# Interferon Alpha is Stable for at Least Twenty Days in Dried Blood Spots
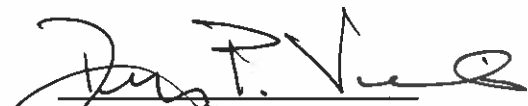
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Thesis submitted to the Faculty of the
University of Michigan – Flint

in partial fulfillment of the requirements for the degree of

Master of Science in Biology

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July 2019

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## Acknowledgements

To begin, I am truly grateful to Dr. David Duriancik, my thesis advisor. Your encouragement, generosity, advice, and support throughout this project has been invaluable. Thank you for all the work that you put in to help me succeed. You always go above and beyond for your students, and it does not go unnoticed. This was an amazing learning experience and I am so thankful I had you to guide me through it.

I also express my warmest gratitude to Joshua Cunningham for your help and work with the Bradford Assay as well as to the members of my committee, Dr. Jill Slater and Dennis Viele, for their feedback and support. You are both wonderful professors, and I am so lucky to have known you throughout my University of Michigan education.

To my friends especially Rachelle Rowbatham, Aaron Burch, Nicholas Potochick, and Doug Wilkinson, thank you all your help whenever I had a question or concern. Thank you for steering me in the right direction and being there to listen whenever I felt like giving up. I appreciate you more than you know.

Thank you also to my family and friends for all your love and support through this journey- especially my parents Lynn and Bill Thomas, my husband Nathan Cooper, my sons Caden and Benjamin Cooper, and my sister Natalie Novik. You are truly wonderful, and I could not have done this without you.

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## Abstract

Dried blood spot (DBS) cards are a simple, inexpensive, and convenient method for storage and analysis of blood samples. DBS samples have been used for the analysis of a variety of markers such as proteins, enzymes, cytokines, nucleic acids, fatty acids, and drugs. One of the beneficial properties of these cards is their ability to be stored for long periods of time with little to no deterioration or degradation of analytes, especially when stored in colder temperatures. The primary objective of this study was to determine the stability of a cytokine, interferon-alpha (IFN-α), in DBS cards stored at room temperature for 20 days without any additional treatments or additives. Blood samples were spiked with IFN-α standard and stored on DBS cards in the dark at room temperature with desiccant for up to 20 days. Concentration of IFN-α was measured using an ELISA immunoassay. Total protein extracted was also measured by a Bradford Assay to account for any decreases in concentration. Total protein and IFN-α were quantitated and analyzed using Kruskal-Wallis, the Bonferroni Procedure, and One-Sample-T-Tests. IFN-α was detectable in all spiked samples, and little to no degradation of the protein was observed at day 20. Total protein extracted from DBS ranged from 30-50%. Concentrations of IFN-α were as expected based on the dilution of the standard after accounting for protein extraction. Improved protein extraction methods will be assessed to improve the assay. These data provide evidence of the feasibility of assessing IFN-α using DBS technology which may facilitate international research projects.

**Introduction**

Dried blood spot (DBS) cards are a simple, inexpensive, and convenient method for storage and analysis of blood samples. Utilized since the 1960s for testing metabolic processes in newborns, DBS cards are being used more in research for the analysis of a variety of markers such as proteins, enzymes, cytokines, nucleic acids, fatty acids, and drugs (Gibson et al., 2017). One of the beneficial properties of these cards is their ability to be stored for long periods of time with little to no deterioration or degradation of analytes (Chapman, 1924; Hannon & Therell, 2014).

Utilization of DBS cards allow for research to be performed in geographical locations and populations that are generally difficult to access. While protein degradation is reduced when DBS cards were stored at colder temperatures, these cooling/freezing techniques may not always be available in specific geographical areas of study (Skogstrand et al., 2008). Transport, handling, and storing of blood specimens all affect the stability and detection of analytes. It is crucial to determine the effect of specific transport, processing, and storage conditions on quantitation of analytes of interest.

The primary objective of this study was to determine the stability of a cytokine, interferon alpha (IFN-α) in blood samples stored on DBS cards at room temperature for 20 days without any additional treatments or additives. Some samples of blood were "spiked" with the cytokine IFN-α to monitor stability. It was hypothesized that there will be little to no degradation after this 20-day period.

### Background on Dried Blood Spot Cards

Blood is the most widely collected liquid sample for diagnostic and clinical laboratory testing (McMorran et al., 2016). Venipuncture, the traditional method for blood collection, is an invasive technique. It can be difficult to perform on several populations in regards to age and size of patients, previous IV drug use, and creates discomfort and anxiety for some subjects. There is also a risk of bloodborne illness transfer. Reliability and reproducibility of analytes is dependent on instant separation of plasma or serum from blood cells along with immediate storage at low temperatures (Zander et al.,2014). The necessity for centrifugation and refrigeration creates logistical and cost concerns in terms of transport and storage, especially in geographical areas where laboratory resources are unavailable. A strategy to overcome these restrictions and simplify the blood sampling and sample pretreatment procedure is the use of dried blood spot (DBS) cards (López de las Hazas et al., 2016).

Filter paper has been used for diagnostic blood testing since the 1900s including determining glucose concentration from eluates and disease diagnosis such as syphilis (Hannon & Therell, 2014; Schmidt, 1986). However, the analysis of metabolites in DBS was first introduced by Robert Guthrie in 1963 to screen newborns for congenital metabolic diseases (Chapman 1924; Guthrie & Susi, 1963). Since then, DBS cards have been implemented in many different applications from clinical monitoring to single analyte or profiling several types of metabolites (Zukunft et al.,2013). Utilization of DBS cards offers advantages over traditional venipuncture blood sample collection. DBS involve obtaining a few drops of blood by finger or heel prick placed onto filter papers cards. Blood collection for DBS is simple and minimally invasive to perform. Rather than a venous puncture, patients and volunteers can perform the blood collection themselves (Leichtle, et al., 2010). DBS samples also have minimal risk of bacterial contamination or probability of hemolysis (Chapman, 1924; Hannon, 2014). Sample volumes for DBS are much lower than a venous collection (Zukunft et al., 2013). This allows for

easier storage in facilities such as blood banks and other research laboratories. Specimens are stored in low gas-permeability plastic bags with desiccant added to reduce humidity and may be kept at ambient temperatures (Sharma et al., 2014). DBS also allow for easier transportation since specimens can be shipped by mail or other carrier with no reasonable expectations of occupational exposure to blood or other potentially infectious material (Center for Disease Control and Prevention, 1995; Knudsen et al.,1993). Most importantly, the DBS are stable under ambient conditions and can be preserved for long periods of time with little to no deterioration of the analytes (Chapman, 1924; Hannon & Therell, 2014; McMorran et al., 2016). These properties of the DBS method have provided an alternative to plasma as the analytical matrix for storing and testing blood analytes (Andriamandimby et al., 2013; Smit et al., 2014; Tretzel et al., 2014).

