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The Role of Influenza Infection on Cell Metabolism

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Introduction

Influenza viruses, which are members of the *Orthomyxoviridae* family, are present in four genera, of which A and B are responsible for human epidemics (McAuley et al., 2019). Influenza A is primarily res periodic pandemics due to the mutation rate whereas A and B cause seasonal epidemics (Simonsen, 1999). The viral structure consists of a lipid envelope that contains the glycoproteins Hemagglutinin (HA) and neuraminidase (NA) that project from the surface which allow for infection and entrance into host cells in the respiratory tract. The M1, matrix protein forms a layer underneath the lipid envelope. Enclosed within the lipid envelope are the eight negative sense segmented RNA strands which constitute the viral genome and are each wrapped around a nucleoprotein (NP) protein. The nuclear export protein (NEP) which facilitates the export of newly synthesized progeny virus genomic RNA segments from the host cell nucleus is also contained within the lipid envelope (O'Neill, Talon, and Palese, 1998).

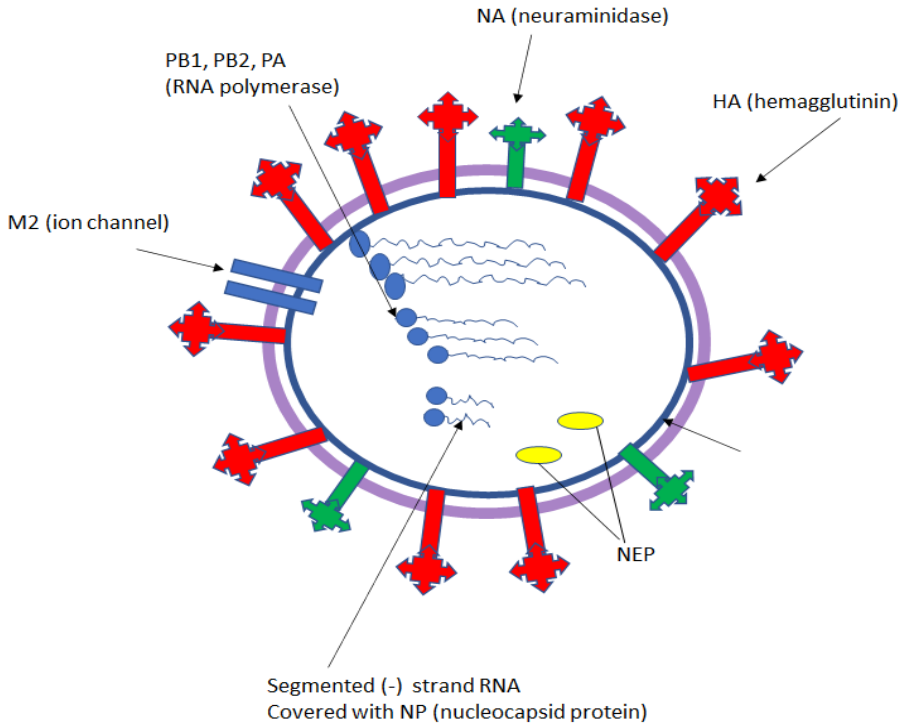

Fig. 1 - Structure of Influenza Virus

Infection of host cells occurs through the binding of HA to specific sialic acid residues on the host cells generally followed by clathrin-mediated endocytosis. The low pH within endosomes triggers conformational changes which then cause viral fusion and activates the M2 proton channel acidifying the interior of the viral particle. These changes result in therelease of vRNPs (RNA segments) into the cell cytosol. The vRNPs contain a nuclear localization signal that allows them to enter the nucleus where replication will occur (Norkin, 2010).

Within the nucleus, the virus polymerase complex composed of three different proteins works to transcribe viral RNA segments which can then be translated to replicate the viral machinery and generate progeny virus molecules. Since viral RNA segments do not code for the 5' methyl cap that mature human RNA must have in order for translation to occur, a cap snatching mechanism is performed by the viral polymerase in which an mRNA segment containing the 5' methyl cap is cleaved and used as a primer for transcription ensuring mature mRNA (Norkin, 2010).

NA functions in the final stage of infection to remove sialic acids from the host cell and newly synthesized HA and NA to prevent virion aggregation and viral binding back onto the dying host cell (McAuley et al., 2019). The envelope and associated proteins surround the M1 protein which contains the NS2 protein and the segmented RNA genome that associates with NP protein and the RNA-dependent RNA polymerase (Norkin, 2010).

The segmented genomes and error prone RNA polymerase lead to antigenic shift and antigenic drift during the infection cycle. Antigenic shift refers to the genetic alteration of viral components that can occur through horizontal gene transfer between the 8 RNA segments of different strains. Antigenic drift is a slower mutation process that leads to different strains due to

an accumulation of small mutations over time. Together these mechanisms lead to seasonal epidemics and the potential for worldwide pandemics (Harrison et al., 2006).

