**DNR 27**	**DNR 28**
1		Synthroid	Prilosec	Ibuprofen	Synthroid, Relpax	Lexapro, Hydrocortisone	
2		Synthroid	Prilosec		Synthroid, Relpax		
3		Synthroid	Prilosec		Synthroid, Relpax		
4		Synthroid	Prilosec		Synthroid, Relpax		
5		Synthroid	Prilosec		Synthroid, Relpax		
6		Synthroid	Prilosec				
7		Synthroid	Prilosec				
8		Synthroid	Prilosec		Synthroid, Relpax		
9		Synthroid	Prilosec		Synthroid, Relpax		Ibuprofen
10		Synthroid	Prilosec	Zyrtec	Synthroid, Relpax		Ibuprofen
11		Synthroid	Prilosec	Zyrtec	Synthroid, Relpax		Ibuprofen
12		Synthroid	Prilosec		Synthroid, Relpax		
13		Synthroid	Prilosec				
14		Synthroid	Prilosec				
15		Synthroid	Prilosec, Ibuprofen			Ibuprofen	
16		Synthroid	Prilosec				
17		Synthroid	Prilosec, Ibuprofen				
18		Synthroid	Prilosec, Ibuprofen	Zyrtec			
19		Synthroid	Prilosec				
20		Synthroid	Prilosec, Ibuprofen				
21		Synthroid	Prilosec, Ibuprofen				
22	Ibuprofen	Synthroid	Prilosec				
23		Synthroid	Prilosec			Ibuprofen	Ibuprofen
24		Synthroid	Prilosec, Ibuprofen				
25		Synthroid	Prilosec				Ibuprofen
26		Synthroid	Prilosec				
27		Synthroid	Prilosec	Zyrtec			
28		Synthroid	Prilosec	Zyrtec			
29		Synthroid	Prilosec, Ibuprofen	Zyrtec			
30			Prilosec	Zyrtec			