While there are numerous benefits to the use of DBS cards, there are still concerns and limitations. While low blood volume is a distinct advantage of DBS, it may also be a concern because it can only be coupled with highly sensitive analytical techniques (Sharma et al., 2014). DBS sampling is also inadequate for air sensitive or volatile analytes (Li & Tse, 2010; Majumdar & Howard, 2011). Therefore, suitability of DBS for each analyte of interest should be determined empirically. There are other concerns with uneven distribution of analyte on the DBS card from factors such as card material and hematocrit level effect. Hematocrit value, the measure of packed cell volume in blood, affects the size, spread, and diameter of DBS sample. Hematocrit values affect the spread of blood on the DBS card and can alter results with significant inter-subject variability. Blood samples with different hematocrit values can cause different spot sizes due to differences in viscosity and blood spot diffusion on the card resulting in measurement variability (Sharma et al., 2014).

Several strategies have been developed to address differences in hematocrit and spot variability. The easiest approach is the analysis of volumetrically applied DBS that are obtained

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by pipetting or using precision capillaries or other micro-sampling devices and deliver a fixed amount of blood to filter paper (Li et al., 2011; Youhnovski et al., 2011). Because samples with different hematocrit values alter spot sizes, blood samples can be spotted on pre-cut dried blood spots (PCDBS). Utilization of PCDBS will nullify the effect of the hematocrit by extracting the whole spot without punching a specific area (Youhnovski et al., 2011).

Spot homogeneity and compound degradation are other problems especially in long-term storage (Sharma et al., 2014). Amino acids and derivatives, such as carnitine levels, have been reported to vary in concentrations in long-term studies using DBS cards (Strnadova et al., 2007). Certain amino acids and metabolites have also been absent in DBS extracts while detectable in blood or plasma samples creating precautions regarding metabolic profiling (Kong et al., 2011). The drying process of the DBS may also cause concern. The enzymatic and/or nonenzymatic degradation of the unstable analytes may occur during the early drying period of the blood spots (Li, Zhang, and Tse, 2011). Although there are pitfalls, DBS application is still a highly recommended and respected technique.

### DBS in Research

DBS samples can be utilized for testing for a variety of different analytes, including cholesterol, C-reactive protein, glycosylated hemoglobin, antibodies, and several other proteins and cytokines (Ostler et al., 2014). For example, use of DBS cards has proven successful for the monitoring of retinol binding protein (RBP) in Vitamin A deficient (VAD) individuals. Analysis of vitamin A using DBS was first described by Shi et al. (1995) using high performance capillary electrophoresis with laser-enhanced fluorescence detection. Modifications were made and sample preparation methods were developed for the elution of holo-RBP from DBS, ultimately demonstrating the stability of the complex in dried blood which was once thought to be unstable when exposed to air and iron in red blood cells (Ma et al., 1993; Craft et al., 2000). Additional

analytes may have similar stability in DBS, but sampling, storage, and processing conditions must be empirically validated.

Fatty acids can also be accurately measured in DBS treated with proprietary preservatives. Due to the long carbon chain and number of double bonds, poly-unsaturated fatty acids have a high risk of oxidation due to light, temperature, and exposure to air. This risk of oxidation prevented the ability to analyze fatty acids from DBS until recently. Fatty acids have been assessed from DBS that have been coated with preservatives and antioxidants (Marangoni et al., 2004). These preservatives aid in preventing degradation and increase stability of fatty acids. Since applying these modifications, DBS samples have been used in a series of research projects (Sarter et al., 2015, Johnston et al., 2013, Aarsetoey et al., 2011, Monge et al., 2016, Jumbe et al., 2016; Baack et al., 2015, and Wolters et al., 2014). Increased research of fatty acids is due to the interest in omega-3 (n3) fatty acid intake and their role in health and disease. There is growing evidence that n3 fatty acids have beneficial effects in chronic inflammatory diseases including chronic obstructive pulmonary disease (COPD), asthma, rheumatoid arthritis, and inflammatory bowel disease (Calder, 2013; Yates et.al, 2014). Other important roles include prevention of autoimmune disorders, blood clotting, cholesterol metabolism, structure of membrane phospholipids in the brain and the retina, as well as fetal development, cognitive ability, and cardiovascular function (Abedi & Sahari, 2014; Shireman, 2003). The ability to measure fatty acids in DBS would increase access to otherwise unavailable populations with varying n3 fatty acid intake and prevalence of disease.

### Interferon-Alpha

Interferons (IFN) are potent signaling protein cytokines produced by somatic cells during viral infection (François et al., 2005). The presence of IFN in biological samples is frequently used to identify viral infection (Meager, 2002). Interferons were discovered by Isaacs &

Lindenmann (1957) and were named for their ability to interfere with viral replication (Perry et al., 2005). In addition to their role in viral infections, IFN have immune modulating, antiproliferative, and antineoplastic effects. They also act as regulators of growth and differentiation (Lombardi et al., 2018).

The type I IFN is a multi-gene cytokine family that encodes 13 partially homologous Interferon-alpha (IFN-α) subtypes in humans and 14 in mice (Pestka et al., 2004). IFN-α is a key cytokine of the innate response produced by the immune system in response to pathogens and tumor and virus-infected cells (Lombardi et al., 2018). Plasmacytoid dendritic cells release large amounts of IFN-α (Blanco et al., 2001; Bennett et al., 2003) and induce myeloid dendritic cells maturation. Plasmacytoid dendritic cells constitute only 0.2–0.8% of the peripheral blood mononuclear cells but produce large amounts of IFN-α on a per cell basis in response to viruses like the Herpes Simplex Virus (Fitzgerald-Bocarsly et al., 2008; Reizis et al., 2011).

### Interferon-Alpha and Autoimmunity

Type I Interferons play a role in host defense. However, growing evidence suggests that IFN is also a mediator in autoimmune diseases. IFN production is an immediate response protein during viral infections and is transient in duration. Therefore, healthy individuals do not have sustained IFN expression in their system in contrast to individuals with pathological conditions like autoimmune and inflammatory disorders (Di Domizio; Cao, 2013). Systemic lupus erythematosus (SLE) is an autoimmune disorder associated with increased levels and overexpression of IFN-α. Shahin et al. (2011) measured serum levels of IFN-α in Egyptian SLE patients and their first-degree relatives in comparison to unrelated healthy controls to identify individuals at the greatest risk for clinical illness. They noted that patients with SLE had mean concentrations of IFN-α of 65.3 ± 53.4 pg/mL, 19.5 ± 23pg/mL in first-degree relatives, and 5.2 ±5.2 pg/mL in unrelated healthy controls.

Excess serum IFN-α and IFN-response gene expression are most likely the result of excessive plasmacytoid dendritic cells (pDC) activation. PDCs play a role in the production of IFN-α and are the main sources of serum IFN (Fitzgerald-Bocarsly et al., 2008). In response to infection, the plasmacytoid dendritic cells release large amounts of IFN-α and induce mDC maturation (Blanco et al., 2001; Bennett et al., 2003). In individuals with SLE, mDCs are able to activate phagocytosis and present self-antigens to T-cells. The T-cells activated by IFN-α-treated dendritic cells also are enriched for T-follicular helper cells, a recently described cell type which activates B cells and drives antibody production (Cucak et al., 2009). Abnormal IFN presence is not limited to SLE. Over-expression and activation of IFN pathways have been associated with several other autoimmune disorders such as: Type 1 Diabetes (T1D), autoimmune thyroid disease (AITD), rheumatoid arthritis (RA), scleroderma, and primary biliary cholangitis (Lombardi et al., 2018, Pascual, et al., 2006; Stefan et al. 2014; Hasham et al., 2013; Cacopardo et al., 2013).