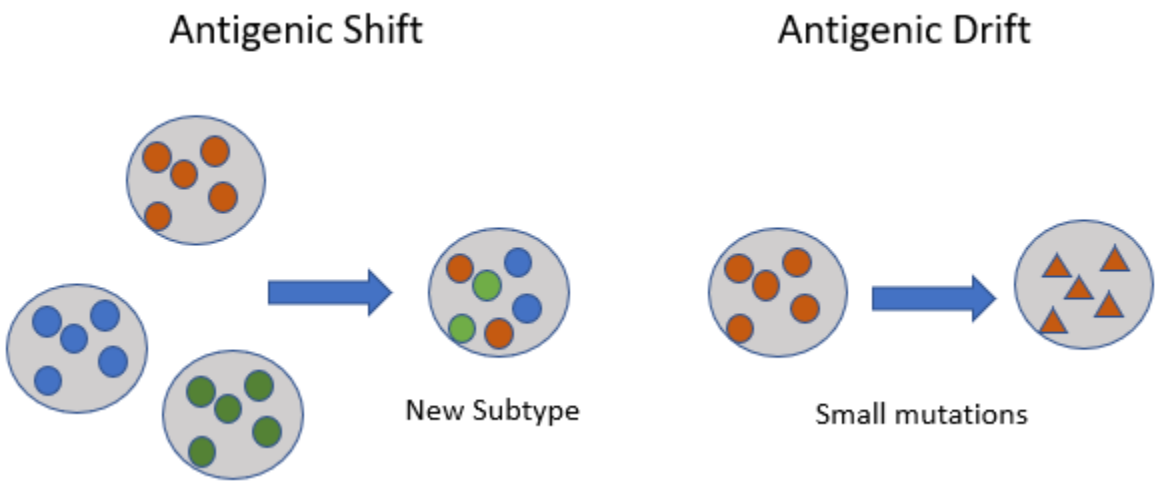

Fig. 2 - Antigenic shift and drift in influenza infection

Currently, there are six licensed antiviral drugs, but three of them target neuraminidase (Kamali and Holodniy, 2013). The potential for the development of resistance to these compounds is high necessitating the identification of new therapeutics to target infection.

Metabolomics is the qualitative and quantitative analysis of all metabolites in a particular specimen. . Untargeted metabolic analysis aims to broadly analyze and identify any significant changes in metabolite levels within samples. This can be used to generate new hypotheses regarding metabolic processes that may be involved in tissue injury or pathology. Typically, this is done through liquid chromatography-mass spectrometry (LC-MS). Specifically, for this study, liquid-liquid extraction will be performed to obtain metabolites for analytical analysis followed by separation using liquid chromatography after which the mass spectrometer can be used to ionize metabolites and separate them by charge and mass (Zhou et al., 2011).

In this project, I will be using untargeted metabolomics to examine a mouse model of infection and reinfection with two strains of influenza H1N1 (PR8) and H3N2 (X31). This project also examines mice with genetic knockout of immunomodulatory genes; however this project will focus on analyzing WT mice who receive one infection of H1 or sequential infections with H1 and H3 viruses. This particular study was performed as a part of an overall goal to identify new host factors involved in sustaining influenza infection by combining a systems approach to analyzing infection using genomic, proteomic, and metabolomic technologies (Norkin, 2010).

Materials and Methods

Mouse Infection

All samples were prepared at Mount Sinai School of Medicine and studies were approved by the Institutional Care and Use Committee at that institution. Lung samples were harvested from wild-type mice that received the mouse adapted influenza strain,PR8 (males and females) alone or a double infection consisting of PR8 and X31 strains of influenza infection (females only) over the course of 42 days. Lung samples were harvested at 3 days post infection (males and females) and 5 days post infection (females). The virus stock was prepared and titrated to be 0.1LD50 sublethal dose for PR8 (H1N1) and 2.5LD50 or 1000 PFU for the X31 (H3N2). Lung samples were homogenized in PBS and frozen in PBS/MeOH (90/10) and stored at -80 degrees C until analysis.

