SUMMER 1 REFLECTIVE REPORT

TEA STAPAR, 2023 LAIDLAW SCHOLARS COHORT

In the summer of 2023, I had the pleasure of conducting my research project in the lab of Prof. Kenneth Hun Mok, in the School of Biochemistry and Immunology at Trinity Biomedical Sciences Institute (TBSI), Trinity College Dublin. My research project involved using a technique called NMR metabolomics to investigate whether a novel chemotherapy called BAMLET (**B**ovine **A**lpha-lactalbumin **m**ade **le**thal to **t**umour cells) could be used as a therapy for the treatment of osteosarcoma. Throughout my research project, my goal was to evaluate whether there are changes in the metabolite levels between osteosarcoma cells that were treated with the chemotherapy and ones to which only water was added. This information was then used to evaluate whether BAMLET had tumoricidal effects on osteosarcoma cells.

The structure of my research project consisted of three parts. The first part of the project involved culturing the osteosarcoma cell line (U2OS cell line) to acquire enough cells to carry out the different experiments. As cell culturing was something I had never done before, Dr. Min from the School of Biochemistry and Immunology kindly taught me and guided me through the different procedures that had to be followed. This involved: 1) Changing the media of the cell lines every two days to give them sufficient nutrients to grow. 2) De-plating the cells once they have reached confluency (when they have covered the entire surface of the plate) and moving them into different dishes to give them more room to grow. 3) Treating half of the cells with the chemotherapy for different lengths of time and leave the other half to act as controls by only adding water into their plates. By the end of the cell culturing process, the total number of cells that had been cultured was roughly 8 million. Throughout the process, Dr. Min and I decided to separate the cells into three different sets, where each set would be subdivided into the same categories to allow for a meaningful analysis at the end of the project. As mentioned, each set was divided into different categories based on the different lengths of time the cells were either exposed to BAMLET or water, and they were as follows:

1. 0h control
2. 2h BAMLET treated cells
3. 2h water control

4. 24h BAMLET treated cells
5. 24h water control

To ensure the cells were only exposed to BAMLET at specific time lengths, they were rapidly cooled with liquid nitrogen after the time period had elapsed to stop any reactions from occurring.

The second stage of the project involved preparing the samples and putting them into NMR tubes. In this process, I was helped and guided by my supervisor, Prof. Mok, who taught me the correct procedures and supervised my work. As my project was only concerned with the levels of metabolites, this meant that I had to remove everything else from the cell samples, such as any cell components and proteins, lipids etc. that would interfere with the analysis of my samples. Firstly, the cells were thawed, and an organic solvent was added to the cell plates to cause the cells to detach from the cell plate surface. A cell scraper was then used to remove all the cells from the surface to be transported to centrifuge tubes. As mentioned before, my project only focused on the metabolites, so to remove all the unwanted components from my samples, different filters were used while centrifuging the cells in order to be left with just metabolites. The cells were first centrifuged with a 0.33 µm filter well, which allowed for the removal of different cell components such as the cell membrane. Afterwards, the cells were centrifuged using a molecular weight 10,000 cut-off point, which meant that only molecules which had a molecular weight below 10,000 could go through the sieve, which helped remove different proteins that were unwanted.

The third stage consisted of running the samples in the NMR spectrometer at different settings. Each sample had 4 different scans recorded. The samples were run at different signal sensitivities and for different lengths of time. The process of learning how to run samples in the NMR spectrometer was really enjoyable yet challenging, as there were many procedures to be followed. The entire process was very time consuming as one of my samples was scanning for 20 hours at a time. After all the scans were done, I carried out multivariate and pairwise analysis for all my samples and graphs were generated to represent my results.

Firstly, the raw data had to be filtered and prepared in order to be statistically analysed. This included using a software called NMRProcFlow where the signals for each sample were normalised. Afterwards, the data was uploaded into MetaboAnalyst 5.0 where statistical analysis was carried out. For multivariate analysis, one-way ANOVA graph (Figure 1) was generated to see which peaks in the NMR spectra were statistically significant.