IFN-α expression in autoimmune diseases results in different gene signatures than those produced by viral infections and dysregulated IFN-α levels in SLE activate a panel of IFN-regulated genes, commonly referred to as an IFN signature (Bezalel et al., 2014; Kyogoku et al., 2013). This IFN signature frequently correlates to disease severity. IFN-α can be used as a marker when identifying persons at risk for disease development; downregulation of IFN-α can be a therapeutic approach to prevent disease flares and potential organ damage (Bauer et al., 2009; Shahin et al.,2011).

### Cytokine Stability in Research

Information on the stability and detection of cytokines in DBS relative to blood sample handling and storage conditions is therefore of great importance (De Jongh et al., 1997). There are studies that monitor the effects of handling blood samples spiked with cytokines (Flower et

al., 2000, Thavasu et al., 1992). Thavasu et al. (1992) spiked blood with several types of cytokines: TNF-α, IL-6, IL-1α, IL-1β, IFN-α and IFN-γ. Whole blood collected from healthy volunteers was spiked with individual cytokines and assessed for their recovery and stability in serum, or plasma from the whole blood. The samples were subjected to timed separation and stored at 4 °C and room temperature. They found that all analytes except IL-1α decreased in concentration after storage, especially at room temperature. Skogstrand et al. (2008) measured the effects of endogenous cytokines in DBS and blood samples with different handling and storage conditions. They found that the measurable concentrations of cytokines were rather stable in DBS samples stored at different temperatures for many days with reference to DBS samples frozen immediately after preparation. Storage of DBS samples at low temperature appeared to provide the best results. They, however, did not measure the concentration of IFN-α. After reviewing previous literature, there are no known studies that have monitored the stability of IFN-α on DBS cards.

Although studies have shown success with DBS placed in lower temperatures, access to proper laboratory resources and techniques may not be available specifically in geographical regions such as Arctic native populations. It is important to know how this may affect the concentration of analytes in serum/plasma, and DBS samples. The primary objective of this study is to determine the stability of interferon alpha (IFN-α) in blood samples stored on DBS cards at room temperature for 20 days without any additional treatments or additives. It was hypothesized that there would be little to no degradation after this 20-day period.

**Methods**

*Participants*

The study was conducted at the University of Michigan-Flint. This was considered an exempt case study (IRB #HUM00163721). Three subjects were verbally recruited to participate

in this study.  The subjects consisted of two male participants and one female between the ages of 23-37 and in good health. Once recruited, each subject performed the blood collection and donated heparinized microhematocrit tubes of whole capillary blood obtained from a fingerstick. The protocol for blood collection was adapted from Adjepong et al., 2018 in which a single lancet was used to puncture the tip of each subject's middle finger to obtain drops of blood. After the initial puncture, the first drop of blood was discarded (Adjepong et al., 2018). Subsequently, each participant filled 18-20 capillary tubes of blood. The capillary tubes were combined into one microcentrifuge tube per subject prior to aliquoting blood for processing.

### Blood processing

One aliquot of approximately 600uL whole blood per subject was centrifuged at 2000RPM for 10 minutes at room temperature and 300uL of plasma was separated into two microcentrifuge tubes. One 150uL plasma aliquot was spiked with 2.0uL of 10,000pg/mL IFN-α standard (PBL Assay Science) while the other contained 150uL of plasma (un-spiked). The final concentration of IFN-α in spiked plasma should have been 131.6pg/mL. Both plasma aliquots were stored at -20C. The remaining whole blood volume was measured and aliquoted to un-spiked or spiked blood tubes. 24uL of IFN-α standard was mixed with 16uL of remaining blood for the spiked samples. The final IFN-α concentration, at this step, should have been 6,000pg/mL. Spots of 10-20uL, which would contain 120-240pg IFN-α respectively, were then individually pipetted onto 6 Whatman 903 Protein Saver DBS cards (GE Healthcare). All un-spiked samples were spotted on the DBS cards with 20uL, however there was an issue with blood collection where subject A had an excess of blood while subjects B and C were in a shortage. For these reasons, subject A was spotted with 20uL while subjects B and C were spotted with 10uL for the spiked samples. Once all spots were placed on the DBS cards, the cards were dried overnight at room temperature in the dark and subsequently placed into individual Ziploc bags with desiccant packets at room temperature for storage.

### *Dried Blood Spot Elution*

The elution of protein from DBS occurred on Days 1, 10, and 20. The protocol for obtaining blood and utilization of DBS cards was adapted from Adjepong et al., 2018. One 6mm hole punch was obtained from the center of the card. The hole punch was cleaned with an alcohol swab in between punches of DBS samples. The DBS punches were added to a micro-centrifuge tube with 500 μL of Dulbecco's Phosphate-Buffered Saline (dPBS; Sigma) placed on shaker for 1 hour at room temperature and then stored at -20C. The final concentration of IFN-α should have been 120p-240pg/mL for 10-20uL spots, respectively. These steps were repeated for Day 10 and 20 for all DBS.

### *Interferon- alpha Quantitation*

Samples were analyzed the same day using an immunoassay (ELISA) for IFN-α in both un-spiked and spiked plasma and DBS samples. The Verikine Human Interferon Alpha Multi-Subtype Serum ELISA kit (PBL Assay Science, Piscataway, NJ) was used according to manufacturer instructions. The limit of detection of the ELISA kit was 12.5pg/mL with less than 8% coefficient of variation for both inter- and intra-assay variability, and greater than 90% spike recovery from serum. 50ul of diluted sample was added and incubated for 1 hour in 48 wells of a 96-well plate. After 1 hour, the plate was washed one time with diluted wash buffer. Addition of 100uL/well of a diluted antibody solution was incubated for 1 hour. The wells were then washed three times. Horseradish peroxidase (HRP) conjugate solution was added at 100uL/well for 1 hour followed by another four washes. Tetramethyl-benzidine (TMB) was added 100uL/well and incubated for 15 minutes at room temperature in the dark. The reaction was inhibited by adding 100uL of stop solution. Absorbance was read at 450nm using a Biotek microplate reader. The IFN-α concentration was determined based on the standard curve generated by diluted IFN-α standard absorbance and four-parameter curve fit.

### Total Protein Quantitation

Total protein was measured the same day for all spiked and un-spiked samples using a Bradford Assay (Thermo Scientific, Rockford, IL) according to manufacturer instructions. Dilution of 1:100 in dPBS for serum and 1:10 for DBS was performed in preparation for diluted bovine serum albumin standards. Standards and samples were pipetted into microplate wells (Thermo Scientific 48-well plate) followed by addition 250uL of Coomassie Reagent to each well and placed on a shaker for 30 seconds. After ten minutes of incubation at room temperature, the absorbance was read at 595nm using a microplate reader and a standard curve was generated using a four-parameter curve. All samples were measured in duplicate for both un-spiked and spiked IFN- α and total protein samples.

