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META-ANALYSIS OF DOLPHIN AND HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS REVEALS INFLAMMATORY SIGNATURES ASSOCIATED WITH EXPOSURE TO HIGH LEVELS OF PERFLUOROALKYL SUBSTANCES

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Abstract - Currently a fundamental gap exists on how persistent organic pollutants affect humans especially those who consume high levels of seafood from contaminated coastal waters. There is an urgent need to better understand effects of these contaminants on human health in coastal communities. Elevated contaminant levels in coastal seafood will give rise to increased contaminant burdens in humans who consume coastal seafood and given the toxicity of these contaminants increase risk for immune system disorders. In this paper we describe how we assessed the effects of contaminant exposures using high-throughput RNA sequencing (RNA-seq) in peripheral blood mononuclear cells from two species, dolphins and humans. This paper presents an integrated systems biology approach to understand the adverse effects of one of these CECs PFAS on both species.

Keywords - One Health, PFASs, PBDEs, PBMC, Systems Biology.

I. INTRODUCTION

One Health is an emerging paradigm that recognizes that the health and well-being of humans, animals and ecosystems are interconnected. The recognition that environmental factors can impact human health is not novel with origins dating back to Ancient Greece and the Greek physician Hippocrates [1]. The recognition that blood cells can act as sentinels of disease and be exploited for the diagnosis and prognosis of illness is a more recent finding. Peripheral blood is a perfect surrogate tissue as it is relatively easy to obtain and provides a large biosensor pool in the form of mRNA transcripts. Perturbations in macro- and micro-environments manifest as changes in the levels of these gene transcripts [2]. This data can provide key insights into the effects of environmental exposures and the development of disease. High-throughput or next generation sequencing has been exploited by biomedical researchers for over a decade at this point [3]. It has transformed biomedical research facilitating more in-depth genomic analyses and novel biomarker discovery than in any other period of scientific research. One of the most prominent applications of next generation sequencing is ribonucleic acid sequencing, more commonly known as RNA-seq [4, 5]. RNA-seq allows rapid interrogation of the transcriptome, which is essentially the totality of messenger RNA within a cell or a population of cells at a given moment in time [6, 7]. An RNA-seq experiment results in the generation of millions of short sequence reads enabling quantitative measures of gene expression from the cells being studied. The ultimate goal of RNA-seq is biomarker discovery. The output of RNA-seq experiments circumvents many of the issues presented by earlier technologies such as DNA microarrays. The short (digital) sequence reads provide a measure of abundance of a given mRNA. This approach is unbiased and not dependent on pre-selection of DNA probes that target the transcripts being studied. Today RNA-seq has matured into a technology that enables a sensitive and accurate measurement of gene expression, particularly in the context of low abundance transcripts [4, 8].

In the twentieth century, an abundance of both organic and inorganic contaminants has been introduced into the environment. Many of these are industrial chemicals with structural similarity to steroid hormones. These contaminants bind steroid nuclear hormone receptors or key enzymes that regulate steroidogenesis and thus disrupt normal endocrine physiology in humans and wildlife. These endocrine disruptors (EDs) have been labeled contaminants of emerging concern (CEC), because they have the potential to cause adverse effects to human and ecosystem health [9-11].

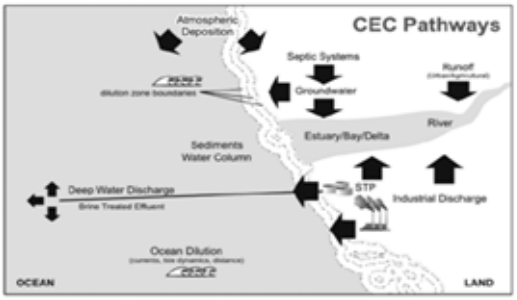

Figure 1 Potential sources and pathways for CEC introduction into the aquatic environment.

CECs are discharged into rivers, lakes, and the ocean, spreading between water columns (interstitial waters that interface with sediment) and solid phase (sediment and particulates in suspension). They subsequently migrate from the water column to sediments where they interact with organic and inorganic particles, ultimately settling in river and estuarine beds and the ocean floor [12-14](Figure 1).

Perfluoroalkyl substances (PFAS): PFAS are a family of incredibly resilient fluorine-containing chemicals which make materials stain- and stick-resistant. The two most commonly found contaminants are perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS)[15]. Data from animal studies of PFOA indicate that exposure leads to the development of tumors. Neonatal death has also been linked with PFOA exposure. Additionally toxic effects on the immune, liver, and endocrine systems have been reported [16].