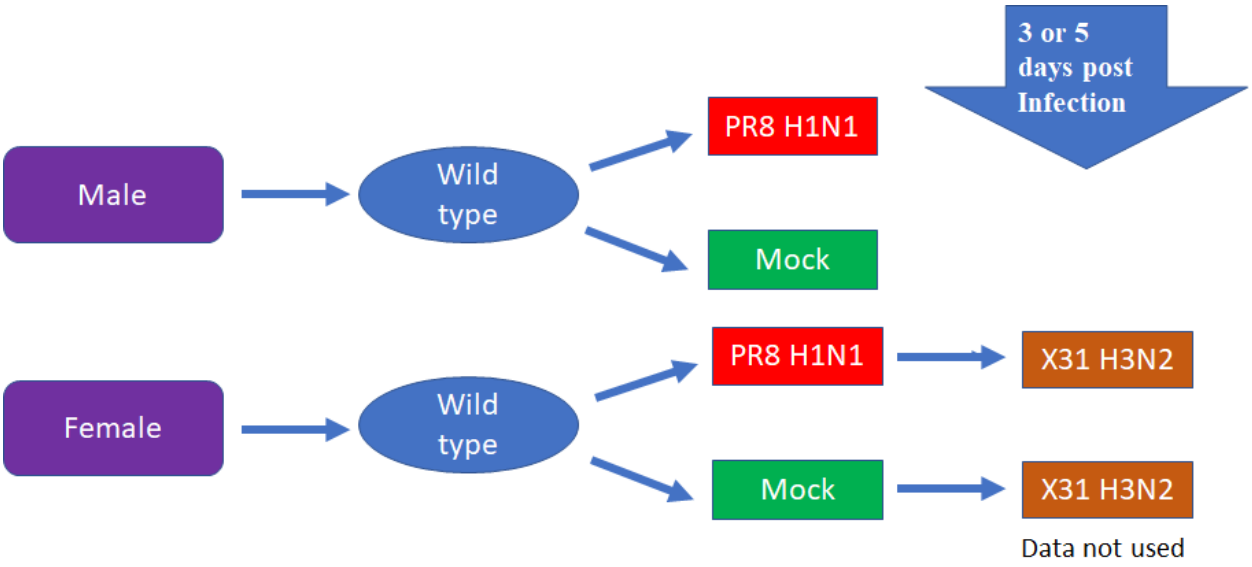

Fig 3 - Treatment scheme

Metabolite Extraction

Extraction of protein from frozen tissue samples was done using a modified Bligh and Dyer liquid/liquid extraction technique after sonication of samples (Bligh and Dyer, 1959)

0.1 mL of MetOH was added to each small sample after which the sample was vortexed for 30 seconds then frozen in liquid nitrogen and thawed subsequently. The solution was then sonicated and the sonicator cleaned with isopropanol and water between uses. This freeze-thaw and sonication cycle was repeated three times for each sample.

For each sample, 0.750 mL of CHCl3:MeOH was added, then 0.350 mL CHCl3. The sample was then vortexed and kept at -20 degrees C for 1 hour. The sample was then centrifuged at 5,000 rpm in a table-top centrifuge for 5 min at 4 degrees C to give a two-phase system. Each phase was recovered separately into new tubes taking care not to agitate the protein disk in the

interphase. To the protein disk that was separated, the organic and inorganic layer isolation were performed again. All samples were then concentrated through SpeedVac and removed as soon as they were dried.

LC-MS

Samples were run using hydrophilic interaction liquid chromatography-mass spectrometry with an ACQUITY UHPLC BEH amide column (Waters) attached Orbitrap ID-X Tribrid Mass Spectrometer (Thermo Fisher). Bioinformatic analysis will be done using MIDTOD—an in-house software which performs automated, high throughput statistical analysis on datasets and identification of metabolites through proteomics and RNA-seq data. It then uses this data to analyze potential relationships between metabolites in this case.

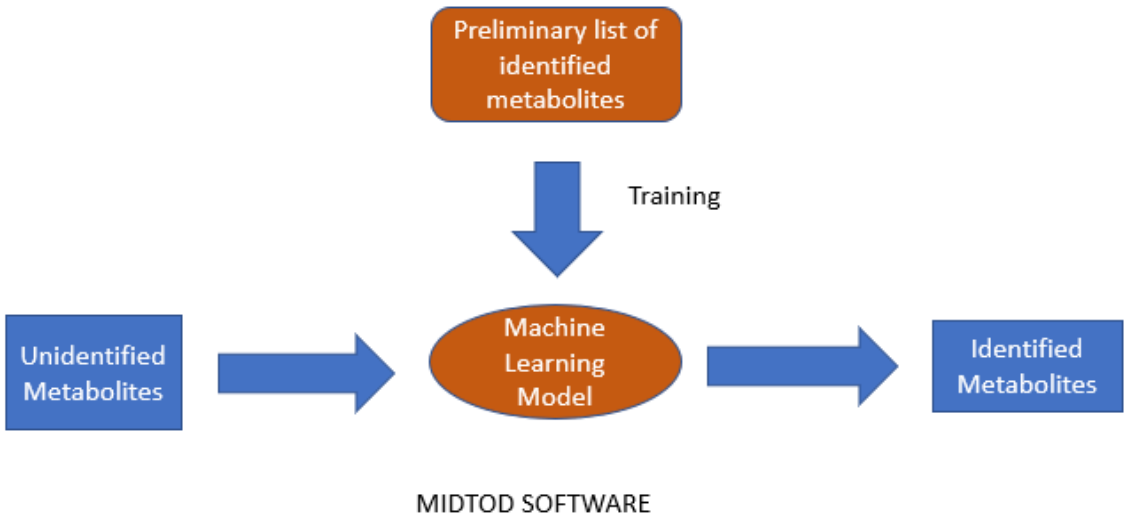

Fig. 4 - MIDTOD workflow

Statistical Analysis

Statistical tests and analyses were performed on the open-source software Metaboanalyst (https://www.metaboanalyst.ca/MetaboAnalyst/home.xhtml) in order to understand the effect of infection and reinfection as well as sex differences on wild type mice. PCA analysis, a multivariate method was used in order to capture relationships not just between test groups but also between individual molecules (Xiao, 2012). Through volcano plots which are based on fold-change analysis as well as t-tests, analysis combining both significance and direction of variance can be performed.

Results

Within the wild type group, mice were all initially infected with PR8 or were subjected to a mock infection. 28 days after recovery, a group of mice were infected with X31. Samples were taken from different groups of mice at day 3 and day 5 after infection. Five different comparisons were made between the datasets as listed and defined in the table below.

In order to account for sex differences, WT male and female mice infected with PR8 initially and samples taken at 3DPI (3 days post infection) were compared using multivariate statistical analysis. The rest of the comparisons were made within the sex group to minimize variation due to sex differences.