### Statistical Analysis

All quantitative values were expressed as the mean of the group ± standard error of the mean (SEM). Statistics for IFN-α concentration in DBS samples and Total Protein from the Bradford Assay were analyzed using the non-parametric Kruskal-Wallis Test with Bonferroni Procedure post-hoc test in SPSS. A One-Sample-t Test was also performed for DBS and plasma samples to compare the DBS card concentrations against the expected concentration. P values were considered significant if $p \leq 0.05$.

### Results

### IFN-α Concentrations in Plasma and DBS

IFN-α concentrations in DBS was determined by ELISA (Table A1). Absorbance was measured using a Biotek microplate reader at 450nm. All Samples (n=3) were measured in duplicate. As displayed, all un-spiked samples (-IFN-α) were below the limit of detection (12.5pg/mL) for IFN- α concentration. All spiked samples (+IFN-α) were above the limit of

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detection. The plasma concentrations were adjusted for dilution and subjects A, B, and C ranged between 7-10,000 pg/mL with a mean of 9,138.47 $\pm$ 349.08pg/mL. Mean concentrations for DBS samples (not accounting for dilution) were similar over the 20 days (day 1; 64.63 $\pm$ 5.62pg/mL, day 10; 77.84 $\pm$ 0.67pg/mL, day 20; 73.23 $\pm$ 4.92pg/mL). The concentrations of DBS samples were analyzed using the non-parametric Kruskal-Wallis test (Table 1). There was no significant change in IFN-α concentration in DBS over Days 1, 10, and 20 (Kruskal-Wallis: $\chi^2$=3.59, df=2, p>0.05). The concentration of IFN-α was then compared individually against the expected 120pg/mL concentration. There was significant change in IFN-α concentration in DBS samples at Day 1 (One-Sample T-Test t=-9.85, test value=120, df= 5, p<0.05), Day 10 (One-Sample T-Test t=-62.86, test value=120, df= 5, p<0.05), and Day 20 (One-Sample T-Test t=-9.50, test value=120, df= 5, p<0.05) indicating loss in concentration. Plasma concentration was also analyzed against the expected concentration. There was no significant decrease in concentration from plasma (One-Sample T-Test t=-2.47, test value=10,000, df= 5, p>0.05).

**Table 1: Kruskal-Wallis Test for IFN-α Concentration in Spiked DBS***

|  | Day | N | Mean + SEM | Mean Rank | Chi-Square | P Value |
|---|---|---|---|---|---|---|
| **IFN-α Concentration in DBS** | Day 1 | 6 | 64.63 $\pm$ 5.62 | 6.67 | 3.59 | 0.17 |
|  | Day 10 | 6 | 77.87 $\pm$ 0.67 | 12.50 |  |  |
|  | Day 20 | 6 | 73.23 $\pm$ 4.92 | 9.33 |  |  |

*No significant difference in IFN-α and Days 1,10, and 20.

*Protein Quantitation and Recovery of IFN- α*

Total protein was measured to examine if any loss of IFN-α from DBS cards would be accounted for by differences in total protein extracted. Total protein in un-spiked (-IFN-α) and spiked (+IFN-α) samples were measured by the Bradford Assay (Tables A6-A7). Means were

calculated for both -IFN-α (Table 2 & 4; day 1, 3,666.50 ± 218.48ug/mL; day 10, 4,772.67 ±

598.51ug/mL; day 20, 4,839.33 ± 178.18ug/mL) and +IFN-α (day 1, 19,446.17 ± 3343.34ug/mL;

day 10, 25,980.00 ± 3145.17ug/mL; day 20, 19,509.00 ± 3085.65ug/mL) DBS samples. There

was a significant difference in total protein extracted in the un-spiked (-IFN-α) DBS samples

(Kruskal-Wallis $\chi^2$ = 6.38, df=2, p<0.05). After completion of the Kruskal-Wallis test for -IFN-α

DBS samples, a post-hoc test was performed as a pairwise comparison to measure significance

between each day (Table 3). Significant values were adjusted by the Bonferroni correction for

multiple tests (Table 4). It was determined that there was no significant difference from Day 1-

Day 10, Day 10-20, or Day 1-20 (Bonferroni Correction: p>0.05). There was not a significant

difference in total protein extracted in the spiked (+IFN-α) DBS samples (Kruskal-Wallis $\chi^2$

=2.70, df=2, p>0.05).

**Figure 1: Total Protein Concentrations for Un-Spiked DBS Samples**
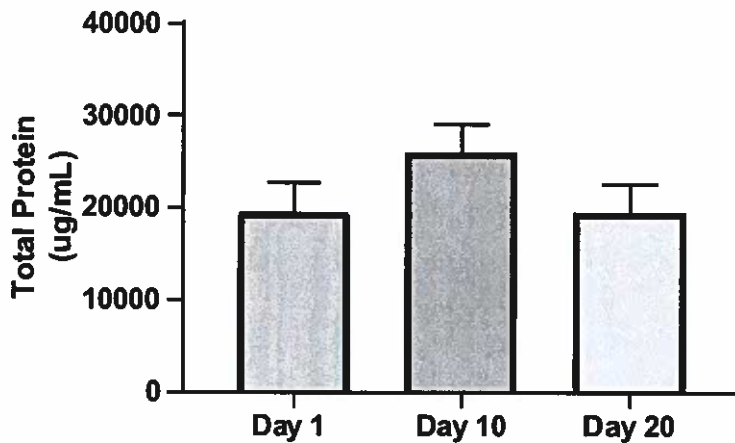
**Table 2: Kruskal-Wallis Statistics for Total Protein in Un-Spiked DBS Samples***

|  | Day | N | Mean + SEM | Mean Rank | Chi-Square | P Value |
|---|---|---|---|---|---|---|
| **Total Protein Extraction Un-Spiked (-IFN-α) DBS** | Day 1 | 6 | 3666.50 ±218.48 | 5.42 | 6.38 | 0.04 |
|  | Day 10 | 6 | 4772.67 ±598.51 | 9.92 |  |  |
|  | Day 20 | 6 | 4839.33 ±178.18 | 13.17 |  |  |

*There was a significant difference in Total Protein for Un-Spiked DBS samples and Days 1,10, and 20.

**Table 3: Post Hoc- Bonferroni Correction for Total Protein in Un-Spiked DBS Samples***

| Sample1- Sample 2 | Test Statistic | Std. Error | Std. Test Statistic | Sig. | Adj. Sig. |
|---|---|---|---|---|---|
| **Day 1-Day 10** | -4.50 | 3.08 | -1.46 | 0.14 | 0.87 |
| **Day 1-Day 20** | -7.75 | 3.08 | -2.52 | 0.01 | 0.07 |
| **Day 10-Day 20** | -3.25 | 3.08 | -1.06 | 0.29 | 1.00 |

*Significant values were adjusted using Bonferroni correction for multiple tests. There was no significant

difference in Total Protein for Un-Spiked DBS samples and Days 1,10, and 20.

**Table 4: Kruskal-Wallis for Total Protein in Spiked DBS Samples***

|  | Day | N | Mean + SEM | Mean Rank | Chi- Square | P Value |
|---|---|---|---|---|---|---|
| **Total Protein Extraction Spiked (+ IFN-α) DBS** | Day 1 | 6 | 19446.17 +3343.34 | 8.17 | 2.70 | 0.26 |
|  | Day 10 | 6 | 25980.00+ 3145.17 | 12.42 |  |  |
|  | Day 20 | 6 | 19509.00 + 3085.65 | 7.92 |  |  |

*There was no significant difference in Total Protein for Spiked DBS samples and Days 1,10, and 20.