Some of the highest PFAS found globally in marine mammals have been observed in bottlenose dolphins in Charleston, SC, USA[17, 18]. The PFAS levels measured in plasma of resident Charleston dolphins are on the same order of magnitude as that of occupationally exposed humans with higher body burdens of specific PFAS compounds in dolphins inhabiting areas with greater developed land use [19]. This suggests an urban environment that contributes a significant source of these compounds to both dolphins and the fish that they and humans consume.

Geographical differences exist in temporal trends of PFAS contamination [20, 21] and habitat and diet are prominent factors that influence PFAS concentrations in marine mammals[22, 23].Non-point source pollution originating from urbanized sites was reported in aquatic ecosystems as a key factor for PFAS[24]. As PFAS are considered toxic and studies with these chemicals have been linked to poor health outcomes [18], this indicates degraded ecosystem health.PFAS exposure impacts dolphin health as well as that of humans exposed to these chemicals through seafood ingestion and utilization of coastal waters in these areas.

Diet and seafood: Seafood is a major source of perfluoroalkyl substances (PFAS) which are endocrine disruptors and immune-modulators[25-28]. A recent study examined the concentrations of 11 PFASs in muscle and whole fish for six species collected from Charlestonto assess potential health risks to humans and wildlife. Across all species and capture locations, total PFAS levels in whole fish were significantly higher than in fillets by a factor of two- to threefold. The most abundant compound in each fish species was perfluorooctane sulfonate (PFOS). Unlike whole fish, PFAS levels in fillets varied significantly by sampling location, reflecting levels of PFASs contamination in these systems. In whole fish, differences in relative concentrations of PFOS, perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA) occurred by capture location, suggestive of different sources. PFOS levels exceeded screening values to protect mammals in 83% of whole fish examined and represent a potential risk to wildlife predators such as dolphins[29].

Predictors of PFAS serum concentrations and adverse health outcomes in humans remain to be clearly identified. Subsistence fisherman and those who consume seafood have higher body burdens of these contaminants than other populations[27, 28]. The African American Gullah population of Coastal Carolina have elevated levels of PFAS and polybrominated diphenyl ethers (PBDEs), which has been associated with markers of autoimmunity[30, 31]. Systemic lupus erythematosus (SLE), also known simply as lupus, is an autoimmune disease in which the body's immune system inadvertently attacks healthy tissue in many parts of the body. Similar to other African American communities, Gullah women are disproportionately affected by SLE, with a higher incidence, prevalence, severity and onset at a younger age, compared to other racial/ethnic groups[32] (20). With local seafood consumption being a dietary staple, potential pollutant contamination of their seafood is of considerable concern[33-35].

Although it is established that dolphins that are resident in the Charleston nearshore environment accumulate extremely high levels of these chemicals, little is known about exposures in humans living adjacent to these areas, and particularly those consuming local seafood.No information exists to date on transcriptomic alterations in peripheral blood as a consequence of increased CEC bioavailability.

The objective of this study was to examine using RNA-seq the peripheral blood mononuclear cell (PBMC) transcriptomes of both dolphins and humans from this geographical area and compare and contrast the genomic signatures.

II. METHODS

A. Selection of human study participants

The SLE in Gullah Health (SLEIGH) study, is a longitudinal cohort of Gullah African Americans that was started in 2003 to investigate potential genetic and environmental factors in the development of autoimmunity [34]. In order to investigate the effect of PFAS on gene expression in human PBMCs, we accessed the patient cohort enrolled in this study. This study protocol (and our request for subjects) was reviewed and approved by the MUSC Institutional Review Board. For this study, we excluded lupus patients and selected individuals with high fish consumption and elevated PFAS levels to compare with those with low fish consumption and low PFAS levels. Fish consumption levels were defined as high if greater than 2-3 times a week and low if less than once a month.

B. Collection of Human Blood Samples:

Aliquots of blood were collected using heparinized blood tubes and the plasma separated (n=8). Plasma destined for PFAS analysis was collected from centrifuged blood and stored in 5 mL cryovials.

C. Isolation of dolphin PBMCs

Blood samples were collected from free-ranging Atlantic

Bottlenose dolphins (Tursiops truncatus) during the Dolphin Health and Risk Assessment Project conducted in August 2013 as outlined in [36](n=9). Dolphins were temporarily captured and released in the estuarine waters of Charleston (SC, USA) to assess their clinical and immune status, disease and contaminant exposure. This study was carried out in strict accordance under National Marine Fisheries permit no. 14352–02.