More specifically regarding multivariate analysis, a Principal Component Analysis (PCA) plot was used to assess variation between datasets with respect to their overall metabolic profile. The datasets are divided into different principal components that have similar levels of variation within their respective test group. PC 1, containing the highest amount of variation was plotted on the x axis and PC 2, with the second highest variation was plotted on the y axis. Both groups are then shown on the plot and the level of overlap between them would then indicate the similarity between the most variable parts of the data (Holland, 2019).

Initially we examined host metabolic response to infection by comparing male and female mice that were infected with PR8 versus mock controlsThe PCA plot for the WTM infection (IFN) vs. mock shows minimal overlap between the groups indicating significant differences in metabolite peaks due to the infection process **(Figure 5**). In contrast to males infected with influenza the PCA plot for WT females infected with PR8 versus mock infection shows significantly more overlap between the groups, indicating less metabolic differences in the context of infection (**Figure 6**).. Respective volcano plots for these groups indicated that WTM

rats subjected to this condition had more significantly different metabolite values both in terms of upregulation and downregulation than the female group. This led us to compare sex specific differences in the metabolic responses between male and female mice infected with PR8. show minimal overlap of the groups in the PCA plot. This indicates that there are sex-specific differences in response to comparison and any further comparisons must take this into account (**Figure 7**). Metabolic The PCA plots for female mice that received the double infection versus a single infection showed more overlap in their metabolic profiles (**Figures 8 and 9**). This indicates that metabolic alterations may be more associated with on-going rather than past infection. The volcano plots of both these groups also showed that the most significant metabolic changes involved downregulation of metabolic pathways. These data point to the potential for metabolic compensation after one infection that leads to a suppression of metabolism in subsequent infections..

We then examined individual metabolite changes within the groups. Within the WTF infection (IFN) vs. mock (1-methyladenosine was significantly lower in the mock group compared to the infection group. In the WTF PR8 and 5DPI X31 group comparison, propionylcarnitine was significantly lower in the X31 group (**Figure 10**).

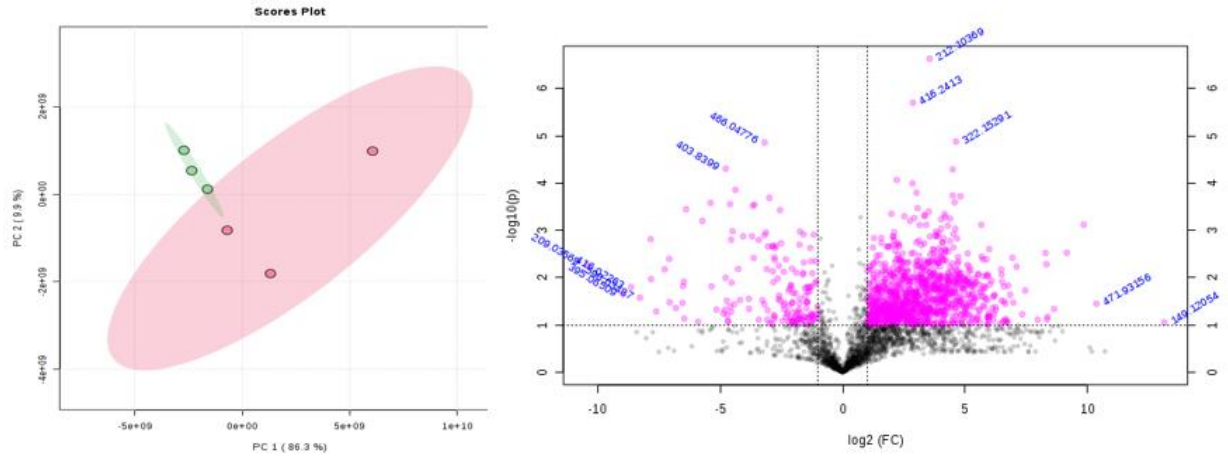

Fig 5: PCA and volcano plots for wild type male mice that were either given the mock or real PR8 infection initially and are part of the 3DPI PR8 group are compared