A percentage was calculated to obtain the total protein extracted from DBS cards (Table A8). Averages were taken from total protein concentrations that were spiked with IFN-α for DBS cards and divided by the average concentrations of serum then multiplied by 100% to get a percentage value. There was a range of 30-55% recovery in all three days (day 1,

32.52±1.71%; day 10, 46.19±5.42%; day 20, 33.36± 1.79%). Concentrations of IFN-α on DBS cards determined from the ELISA were recalculated adjusting for the total protein extracted percentage (Table A9). Adjusted concentration of IFN-α found on the DBS cards for days 1, 10, and 20 yielded concentrations closer to the expected 120pg/mL concentration (day 1, 111.80±11.13pg/mL; day 10, 119.81±4.60pg/mL; day 20, 122.09±12.78pg/mL).

The Kruskal-Wallis Test was repeated for the DBS samples with the adjusted concentrations (Table 5). There was not a significant difference in IFN-α concentration for the spiked DBS samples (Kruskal-Wallis $\chi^2$= 0.62, df=2, p>0.05). The concentration of IFN-α was then compared individually against the expected 120pg/mL concentration. There was also no significant difference in IFN-α for the spiked DBS samples at Day 1 (One-Sample T Test t= -0.73, test value= 120, df=2, p>0.05), Day 10 (One-Sample T Test t=-0.04, test value= 120, df=2, p>0.05, and Day 20 (One-Sample T Test t=0.16, test value= 120, df=2, p>0.05) when compared to the expected concentration.

**Table 5: Kruskal-Wallis for Adjusted IFN-α Concentration in Spiked DBS\***

|  | Day | N | Mean + SEM | Mean Rank | Chi-Square | P Value |
|---|---|---|---|---|---|---|
| **IFN-α Concentration in DBS Readjusted** | Day 1 | 3 | 111.80 + 11.13 | 4.00 | 0.62 | 0.73 |
|  | Day 10 | 3 | 119.81 + 4.60 | 5.67 |  |  |
|  | Day 20 | 3 | 122.09 + 12.78 | 5.33 |  |  |

\*There was no significant difference in IFN-α for Spiked DBS samples and Days 1,10, and 20.

**Discussion**

*Significance of Results*

The un-spiked samples for DBS and serum samples had no detectable IFN-α. This was expected since healthy individuals should have low, if any, concentration of circulating IFN-α. The IFN-α standard used for spiking samples was at a stock concentration of 10,000 pg/mL.

The mean plasma concentration was 9,138.50 ± 295.00pg/mL which was not significantly different than the 10,000 pg/mL standard concentration. It was expected that there would be little to no degradation of IFN-α, and therefore, little to no change in concentration of IFN-α for the spiked DBS samples. Originally, there was a significant difference in IFN-α concentration of the spiked-DBS samples compared to the expected concentration of 120pg/mL. However, after analysis of the Bradford Assay, the total protein recovery was extremely low ranging from 30-55% recovery. Concentrations of IFN-α were then re-calculated for the spiked DBS samples after accounting for the percent recovery of total protein. After the adjustment to IFN-α concentration, statistical analyses were repeated. There was no significant difference in the adjusted IFN-α concentration for spiked DBS samples from Day 1-10, 10-20, and 1-20 and when compared to the expected 120pg/mL concentration. Although variation within the DBS card days did complicate the analyses, the general trend of no IFN-α standard degradation was supported by the data analyses. This allows us to conclude the IFN-α on DBS cards remained stable at room temperature for the 20-day trial period.

### Limitations of Study

Although our conclusion was that little degradation occurred between days 1-20 on the DBS cards, there were limitations to the study including lack of sample size and comparisons of analytes, variation in blood analyte concentrations, and protein extraction/elution efficiency. Limited sample size and high variability resulted in low statistical power. The objective of the study was to determine stability of IFN-α over 20 days. A post-hoc analysis of statistical power was performed using G*Power 3.1.9.2. Protein concentration-adjusted IFN-α concentrations combined across all days was 117.9 ± 15.9 (mean ± standard deviation) which calculates an effect size of 0.132. The 0.132 effect size means of 120.0 and 117.9, alpha of 0.05, and power of 0.8 would require 452 subjects to detect a significant difference in a two tailed, one sample t-test. An effect size of 3.26 would be required to detect significance with 3 subjects. This effect

size is extremely large. Due to lack of time and budget restrictions, running another immunoassay with more subjects was not an option. However, a larger sample size of healthy subjects could potentially yield statistically significant differences and would have allowed for parametric analysis to take place.

Although we did not observe declines in IFN-α concentrations over a 20-day period, it would also be beneficial to compare the concentrations of IFN-α against another Type I IFN to determine the effect of DBS storage and processing on multiple isoforms. The standard did contain multiple isoforms of human IFN-α including IFN-αA, IFN-α2, IFN-αD, IFN-αB2, IFN-αC, IFN-αG, IFN-αH, IFN-αI, IFN-αJ1, IFN-αK, IFN-α1, IFN-α4a, IFN-α4b, and IFN-αWA. After review of current literature, we concluded that the isoforms of IFN-α associated with autoimmune disorders are unknown. Specific isoforms of serum IFN-α subtypes associated with initiating or exacerbating SLE and other autoimmune diseases is also currently unknown (Kuruganti et al., 2014). The lack of research on isoforms and autoimmune association could be due to low abundance of IFN-α in circulation as well as lack of adequately specific antibodies to distinguish between the IFN-α subtypes (Reynolds et al., 2019).

The ELISA kit purchased utilizes a combination of monoclonal and polyclonal antibodies to detect a range of IFN-α subtypes. Monoclonal antibodies only recognize and bind to a single epitope on the antigen, versus polyclonal antibodies that recognize several epitopes on any one antigen. Because the kit was a combination, it is unknown which epitope of IFN-α the antibody was binding. The epitope may have been intact even though the protein was degraded. The structure of IFN-α contains a disulfide bond which is a common site of protein degradation. The antibody epitope may not include the disulfide domain and would still bind IFN-α. However, this does not affect the application of our results. Utilizing DBS to detect IFN-α in populations is still possible if the epitope recognized by the anti-IFN-α antibody remains stable.

Another limitation was variation in analyte concentration of IFN-α among the 20 days. Sources of error contributing to variability could be blood sampling techniques, pipetting error, percent of spot punch prior to extraction, and issues with blood clotting. The variability and low number of samples also contributed to low statistical power.

Blood sampling techniques contribute to variability of blood analyte variability. Even with venipuncture blood collection, the variability due to sampling can be significant. Blood sampling for DBS applications is usually performed with a finger or heel stick and the drop applied directly to the DBS card. However, for this study, sample volume measures were important to monitor for accurate dilution calculations. The subjects in this study sampled their own blood which created variability in the volume of blood collected and the mixing of the blood with the anticoagulant and blood clotting. Differences in blood volumes collected and spotted were accounted for in all calculations. However, higher volumes of blood sampled could have beneficial factors such as stabilizing factors including antioxidants and protein-protein interactions that would reduce protein oxidation and preserve cytokine levels or detrimental effects such oxidative factors including iron that would increase the degradation of cytokines. Blood clotting would also affect the volume, protein concentration, and other unknown factors that could increase the efficiency of this technique. However, blood clotting would not be an issue if the finger or heel stick drop of blood was applied directly to the card, though differences in blood volume would still remain a concern.