During the sampling process, each animal received a physical examination, including full body photo-documentation, diagnostic ultrasound, blood and urine collection, blubber and lesion biopsies, and microbiologic and cytologic sampling. Once restrained, blood samples were collected from the periarterial venous rete in the flukes using a 19-gauge needle, 1.9 cm butterfly catheter with a vacutainer attachment.

Whole blood was collected in 10 ml ethylenediaminetetraacetic acid vacutainers. Peripheral blood mononuclear cells were isolated following lysis of red blood cells after treatment with Becton Dickinson (BD, San Jose, CA, USA) Pharm Lyse solution as per the manufacturer's instructions. PBMCs were isolated within 12 h of sample collection and cryopreserved in freezing media (90% fetal bovine serum, 10% dimethyl sulfoxide [DMSO]).

D. Organic analytical chemistry analyses

Concentrations of PFAS, in blood plasma (humans and dolphins) were determined using established methods employing pressurized fluid extraction, chromatography cleanup and analysis by gas chromatography mass spectrometry, as described in detail previously [30, 36].

RNA-seq Studies: Paired end sequencing was performed on the RNA (8 human samples and 9 dolphin samples respectively). 100-200 ng of total RNA from each sample was used to prepare RNA-seq libraries using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA), following the protocol described by the manufacturer. High throughput sequencing (HTS) was performed using an Illumina HiSeq2500 with each sample sequenced to a minimum depth of ~25 million reads.

Data were subjected to Illumina quality control (QC) procedures (>80% of the data yielded a Phred score of 30). Secondary analysis was carried out on an OnRamp Bioinformatics Genomics Research Platform (OnRamp Bioinformatics, San Diego, CA)[37].

OnRamp's advanced Genomics Analysis Engine utilized an automated RNA-seq workflow to process the data, including (1) data validation and quality control, (2) read alignment to the human genome (hg19) or dolphin genome (turTru2) using TopHat2[38] (3) generation of gene-level count data with HTSeq, and (4) differential expression analysis with DEseq2[39], which enabled the inference of differential signals with robust statistical power. [Genomics Research Platform with RNA-seq workflow v1.0.1, including FastQValidator v0.1.1a, Fastqc v0.11.3, Bowtie2 v2.1.0, TopHat2 v2.0.9, HTSeq v0.6.0, DEseq v1.8.0].

The resulting SAM files were sorted and inputted into the Python package HTSeq to generate count data for gene-level differential expression (DE) analyses. In order to infer differential signal within the data sets with robust statistical power, we utilized DEseq2 [35]. Transcript count data from DESeq2 analysis of the samples were sorted according to their adjusted p-value or q-value, which is the smallest false discovery rate (FDR) at which a transcript is called significant. FDR adjustment is needed with large data sets such as RNA-seq and was calculated using the Benjamini-Hochberg multiple testing adjustment procedure. Statistical analysis of pathways and gene ontology (GO) terms was carried out using this sorted transcript list as described by us previously [36, 37] and using Ingenuity Pathway Analysis (Qiagen, Valencia, CA) and the ToppGene Suite [38]. Area-proportional Venn diagrams were created using BioVenn [39].

III. RESULTS

PFOS and PFOA levels

In the human PBMC samples examined,PFOS and PFOA levels ranged from 14.1 (low) to 125.3 (high) and 3.8 (low) to 12.6 (high) ng/ml respectively. Samples were stratified to allow a comparison of individuals with high total PFOS levels defined as >73.85 ng/ml and low total PFOS levels defined as < 25 ng/ml total PFOS levels. Three females and one male were classified as having high and one male and two female were classified as having low total PFOS levels respectively.

For dolphin PBMCs, total PFOS levels ranged from 288 to 1,833 ng/ml. PFOA levels ranged from 6.34 to 55.4 ng/ml. Samples were stratified to allow a comparison of individuals with high total PFOS levels defined as >1,234 ng/ml and low total PFOS levels defined as < 288 ng/ml total PFOS levels.

Four males and one female were classified as having high levels and three males and one female were classified as having low total PFOS levels respectively.