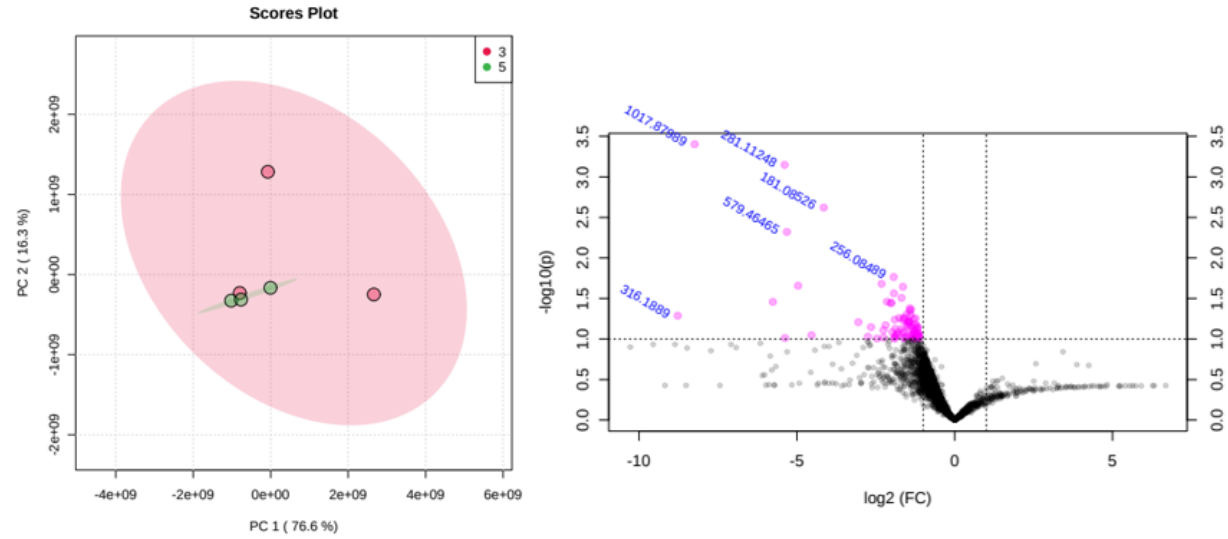

Fig 6: PCA and volcano plot of wild type female mice that were either given the mock or real PR8 infection. Lungs were harvest at 3 days post infection..

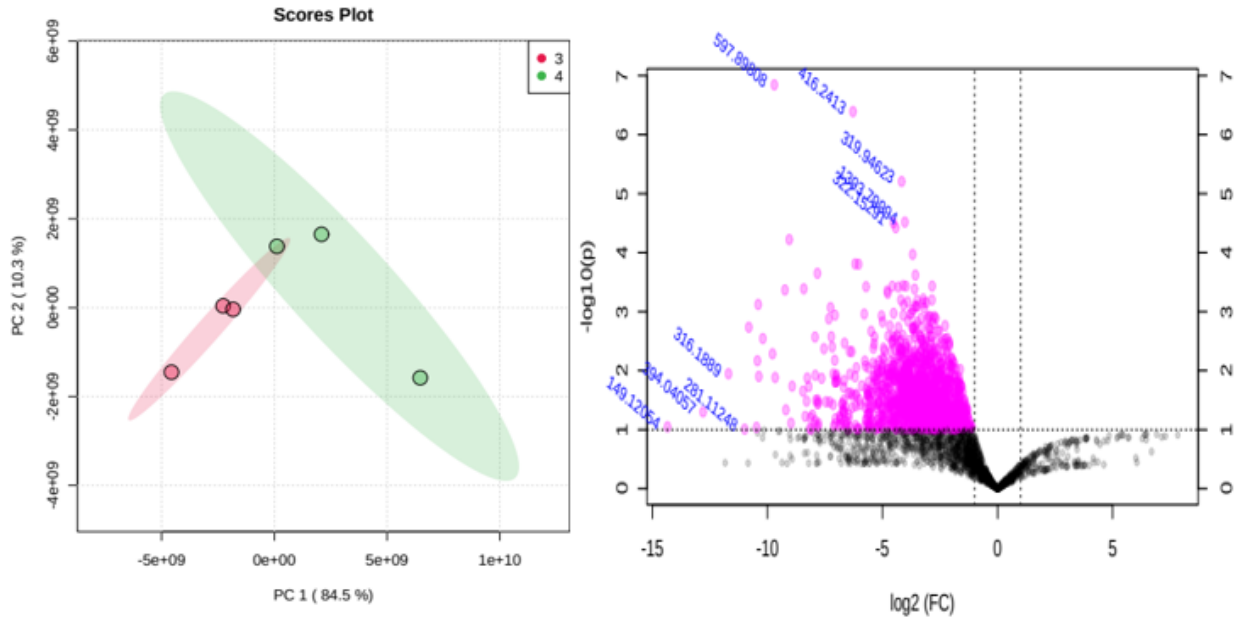

Fig 7: PCA plot and volcano plot for wild type male and female mice that were initially infected with PR8. Lungs were harvested at 3 days post-infection.