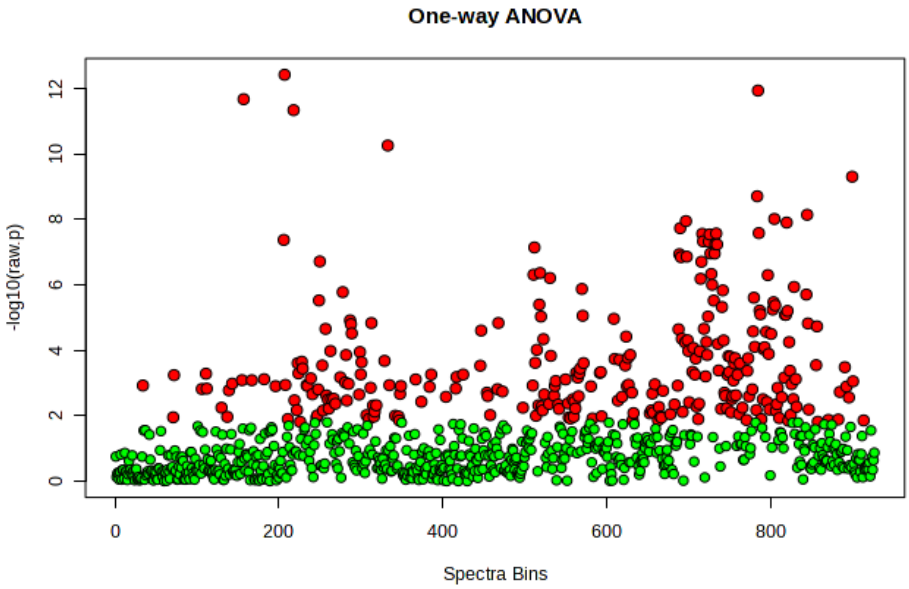

Figure 1: One-way ANOVA table showing significant points (peaks) coloured in red.

Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) were also used for multivariate analysis. PCA explains variance between features in samples without taking into account the labels that have been assigned to samples. PLS-DA is different to PCA because it relies on extraction techniques which are capable of predicting class membership. The predictiveness of the model can be estimated by the Q2 value, which is generated while doing PLS-DA analysis. If the Q2 value is above 0.4, that means that the model is predictive. The PCA graph (Figure 2) from the multivariate analysis shows clear separation between the groups.

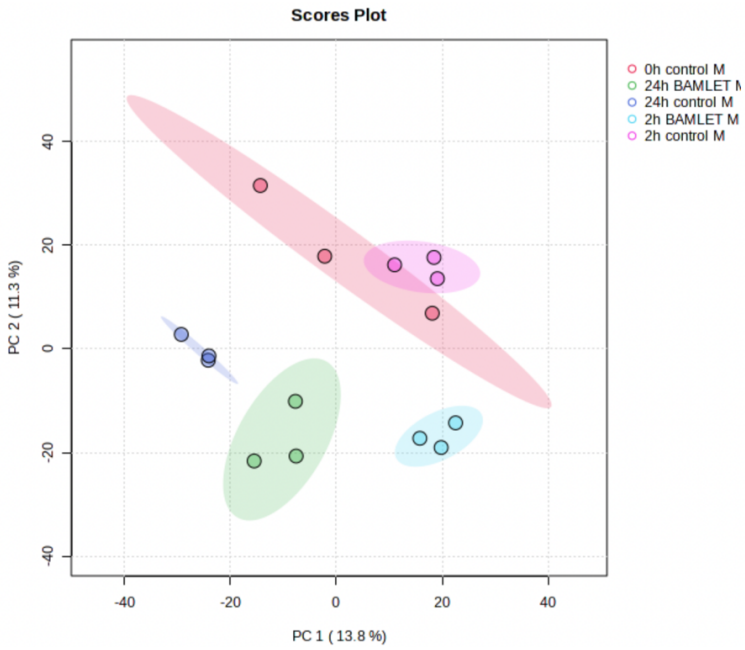

Figure 2:PCA Scores plot shows clear separation between the different experimental groups, meaning they are statistically different from each other.

The PLS-DA Scores plot (Figure 3) from the multivariate analysis shows separation between the different groups, however the Q2 value is very low, showing this is a non-predictive model.