Diffusion and distribution of blood and blood proteins may be affected by subject hematocrit values. Blood samples with different hematocrit values can cause different spot sizes due to differences in viscosity and blood spot diffusion on the card resulting in measurement variability (Sharma et al., 2014). The total protein in spots with 20uL of blood were reduced compared to total protein in spots with 10uL of blood. The difference in protein recovery could be due to varying diffusion rates and excessive spreading of the sample. Although the same

6mm hole punch was used for all DBS samples, the specific area of the hole punch may not have been uniform for each individual sample therefore affecting protein concentration and reducing the amount of protein recovered. If this study were to be repeated, it is in the best interest to make sure the same amount of blood is obtained from all subjects, spotted in equal volumes or utilize PCDBS in order to nullify the effect of the hematocrit issues and aid in sample extraction.

Another limitation was the effectiveness of protein extraction from the DBS cards. Elution of analytes from the DBS is always challenging because of the chance for analyte loss due to ineffective extraction. Poor sample elution is usually due to either incomplete extraction or analyte degradation (Zakaria et al., 2016). In normal plasma protein, the levels range from 60-80g/L. In the spiked serum, the average levels were approximately 60g/L which would be considered on the lower end of the normal range. However, our DBS plasma protein levels yielded were extremely low and completely out of normal range. Modifications of our elution techniques including choice in elution buffer and duration of elution could have created this low yield in protein recovery.

The effects of different elution buffers on DBS samples need to be taken into consideration for protein recovery as well as when working with DBS and ELISA immunoassays (Lee & Li, 2014). If the type or dilution of elution buffer is not appropriate, it can increase the presence of red blood cell lysis and other proteins. This could increase assay background interference and reduce detection of the antibody by the ELISA (Mercader et al., 2006; Villar et al., 2011). Phosphate-buffered saline (PBS) is routinely used in DBS application as a buffer solution (McDade, 2014; Miller & McDade, 2012). Addition of proteins to PBS buffer such as bovine serum albumin, polysorbate 20 (Tween 20, a surfactant), or protease inhibitor may improve assay signal by stabilizing proteins as they come into solution, and by blocking nonspecific binding sites in the assay plate. (McMade, 2014). For our study, we utilized

Dulbecco's phosphate buffered saline (DPBS) as the elution buffer. Although there is no significant difference between the two buffers, DPBS may contain calcium and magnesium ions, which are important for protein-protein interactions or even interactions between protein domains. With the presence of those ions, utilization of dPBS instead of PBS could allow the protein structure to remain more intact.

Duration and temperature are also important factors in elution. Depending on analytes, the protocol can range from 2-4 hours all the way to overnight incubations. While shorter elution periods run the risk of a decline in extraction recovery, analytes from DBS placed into solution for long periods of time are more susceptible to degradation (McDade, 2014). Incubating samples at 4°C during elution may also prevent or attenuate degradation while incubating at room temperature or higher during elution will reduce elution times but may increase rates of sample degradation. Our elution duration was only 1 hour at room temperature. The short extraction duration along with extracting at room temperature serve as rationales for the extremely low protein recovery.

### Future Research Directions

To our knowledge, there have been no studies that monitored the stability of IFN-α on DBS cards. Understanding the stability of IFN-α in DBS can allow for more studies monitoring levels of endogenous IFN-α in individuals with abnormally high levels. The incorporation of subjects known to have viral illnesses or autoimmune disorders could potentially generate different statistical results. Endogenous concentrations of cytokines would be elevated in some patients and we could determine the effect of DBS storage and handling on endogenous type 1 IFN in addition to spiking with the standard. This knowledge could be extremely beneficial when monitoring viral infections such as Hepatitis C, Herpes Simplex virus (HSV), or useful for evaluation and explanation of autoimmune disorders such as SLE and T1D where IFN-α

concentrations are already known to be high (Bennett et al., 2003; Bezalel et al., 2014; Blanco et al., 2001). Shahin et al. (2011) compared serum levels of IFN-α in Egyptian SLE patients. Since SLE is a familial disease, their first-degree relatives (FDRs) were also evaluated and both SLE patients and FDR IFN-α levels were compared to unrelated healthy controls (UHCs). They noted that patients with SLE had mean concentrations of IFN-α of 65.3 ± 53.4 pg/mL, 19.5 ± 23pg/mL in FDRs, and 5.2 ±5.2 pg/mL in UHCs. Examining these levels along with autoantibody levels allowed for identification of individuals at the greatest risk for clinical illness. Based on our results, these levels of IFN-α would not be detectable using DBS unless protein extraction efficiency improved. Using DBS, these tests could be done routinely to monitor the IFN signature, identify persons at risk for disease development, and prevent disease flares or potential organ damage in patients diagnosed with autoimmune disorders.

With knowledge of the stability of DBS cards at room temperature at 20-days, this allows for more analyte measurements from populations that are generally difficult to access for research studies. These difficult-to-access populations may also have reduced technological resources including laboratory equipment as basic as refrigerators. Our data contributes to knowledge that may ease access and allow data collection from these populations. This collection of data is crucial, especially with growing changes in climate, as well as rates of cardiovascular, immune, and nervous system diseases continue to rise. The concomitant rise in disease risk and changes in climate may be due to changes in lifestyle such as dietary intake.

Insufficient intake of EFA leads to deficiency, and may result in impairment of growth, impaired immune function, and poor cognitive development (Jumbe et al., 2016). Even though n3 PUFA supplements such as fish oil are highly consumed as dietary supplements (Albert et al., 2015), the total intake of fish oil in Arctic populations may be decreasing. Due to environmental changes such as climate change and lack of resources, consumption of foods containing n3 FA may grow more difficult and may have a negative effect on health (Kang,

2011). Autoimmune disorders are extremely low in populations with traditionally high intakes of n3, but these populations could see a rise in illnesses such as: SLE, RA, Inflammatory Bowel Syndrome (IBS), and many other disorders due to forced change in diet. More research including measurement and analysis of fatty acid and cytokine concentrations will need to take place, especially in specific regions of the world where blood and DBS storage at low temperatures may not be possible.

To correlate IFN-α concentrations with phospholipid fatty acids and prevalence of autoimmune disease, it would be ideal to measure the same subjects at the same time. Our lab did assess serum fatty acids using similar analytical techniques to OmegaQuant (Tables A13-A16). These fatty acids would be used to calculate the Omega 3 Index which correlates with health and disease. OmegaQuant DBS cards have proprietary preservatives to measure fatty acids, which could affect proteins differently. Our study provides evidence that evaluation of fatty acid analytes in association to IFN-α concentration may be feasible and facilitate collaborative international research projects.