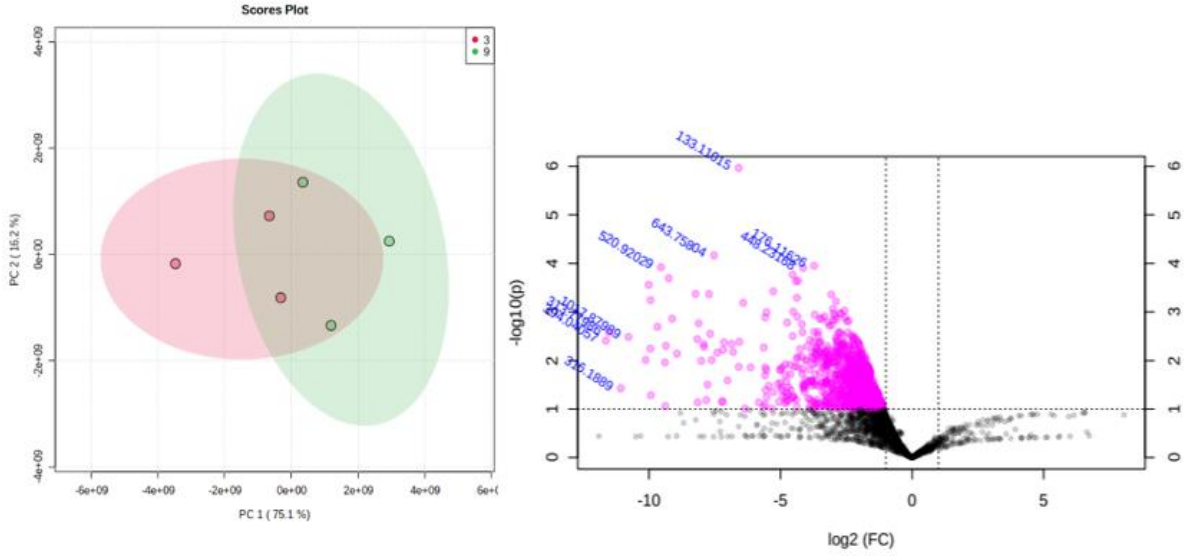

Fig 8: PCA and volcano plot for wild type female mice that received early infection with PR8 followed by infection with X31. Lungs were harvested at 3 days postinfection.

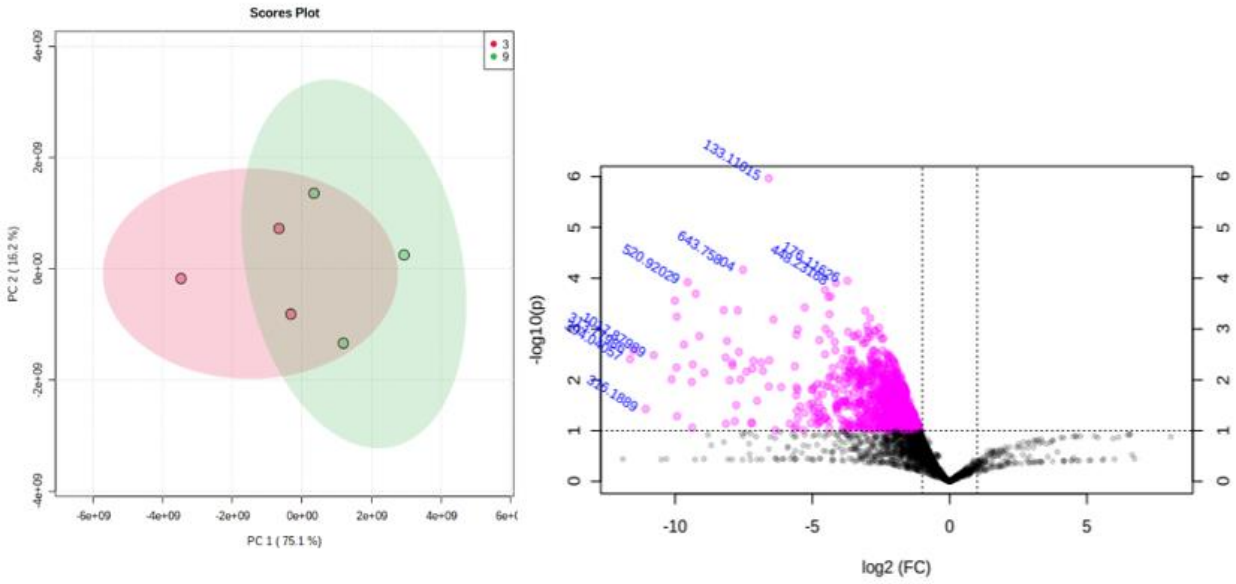

Fig. 9: PCA and volcano plot for wild type female mice that received early PR8 infection followed by X31 infection. Lungs were harvested at 5 days PI.