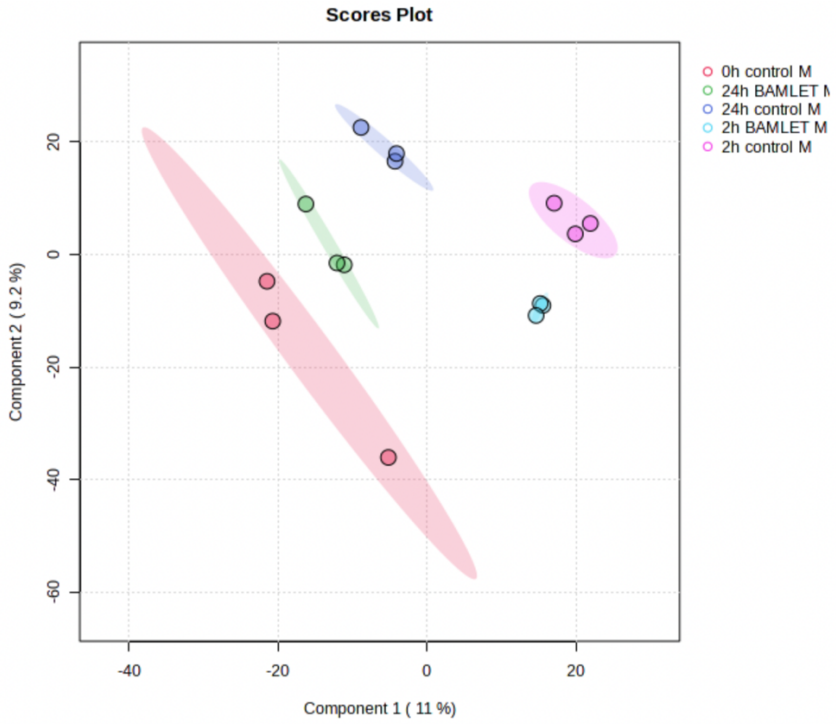

Figure 3: PLS-DA Scores plot from multivariate analysis. Graph shows clear separation in different groups. Q2 value = 0.04 showing that the model is not predictive.

Pairwise analysis was also carried out between the 2h BAMLET samples vs. 2h control samples, and the 24h BAMLET samples vs. 24h control samples. For each, PCA, PLS-DA and volcano plots were generated. Volcano plots are a combination of a Fold Change (FC) analysis and t-tests, which are two techniques used for pairwise analysis that identify significant features in the data. The significant features are found by using both PC analysis, where the threshold for significance was set to 2, and t-tests where the significance was set to a p-value of 0.01. With regards to the 2h BAMLET and control samples, the PCA Scores plot (Figure 4) shows very clear separation between the BAMLET treated samples and the water treated control samples.