Human & dolphin PBMC transcriptome analyses:

For humans and dolphins 169,227,283 and

242,983,541 short sequencing reads were generated respectively. The mapping rate of reads that mapped to the human genome was on average >93%. For dolphin this was slightly lower with >91% mapping. Only uniquely mapped reads were used in downstream analyses, which equated to approximately 70% of all reads. For DE analyses using DEseq2, samples were compared on the basis of low versus high total PFOS levels. We set low serum total PFOS as the control and high serum PFOS as the test. The results of this analyses revealed that 829 genes were DE in humans and 612 in dolphins using a false discovery rate(FDR) q<0.4.

Systems Level Analyses: These data representing DE transcripts in humans with low and high serum PFOS levels were further analyzed through the use of QIAGEN's Ingenuity® Pathway Analysis and the top canonical pathways are presented (Figure. 2A). A similar approach was undertaken to assess DE transcripts indolphins.

Dolphin transcripts were first mapped to their human homologsusing Ensembl homology and then the corresponding human homologs (gene symbols) were inputted into IPA (Figure 2B). A common theme in the PBMC transcriptomes of both species is increased immune response and inflammatory responses. Additionally endocrine signaling was impacted in both humans and dolphins. Specifically in humans, altered endocrine (PPAR Signaling/ Liver X receptor (LXR)/Retinoid X receptor (RXR) Activation), immune system and inflammatory signatures (Toll-like Receptor Signaling, IL-10 Signaling, NF-κB Signaling, Natural Killer Cell Signaling, Interferon Signaling) are clearly evident. In Dolphins, immune (Immunodeficiency Signaling, T Cell Receptor Signaling, CD28 Signaling in T Helper Cells, CCR5 Signaling in Macrophages, CTLA4 Signaling in Cytotoxic T Lymphocytes) and endocrine (Prolactin Signaling/Type I Diabetes Mellitus Signaling) are enriched. When the transcriptomes of both species were compared at a canonical pathway level, of the top ranked 50 pathways, 13 were in common including CCR5 Signaling in Macrophages, EIF2 Signaling and Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells. Analysis of the disease categories revealed Immunological

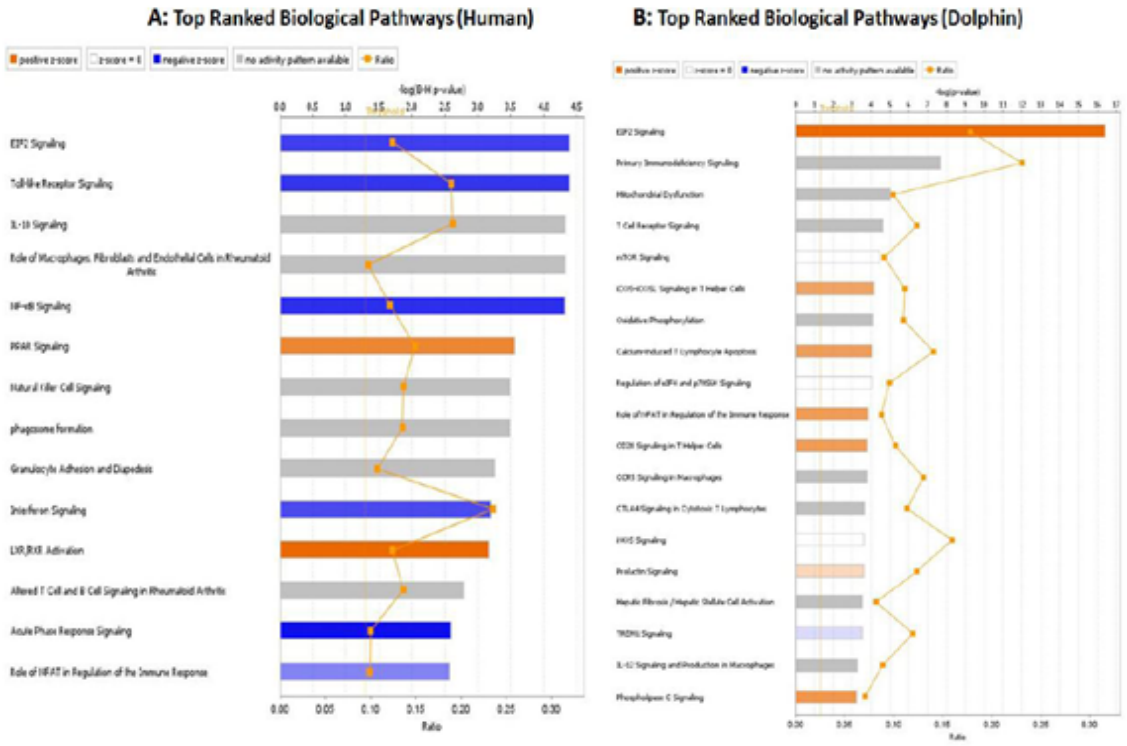

Figure 2: Immune and inflammatory and endocrine canonical pathways enriched in PBMCs from humans and dolphins with high and low PFAS/PBDEs levels.