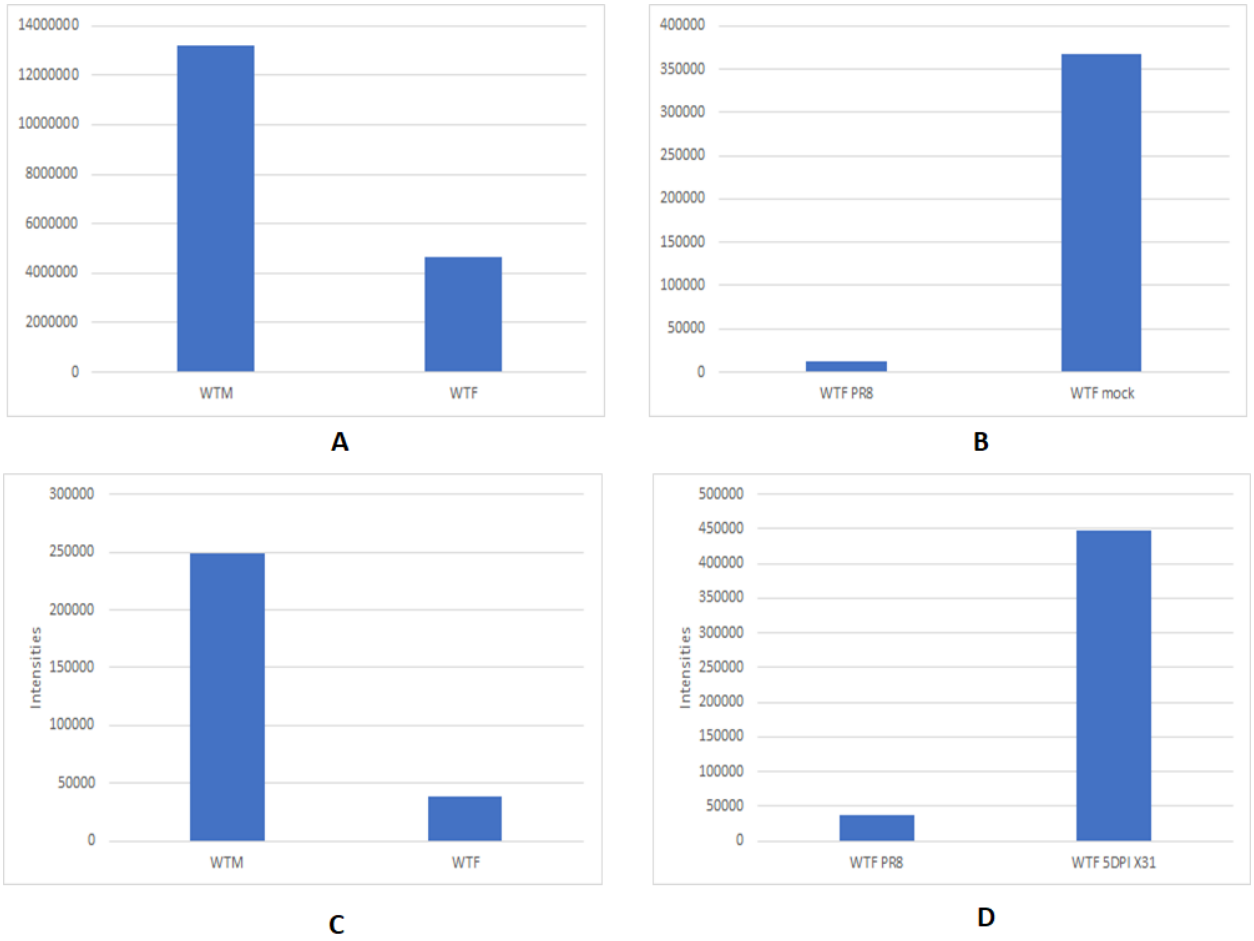

Fig. 10: Methyladenosine and Propionylcarnitine intensities for different test groups of Wild Type Male and Female mice (WTM and WTF).
(A) Methyladenosine intensities for PR8 infected, male vs. female mice and for (**B)** Female PR8 infected mice vs. female with mock infection ; **(C)** Propionylcarnitine intensities for PR8 infected male vs. female mice and for (**D)** Female mice, infected with PR8 vs. X31 reinfection group—samples taken 5 days post infection

Discussion:

Viruses must rely on host metabolic machinery in order to ensure replication and infection--processes that require high amounts of energy. For viruses with limited coding capacities such as the Dengue virus for example, it has been shown that major metabolic pathways such as glycolysis, fatty acid synthesis, and glutaminolysis are altered. It has also been shown that the Warburg effect, or aerobic glycolysis which occurs in cancer cells also occurs as a result of viral infection. This upregulation of glycolysis--an anaerobic process--occurs even

though oxygen is present. This is likely to favor the goal of viral progeny generation by increasing production of biosynthetic molecules although energy output might be compromised (Plaza et al., 2016).

Thus, a better understanding of the relationship between influenza infection and cell metabolism is imperative for diagnosis, prognosis, and development of potential antiviral drugs in humans. Most studies involved in metabolic analysis use techniques such as LC-MS in order to identify and quantify small molecules in metabolic reactions. Through this untargeted approach, potential relationships between influenza and metabolic pathways can be analyzed more broadly as any identifiable and statistically significant metabolite can be recognized. Metabolite extraction is generally a more challenging task due to the chemical diversity and the dynamic properties of the metabolome, however extraction methods and solvents can be selected based on the chemical properties of metabolites if need be (Zhou et al., 2011). In this particular experiment, a modified Bligh and Dyer Liquid-Liquid extraction procedure which generally is used for lipid extraction was used.

Our results demonstrate that sex-specific differences in metabolism may play a role in host responses to viral infection., Previous studies have demonstrated that normal metabolism is different between males and females using a gas chromatography mass spectroscopy (GC-MS) method to profile metabolites. The C57BL/6J strains' female metabolite profile for example showed higher levels of phenylethanolamine and octadecanoic acid together with lower amounts of l-methionine relative to those for males. The metabolite profile of the 129S1/SvImJ strain differed from the C57BL/6J strain which does indicate that the data may not be able to be extrapolated to different strains. 129S1/SvImJ females showed higher levels of lactic acid, citric

acid and oleic acid together and lower amounts of valine,l-norleucine, isoleucine, glyceric acid, serine and l-threonine compared to males (Qiao et al., 2011).

Although all metabolites that were identified and labelled could not necessarily be extrapolated to specific roles within metabolic processes, noting structural and chemical properties may be useful.