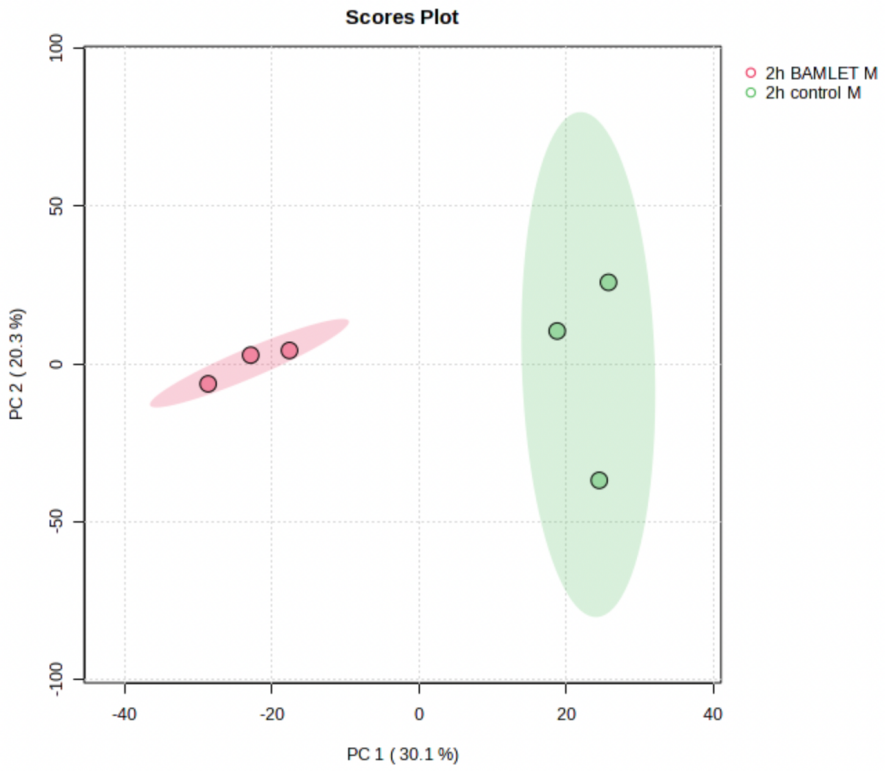

Figure 4: PCA Scores plot of pairwise analysis between 2h BAMLET samples and 2h control samples. Plot shows clear separation between the two groups, showing they are statistically different from each other.

The PLS-DA Scores plot (Figure 5) from the pairwise analysis of the 2hr BAMLET vs. control samples shows distinct separation indicating significant variation between the two groups.

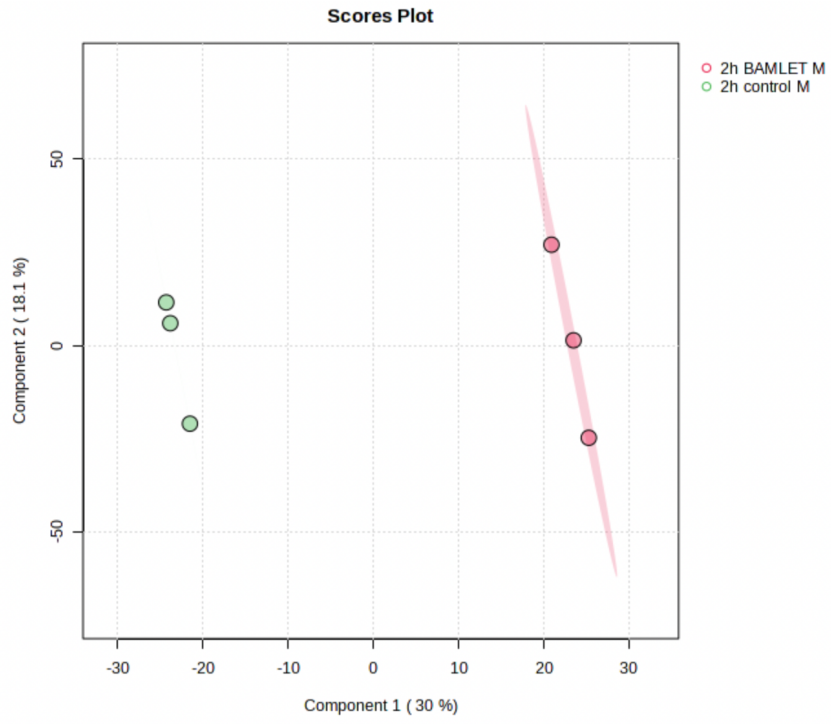

Figure 5: PLS-DA Scores plot of pairwise analysis between 2h BAMLET and 2h control samples. There is significant variation between the samples. Reported Q2 value is 0.41 which means that this is a predictive model.

Figure 6 shows the volcano plot generated from the pairwise analysis between the 2h BAMLET and 2h water control.