Pathway analysis using IPA of the significantly differentially expressed genes from our human and dolphin RNAseq experiments highlight altered endocrine immune system and inflammatory signatures between these 2 groups.The significant canonical pathways for the dataset that are enriched are displayed along the y-axis. The x-axis (top) displays the -log of p-value which is calculated by Fisher's exact test right-tailed (and corrected for False discovery rate (FDR) using the Benjamini–Hochberg

procedure within IPA). Taller bars correspond to increased pathway significance. Orange colored bars indicate that the pathway in question is activated. White bars indicate significant pathways that are neither activated nor inhibited. Gray bars indicate pathways that are significant but no prediction as to activation or inhibition can be made. The orange points connected by a thin line represent the ratio (x-axis bottom). The ratio is calculated as follows: (# of genes in a particular pathway significantly enriched in the RNAseq data set, divided by the total # of genes that make up that pathway and are present in the reference gene set).

Disease/systemic autoimmune syndrome as highly significant in both humans (p-value 4.54E-13) and dolphins (3.23E-24).

IV. DISCUSSION

Human activities have resulted in high concentrations of PFASin ecosystems such as in Charleston, SC via direct discharges from land-based sources (e.g., industrial and municipal wastes), river runoff or drainage, and atmospheric deposition from local and distant sources. These chemicals are very persistent and bioaccumulate[40]in the food-web where they can reach high concentrations in top-level predators and ultimately affect human health. Seasonal impacts on hydrographic conditions are expected to directly influence the availability and toxicological effects of chemical and biological contaminants[41]. Systematic changes in marine hydrographical conditions due to warmer temperatures, reduced salinity and hypoxia may directly impact seafood safety at several levels[42]. Among these concerns are increases in chemical contaminant inputs to marine systems and the consequent exposure level, particularly due to flood events, increased resuspension of sediment-bound contaminants and an increase in their bioavailability and modification of contaminant transport pathways to marine systems. Further, changing their chemical forms to more toxic ones and the consequent exposure level may diminish the species' ability to deal with toxic substances and the different physiological regulation processes involved in the detoxification of hazardous substances. As seafood is an affordable source of essential nutrients and calories with health benefits, new challenges will need to be addressed by public health authorities in regards to seafood safety and maintaining consumers' confidence in eating seafood[43]. Contaminants of Emerging Concern have become a cause of significant human illnesses due to seafood consumption[44]. PFOS concentrations of certain fish species consumed by humans and wildlife (dolphins) from the Charleston Harbor and its tributaries were found to exceed human health and wildlife values. All fish examined revealed PFAS contamination. Consumption of these fish may pose risks as PFAS (especially PFOS) exceeded human screening values for cancer risk in certain species and locations[29].Since persistent organic pollutants are persistent and remain in both the environment and the human body, research on the health aspects of such pollutants is therefore very important.

Our data support the existence of considerable biological differences between low and high contaminant exposures and suggest that overexpression of genes linked to inflammation may contribute to disease in both dolphins and humans. The most striking difference is in the interferon Signaling pathway, negatively regulated in humans and positively regulated in dolphins. This may be explained by the fact that, dolphins lack Mx1 and Mx2, key proteins of the Interferon signaling pathway [45]. The negative regulation of NF-kB signaling is difficult to interpret as its activity as pro or anti-inflammatory is highly contextual [46]. Overall we can conclude that the dolphins exposed to high PFAS levels have an altered immune system compared with those exposed to low levelswhich supports earlier associations of immune and clinical assessments with dolphin PFAS concentrations[47]. Although the diet of humans and dolphins includes much overlap in seafood consumption, unlike humans, the dolphins' exposure to ocean contaminants is continuous so in this regard they represent the worst case exposure scenario.In humans, IL-10 acts as an anti-inflammatory cytokine[48] and an inhibitor of NF-kB activity[49]. IL-17 is also a regulator of NF-kB, and is involved in chronic inflammation and is a major player in the development of autoimmune diseases[50, 51]. When considered together, both the Charleston dolphins exposed to high PFC levels and humans exposed to high PFC levels via fish consumption present altered immune responses.

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