**Conclusion**

We hypothesized that there would be little to no degradation on DBS cards spiked with IFN-α and placed at room temperature for a 20-day period. Adjustments to IFN-α concentration were made after adjusting for total protein recovery. With these adjustments, there were no significant differences in IFN-α concentration from DBS cards stored for 20 days at room temperature. IFN-α stored on DBS cards is relatively stable in room temperature conditions.

As the incidence of autoimmune disorders and cardiovascular and inflammatory illnesses increases, the need to measure and analyze cytokine concentrations and other analytes such as fatty acids will also increase. The utilization of DBS for analyte measurement may be essential in specific regions of the world where blood and DBS storage at lower

temperatures may not be possible. Understanding the stability of IFN-α and other analytes in DBS at room temperatures can allow for measurement of cytokine activity and expression, monitoring of illness/disease development and flares, and collaborative projects internationally in future research.

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**Appendices**

**Table A1: IFN- α Concentration of Individual Samples from ELISA Immunoassay**

| Subject | Serum -IFN-α (pg/mL) | Serum + IFN-α (pg/mL) | DBS D1 -IFN-α (pg/mL) | DBS D1 + IFN-α (pg/mL) | DBS D10 -IFN-α (pg/mL) | DBS D10 + IFN-α (pg/mL) | DBS D20 -IFN-α (pg/mL) | DBS D20 + IFN-α (pg/mL) |
|---------|------|------|------|------|------|------|------|------|
| A | ND* | 9237.20 | ND | 74.98 | ND | 76.34 | ND | 59.65 |
| A | ND* | 9299.20 | ND | 81.81 | ND | 76.56 | ND | 68.81 |
| B | ND* | 9298.40 | ND | 53.68 | ND | 79.69 | ND | 67.72 |
| B | ND* | 8679.60 | ND | 61.09 | ND | 79.71 | ND | 67.10 |
| C | ND* | 10458.80 | ND | 45.28 | ND | 76.38 | ND | 90.86 |
| C | ND* | 7857.60 | ND | 70.94 | ND | 78.56 | ND | 85.24 |
| Mean ± SEM | ND* | 9138.47 ± 349.08 | ND | 64.63 ± 5.62 | ND | 77.87 ± 0.67 | ND | 73.23 ± 4.92 |

*ND (Not Detected) represents values that were below the rate of detection for IFN-α concentration

**Table A2: One-Sample T Test Statistics for Spiked DBS Samples Day 1**

**Test Value= 120**

|  | N | Mean | Std. Deviation | Std. Error Mean | t | Sig (2-tailed) | df |
|---|---|---|---|---|---|---|---|
| IFN-α Concentration DBS Day 1 | 6 | 64.62 | 13.78 | 5.63 | -9.85 | <0.05 | 5 |

**Table A3: One-Sample T Test Statistics for Spiked DBS Samples Day 10**

**Test Value= 120**

|  | N | Mean | Std. Deviation | Std. Error Mean | t | Sig (2-tailed) | df |
|---|---|---|---|---|---|---|---|
| IFN-α Concentration DBS Day 10 | 6 | 77.88 | 1.64 | 0.67 | -62.86 | <0.05 | 5 |

**Table A4: One-Sample T Test Statistics for Spiked DBS Samples Day 20**

**Test Value= 120**

|  | N | Mean | Std. Deviation | Std. Error Mean | t | Sig (2-tailed) | df |
|---|---|---|---|---|---|---|---|
| IFN-α Concentration DBS Day 20 | 6 | 73.23 | 12.05 | 4.92 | -9.50 | <0.05 | 5 |

**Table A5: One-Sample T Test Statistics and Test for Spiked Plasma Samples**

|  | N | Mean | Std. Deviation | Std. Error Mean | t | Sig (2-tailed) | df |
|---|---|---|---|---|---|---|---|

| IFN-α Concentration Plasma | 6 | 9138.47 | 855.06 | 349.08 | -2.47 | 0.06 | 5 |
|---|---|---|---|---|---|---|---|

**Table A6: Total Protein in Un-spiked Samples Measured by the Bradford Assay**

| Subject | Serum - IFN- α (ug/mL) | DBS D1 -IFN- α (ug/mL) | DBS D10 -IFN- α (ug/mL) | DBS D20 IFN- α (ug/mL) |
|---|---|---|---|---|
| A | 24956.10 | 3423.00 | 4081.00 | 4307.00 |
|  | 12069.30 | 3108.00 | 4194.0 | 4405.00 |
| B | 12687.90 | 4541.00 | 6685.00 | 5188.00 |
|  | 2640.30 | 4081.00 | 6483.00 | 5438.00 |
| C | 43834.90 | 3476.00 | 3056.00 | 4861.00 |
|  | 32856.30 | 3370.00 | 4137.00 | 4837.00 |
| Mean ±SEM | 21507.47 ± 6223.71 | 3666.50 ±218.48 | 4772.67 ±598.51 | 4839.33 ±178.18 |

**Table A7 Total Protein in Spiked Samples Recovered by Performance of Bradford Assay**

| Subject | Serum +IFN- α (ug/mL) | Day 1+IFN-α (ug/mL) | Day 10 +IFN-α (ug/mL) | Day 20 +IFN-α (ug/mL) |
|---|---|---|---|---|
| A | 34016.70 | 12110.0 | 17180.0 | 11310.0 |
|  | 26636.40 | 6767.0 | 15880.0 | 10910.0 |
| B | 62329.10 | 23420.0 | 30040.0 | 18714.0 |
|  | 68123.10 | 23420.0 | 32620.0 | 21040.0 |
| C | 83620.50 | 28340.0 | 26680.0 | 25040.0 |
|  | 83382.90 | 22620.0 | 33480.0 | 30040.0 |
| Mean ± SEM | 59684.78 + | 19446.17 | 25980.00+ | 19509.00 + |

| | 9939.42 | +3343.34 | 3145.17 | 3085.65 |
|---|---|---|---|---|

**Table A8. Average Percent Recovery for Total Protein Extraction**

| Subject | Day 1 | Day 10 | Day 20 |
|---|---|---|---|
| A | 31.13% | 54.51% | 36.63% |
| B | 35.91% | 48.03% | 30.47% |
| C | 30.51% | 36.02% | 32.98% |
| Mean ± SEM | 32.52 ± 1.71% | 46.19 ± 5.42% | 33.36 ± 1.79% |

**Table A9. Adjusted Concentration of IFN-α for DBS Cards After Total Protein Extraction**

| Subject | Day 1 + IFN-α (pg/mL) | Day 10 + IFN-α (pg/mL) | Day 20 + IFN-α (pg/mL) |
|---|---|---|---|
| A | 132.39 | 111.27 | 104.92 |
| B | 94.16 | 121.11 | 114.28 |
| C | 108.86 | 127.04 | 147.06 |
| Mean ± SEM | 111.80 ± 11.13 | 119.81 ± 4.60 | 122.09 ± 12.78 |

**Table A10: One-Sample T Test Statistics for Adjusted IFN-α Concentration in DBS Day 1**

|  | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|
| **IFN-α Concentration in DBS Day 1 Readjusted** | 3 | 111.83 | 19.27 | 11.12 |

**Table A11: One-Sample T Test Statistics for Adjusted IFN-α Concentration in DBS Day 10**

|  | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|
| **IFN-α Concentration in DBS Day 10 Readjusted** | 3 | 119.83 | 7.98 | 4.60 |