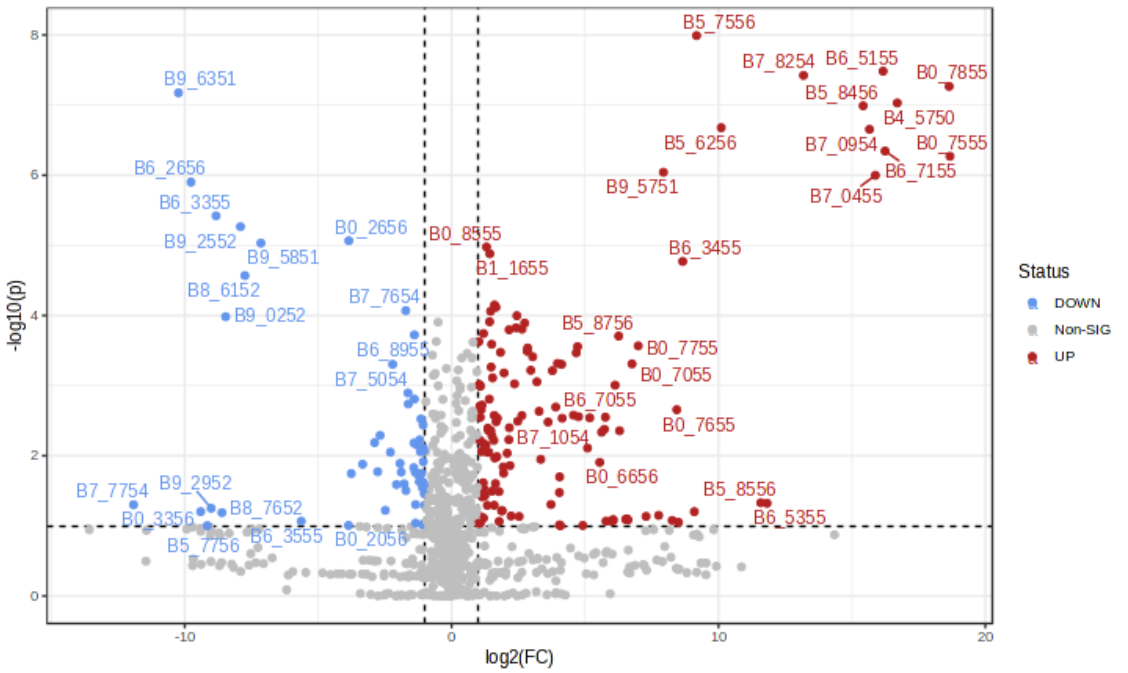

Figure 6: Volcano plot from pair-wise analysis between 2h BAMLET and 2h control samples. The red points (buckets) on the plot are features above the threshold, blue are within the threshold and grey are insignificant.

Pairwise analysis was also carried out in the same fashion for the 24h BAMLET vs. 24h water controls. Figure 7 shows the PCA Scores plot from this analysis, indicating clear separation between the groups.

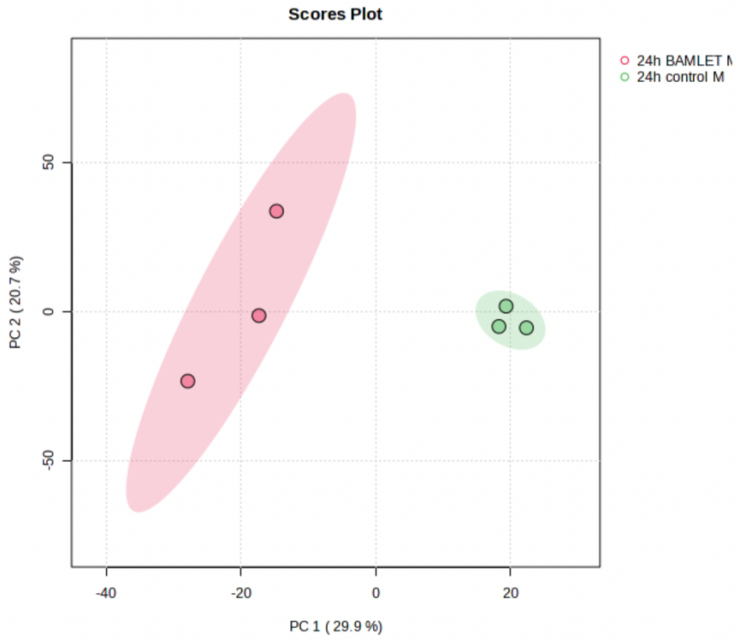

Figure 7: PCA Scores plot from pairwise analysis between 24h BAMLET and 24h control samples. Clear separation between the groups is seen.

Figure 8 shows the PLS-DA Scores plot generated. Significant variation between the samples is seen, showing that there are statistical differences between the two sample groups. While doing the analysis, the model was found to be predictive as well.