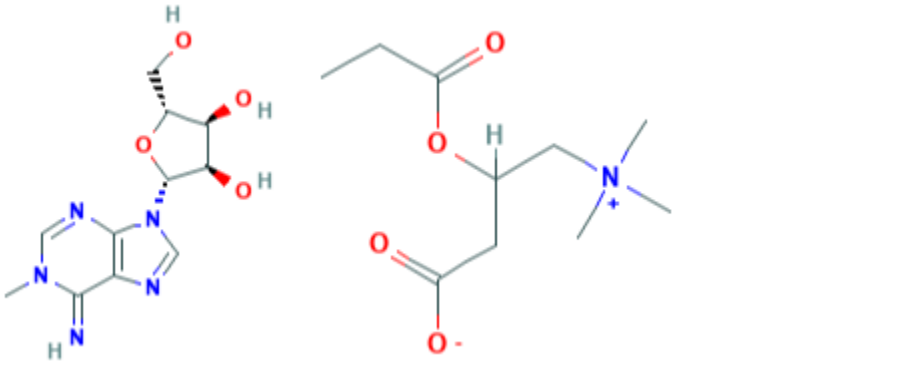

Fig 11: 1-Methyladenosine and Propionylcarnitine
Source: https://pubchem.ncbi.nlm.nih.gov/

Propionylcarnitine was significantly upregulated in the 5DPI X31 group compared to mice receiving a single infection. Propionylcarnitine, a propionyl ester of L-carnitine has a high affinity for the enzyme carnitine acetyltransferase (CAT). This enzyme works in the process of beta-oxidation, or the breakdown of fats for energy release. Specifically, L-carnitine is involved in the carnitine shuttle, a process which transports fatty acid chains into the mitochondria by attaching them to the L-carnitine molecules which act as carriers for the acyl chains. After linking to these carnitine molecules, the chains can be transported into the mitochondria in pieces for beta-oxidation or breakdown. The high affinity of Propionylcarnitine for carnitine acetyltransferase allows for the conversion of propionylcarnitine into carnitine.

Given that influenza virus utilizes host-derived lipids during budding, it has been shown that any interference with peroxisome function or lipid metabolism impairs influenza virus

production. In addition, soluble lipid mediators which originate from glycerophospholipids (GPLs) through phospholipase activity and are to a certain degree metabolized in peroxisomes. Influenza infection decreases GPL levels while increasing odd chain fatty acid chain supporting viral biosynthesis. Beta-oxidation is necessary in the peroxisome indirectly to produce protection D1 which prevents nuclear export of influenza virus RNAs. In addition, the role of peroxisomes was further shown by demonstrating that multifunctional protein 2, which is essential for peroxisomal B-oxidation acts as an antiviral protein by interacting with the influenza virus nonstructural protein 1 (NS1) (Tanner et al., 2014).

One of the major sources of error for this particular study was the loss of one of the three samples within the wild type female group infected with PR8 initially. For metabolomic studies it is suggested that a minimum of three replicates is required to meet the standard for chemistry analysis. With the inability to include this sample in the analysis, the wild type female group with the PR8 infection only contained two samples. In order to avoid the risk of data inaccuracy, more samples for each group should be added in any future experiments as it is suggested in the proposed minimum reporting standard for chemistry analysis that five replicates is preferred (Sumner et al., 2007).

One of the limitations of LC-MS is that there are many factors that can cause data errors such as instrument condition, environment of operation, and sample preparation. These factors can cause a drift in retention times, different intensity values, and on a much smaller scale, m/z values. In addition, although this method can achieve an accuracy of less than 1 ppm, it still cannot distinguish between compounds with very similar molecular weights. Mass-based metabolite identification also cannot discriminate between isomers of different compounds which have the same elemental composition, and therefore mass, but different structures. The

metabolite databases have limited coverage of ions as well, and generally less than 30% of detected ions can be identified as a specific metabolite through this mass-based search. Because of this many ions are left as unidentified or having many different identifications (Zhou et al., 2011).

Conclusion:

In this particular study global metabolomics LCMS data was analyzed to determine the effect of influenza infection on host metabolic processes. Specifically, wild-type male and female mouse lung samples from mice infected with PR8 (H1N1) or both PR8 and a reinfection of X31 (H3N2) were analyzed. By performing LCMS and making five different dataset comparisons it was found that methyladenosine is downregulated in mock infected female mice as compared to PR8 infected mice. It was also found that Propionylcarnitine, a metabolite involved in beta-oxidation and peroxisome function was upregulated in mice reinfected with X31. This could indicate a link between influenza infection and lipid metabolism. The LCMS technique poses challenges that can cause a large number of unidentified metabolites and can result in difficulties when trying to truly identify significant metabolic changes. Tandem mass-spectroscopy could be used in future studies to attempt to limit this. In addition, the potential link to lipid metabolism could be further studied by performing a targeted analysis investigating fat metabolism related metabolites.

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Appendix: Safety Considerations

In order to maximize safety in the lab environment, appropriate personal protective equipment must be used when handling chemicals (ex. safety glasses, gloves, or protective clothing). Chemical waste, specifically the organic solvents used for extraction were handled in a fume hood to prevent inhalation of dangerous fumes and disposed of by environmental health and safety. Contact with chemicals must be minimized as much as possible. Appropriate personal protective equipment such as safety glasses, gloves, or protective clothing was worn for handling of infected mouse samples prior to extraction as well as during the extraction. If a chemical spill occurs causing skin contact, a shower or eyewash station should be used to rinse the chemicals immediately.