**Table A12: One-Sample T Test Statistics for Adjusted IFN-α Concentration in DBS Day 20**

|  | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|
| **IFN-α Concentration in DBS Day 20 Readjusted** | 3 | 122.07 | 22.10 | 12.76 |

**Table A13- Serum Fatty Acid Analysis** ᵃ **Table for Saturated Fatty Acids**

| Sample | Myristic 14:0 | Palmitic 16:0 | Arachidic 20:0 | Stearic 18:0 | Behenic 22:0 | Lignoceric 24:0 |
|---|---|---|---|---|---|---|
| A | 8.3 | 373.8 | 2.8 | 89.0 | 17.3 | 7.0 |
| B | 15.0 | 260.0 | 3.8 | 93.0 | 11.8 | 7.3 |
| C | 6.0 | 297.0 | 3.5 | 95.8 | 13.3 | 10.8 |
| D | 16.5 | 448.3 | 5.5 | 179.3 | 16.5 | 12.5 |
| E | 21.0 | 294.3 | 4.5 | 102.8 | 10.5 | 8.3 |
| F | 32.0 | 703.3 | 6.0 | 132.0 | 11.0 | 13.3 |
| Mean + SEM | 16.5 +3.8 | 396.1+67.4 | 4.3+ 0.51 | 115.3+ 14.3 | 13.4+ 1.2 | 9.8+ 1.1 |

ᵃ Analysis was performed by Jenifer Fenton lab on December 14th, 2018 by Raghav Jain. Units in µg FA/mL serum.

**Table A14- Serum Fatty Acid Analysis • Table for Unsaturated Fatty Acids**

| Sample | Palmitoleic 16:1n-7 | Elaidic 18:1n-9t | Oleic 18:1n-9 | Palmitelaidic 16:1n-7t | Linoelaidic 18:2n-6t | GLA 18:3n-6 | Mead 20:3n-9 | DGLA 20:3n-6 | DTA 22:4n-6 | DPAn-6 22:5n-6 | DPAn-3 22:5n-3 | Nervonic 24:1n-9 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 19.5 | 6.8 | 237.0 | 8.0 | 1.3 | 6.8 | 0.0 | 8.0 | 11.5 | 16.5 | 10.0 | 10.0 |
| B | 14.8 | 3.8 | 203.5 | 8.8 | 1.0 | 8.3 | 0.0 | 14.3 | 3.5 | 6.0 | 6.0 | 10.8 |
| C | 14.3 | 0.0 | 216.5 | 8.0 | 0.8 | 4.3 | 0.0 | 13.0 | 9.5 | 6.8 | 10.0 | 12.0 |
| D | 11.0 | 4.0 | 243.8 | 9.8 | 2.0 | 10.3 | 0.0 | 23.8 | 13.5 | 15.0 | 12.0 | 15.8 |
| E | 23.3 | 3.8 | 187.0 | 8.5 | 1.0 | 7.0 | 0.0 | 29.5 | 9.3 | 7.3 | 9.8 | 14.8 |
| F | 26.3 | 0.0 | 276.3 | 14.0 | 1.8 | 5.3 | 0.0 | 24.3 | 6.8 | 32.3 | 10.3 | 12.5 |
| Mean + SEM | 18.2 + 2.4 | 3.0 + 1.1 | 227.3 + 13.0 | 9.5 + 0.94 | 1.3 + 0.2 | 7.0 + 0.87 | 0.0 | 18.8 + 3.4 | 9.0 + 1.4 | 14.0 + 4.1 | 9.7 + .08 | 12.6 + 0.9 |

• Analysis was performed by Jenifer Fenton lab on December 14th, 2018 by Raghav Jain. Units in µg FA/mL serum.

Table A15- Serum Fatty Acid Analysis – Table for Total Ratio of Fatty Acids

| Sample | Total SFA | Total MUFA | Total PUFA | Total HUFA | HUFA ratio | Total n-6 | Total n-3 | n-6:n-3 ratio | O3I | Total FA |
|---|---|---|---|---|---|---|---|---|---|---|
| A | 498.0 | 281.3 | 724.0 | 147.0 | 0.2 | 683.8 | 39.0 | 17.5 | 4.0 | 1503.3 |
| B | 390.8 | 241.5 | 562.0 | 160.0 | 0.1 | 540.0 | 21.0 | 25.7 | 3.1 | 1194.3 |
| C | 426.3 | 250.8 | 672.8 | 175.5 | 0.1 | 623.3 | 48.8 | 12.8 | 3.3 | 1349.8 |
| D | 678.5 | 285.8 | 1279.5 | 346.5 | 0.1 | 1233.3 | 44.3 | 27.9 | 3.3 | 2243.8 |
| E | 441.3 | 237.3 | 640.0 | 178.0 | 0.1 | 601.3 | 37.8 | 15.9 | 3.0 | 1318.5 |
| F | 897.5 | 329.0 | 1842.0 | 300.5 | 0.2 | 1788.8 | 51.5 | 34.7 | 3.3 | 3068.5 |
| Mean ± SEM | 555.4 ± 80.1 | 270.9 ± 14.3 | 953.4 ± 206.4 | 217.9 ± 34.2 | 0.1 ± 0.02 | 911.7 ± 203.4 | 40.4 ± 4.4 | 22.4 ± 3.4 | 3.3 ± 0.1 | 1779.7 ± 299.6 |

Analysis was performed by Jenifer Fenton lab on December 14th, 2018 by Raghav Jain. Units in µg FA/mL serum.

Table A16- Serum Fatty Acid Analysis •Omega-3 Index and Omega 6:3 Ratios

| Sample | ALA 18:3n-3 | Linoleic 18:2n-6 | Eicosenoic 20:1 | Eicosadienoic 20:2n-6 | Arachidonic 20:4n-6 | EPA 20:5n-3 | DHA 22:6n-3 | Total n-6 | Total n-3 | n-6:n-3 ratio | O3I | Total FA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 2.8 | 566.3 | 0.0 | 0.0 | 74.8 | 6.0 | 20.3 | 683.8 | 39.0 | 17.5 | 4.0 | 1503.3 |
| B | 2.5 | 390.3 | 0.0 | 0.0 | 117.8 | 0.0 | 12.5 | 540.0 | 21.0 | 25.7 | 3.1 | 1194.3 |
| C | 23.3 | 469.0 | 0.0 | 0.0 | 120.8 | 1.8 | 13.8 | 623.3 | 48.8 | 12.8 | 3.3 | 1349.8 |
| D | 6.8 | 914.0 | 1.5 | 0.0 | 256.8 | 0.0 | 25.5 | 1233.3 | 44.3 | 27.9 | 3.3 | 2243.8 |
| E | 15.8 | 438.3 | 0.0 | 0.0 | 110.0 | 0.3 | 12.0 | 601.3 | 37.8 | 15.9 | 3.0 | 1318.5 |
| F | 4.8 | 1529.8 | 0.0 | 0.0 | 190.5 | 4.8 | 31.8 | 1788.8 | 51.5 | 34.7 | 3.3 | 3068.5 |

Analysis was performed by Jenifer Fenton lab on December 14th, 2018 by Raghav Jain. Units in µg FA/mL serum.