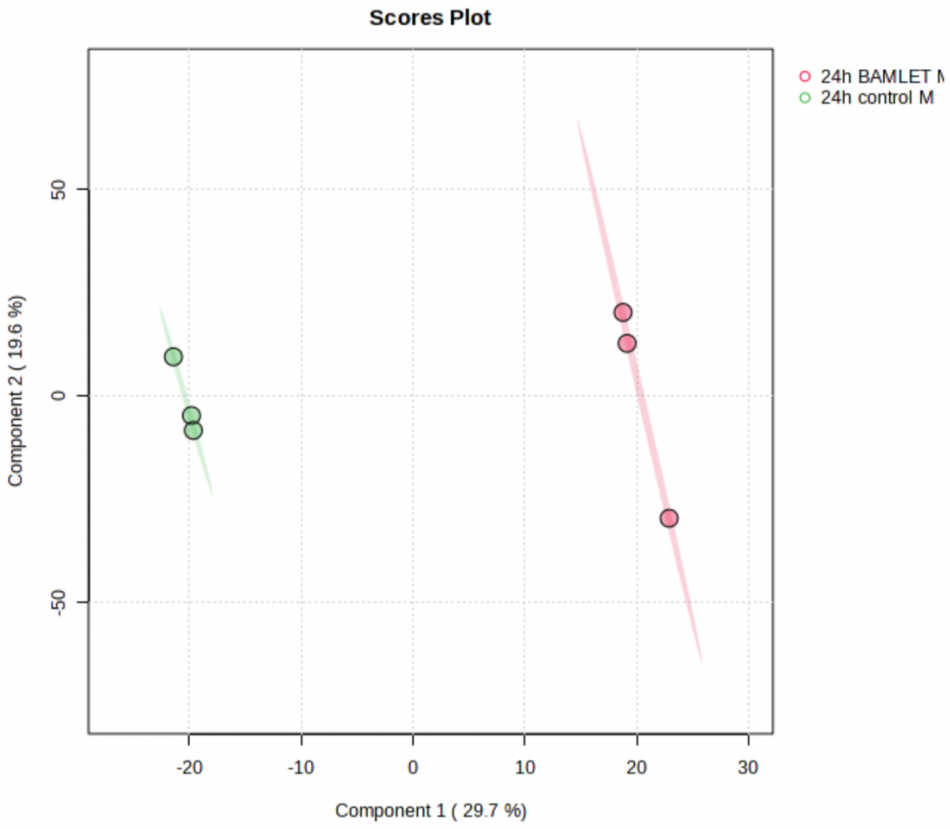

Figure 8:PLS-DA Scores plot of pairwise analysis between 24h BAMLET and 24h control samples. There is significant variation between the samples. Reported Q2 value is 0.38 which means that this is a predictive model.

Figure 9 shows the volcano plot from the pairwise analysis, which indicates the significant peaks in the NMR spectra, corresponding to metabolites which are significantly different between the two groups.

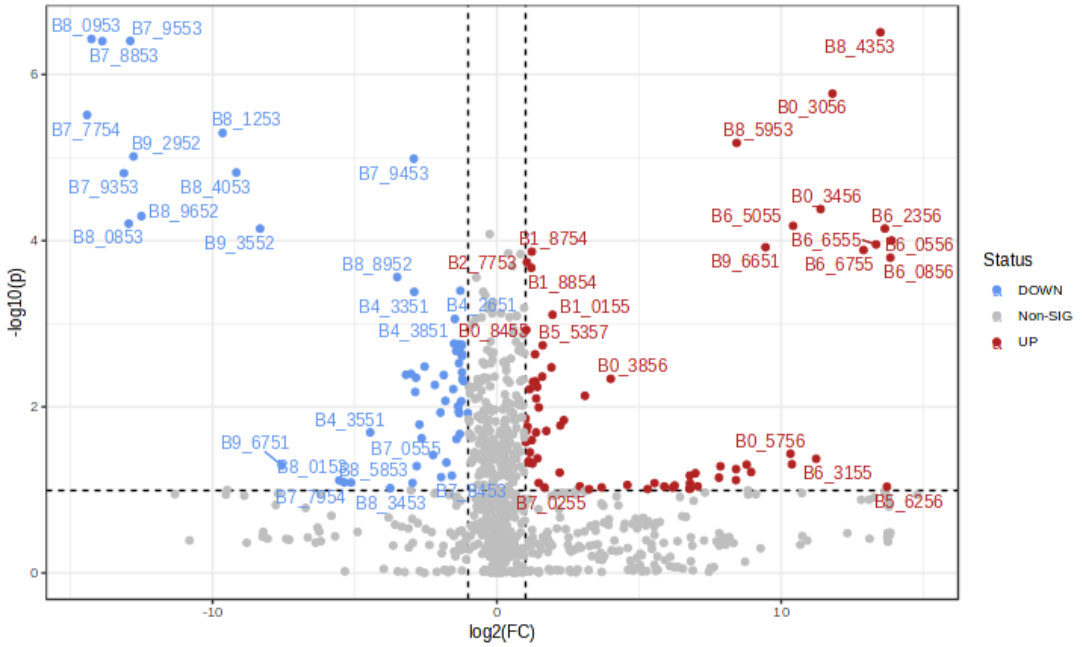

Figure 9: Volcano plot from pairwise analysis between 24h BAMLET and 24h control samples. The red points (buckets) on the plot are features above the threshold, blue are within the threshold and grey are insignificant.

All of the results show there are differences between the levels of metabolites in between the different experimental groups. This information is one step closer to finding out whether BAMLET really is a suitable candidate for the treatment of human osteosarcoma.

I have grown immensely as a person throughout this experience. I have learned many new lab techniques, and I have made many mistakes from which I have learnt invaluable lessons. My research project did not go completely according to plan, and I am glad that this happened because it made me learn how to adapt to change, learn how to improvise in unfamiliar situations and become more resilient in the face of obstacles. I experienced significant technical challenges during my research project, which consisted of the NMR spectrometer room overheating due to AC system failure. This caused the quality of my results to be diminished, and the most frustrating part about it was that I couldn't do much about it. I believe this was a very big learning lesson for me as I find it very difficult to come to terms with the fact that sometimes I can't do anything to solve a problem. It made me learn to not panic at first and believe that things will resolve themselves. But the most important lesson it made me learn was that it is sometimes okay to just do the best that you can in that situation and to move on. Time is precious, and sometimes we just must make do with that we have and continue with the project. Another valuable lesson that I have learnt throughout my research experience was to not give up when things get difficult. I had difficulty with time management during the project as some of the preparation steps for my samples were taking longer than expected. This really took a toll on me as I didn't know what to do at first about this and I felt very helpless. But then I realised, rather than panicking and giving up, I asked for help and managed

to find ways where I could cut back on time and organise my time more efficiently. Asking for help can sometimes be very intimidating and difficult to ask, but we all need help sometimes. None of us know how to do everything ourselves and sharing ideas amongst each other is an important part of learning.

During my research project, I had the opportunity to engage with other Laidlaw Scholars who were also completing their research projects in Trinity Biomedical Sciences Institute. I was happy I got to meet some new people, but it was also great to talk to everyone about how their projects were progressing. The aspect that I enjoyed the most was that we were all able to support each other and help each other out if there were any problems we were facing. I found this environment very comforting as I knew I wasn't the only person who was having problems with my research project. We would see each other almost every day at the TBSI, and each lunch time we would all reflect on how our day was going and if any of us faced any problems. I also had the opportunity to interact with PhD students in TBSI who were very kind and helpful if I had any problems or wasn't familiar with using a piece of equipment in the lab. At first it was very intimidating asking them for help as they were all older and more senior in their research education than me, but all of them were super nice and always happy to answer any of my questions. It was great getting to hear their advice and any tips that they had for how to do an experiment in a more efficient way, and I was able to learn a lot from them which I am very grateful for.

I was fortunate to also have leadership experience throughout my research project as well. I had the opportunity to assist another student in their process of learning how to do NMR spectroscopy. This involved me organising times when to meet with them but also share any tips and offer them help throughout their learning experience. This was a great opportunity for me as it made me feel more comfortable being in leadership portions, as this as something I have previously not been as comfortable with.

Overall, my Laidlaw Research Project has been a very enjoyable and rewarding experience and I am very grateful for all the opportunities I have been given throughout the summer.