MEA Project Report

Establishing Protocols to Assess Mitochondrial Function in Granulocytes and PBMCs from ME/CFS patients

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Research Location:	Nuffield Department of Obstetrics and Gynaecology, University of Oxford.
Duration:	14th June 2016 – 23rd December 2016
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Executive summary

- Using freshly collected blood we were unable to routinely produce a granulocyte layer on the Histopaque gradient used in the Acumen protocol.
- Peripheral blood mononuclear cells (PBMCs) were routinely isolated on a Histopaque gradient from freshly collected blood.
- Other non-gradient approaches showed high levels of neutrophils to be present in fresh blood samples with numbers significantly down when blood was left in the collection tube for 24hrs before processing.
- Granulocytes and PBMCs isolated from the gradient maintain viability following 24hr incubation in cell culture. The inclusion of inhibitors of mitochondrial ATP production had no effect on granulocyte viability but resulted in loss of viability of PBMCs after 7-8 hrs in culture. This suggests the PBMC's are changing over time and adopting a more mitochondrial energy requiring phenotype. This is consistent with PBMC activation over time something that was also observed in the 24 hr blood sample.
- In freshly isolated blood, PBMC's show measurable levels of mitochondrial respiration with the mitochondrial contribution to cellular ATP production of between 37-25%. A longitudinal study of PBMC's isolated from 3 control subjects over 8 weeks showed consistent levels of mitochondrial ATP production and mitochondrial respiration. Mitochondrial respiration levels were low but detectable when large numbers of cells were used.
- In freshly isolated blood samples granulocytes show very little mitochondrial derived ATP production between 14-11% of total ATP levels. Total ATP levels were low and the results varied significantly in the longitudinal control study. Levels of mitochondrial respiration were very low.
- Using ATP as an output of mitochondrial function is problematic when comparing between samples run on separate days. Running consistent standard curves is not always easy even when using a commercial kit. Although mitochondrial respiration levels are low in PBMC's mitochondrial oxygen consumption may give a more robust assessment of mitochondrial function if used on cells isolated from freshly isolated blood samples

Recommendations

- Granulocytes appear to be lost/modified in blood samples stored for 24hrs in a blood collection tube. PMBCs appear to be undergoing activation with changes in mitochondrial function (i.e. sensitivity to respiratory chain poisons) also occurring following culture of freshly isolated PBMC's.

  -We recommend that assessment of mitochondrial function using cellular assays be only carried out on freshly isolated blood within 1-2hrs of isolation.

- PBMC's appear to be a better system to assess mitochondrial function than granulocytes and these should be used in preference to granulocytes. A more straight forward PBMC isolation kit is available using an Accuspin^{TM} 1077 system from Sigma.

- Regarding the data previously reported by Acumen. There is a clear cellular band on the acumen gradient which was determined using morphological assessments to be a neutrophil fraction. These assessments rely on the morphological differences observed in freshly isolated granulocytes and PBMC's. As we demonstrate PBMC's appear to be going through a granulation change in the 24hr blood sample this could potentially result in a different pattern of migration in the Histopaque gradient. Activation of neutrophils during the 24hrs in the blood tube could also affect mitochondrial function.

  - Further examination of cells isolated on the Histopaque gradient on blood samples processed after 24hr is worth exploring. It is important to run a more sophisticated flow cytometry cell classification assessment on isolated cells using surface markers to unequivocally identify the cell types present in the granulocyte and PBMC layers. In light of recent metabolomics studies identifying differences in mitochondrial function between ME/CFS and controls, the studies of Booth and Myhill could still be very relevant if the cells used for the analysis could be put through a more thorough classification.

Introduction

ME/CFS remains a devastating illness with no clear link to the underlying cause of the pathology. Papers published by Norman Booth and Sarah Myhill et al have demonstrated an interesting correlation between the level of neutrophil mitochondrial dysfunction and the severity of disease in ME/CFS patients (the work was conducted by Dr. John McLaren Howard of The Acumen Laboratory) [1-3]. In these papers the authors measure total cellular ATP. They also measure the ability of mitochondria to generate ATP (ADP to ATP conversion efficiency) following depletion of mitochondria ATP with the electron transfer chain (ETC) inhibitor sodium azide. This is an attractive approach to assess ATP rather than relying on steady state levels as steady state levels do not give any indication of supply and demand.

In the Myhill/Booth granulocyte studies cells were isolated from whole blood using a density gradient of HistopaqueTM 1.077/1.119. A granulocyte fraction isolated in this manner will contain neutrophils, basophils and eosinophils. Neutrophils predominate as the most abundant white blood cell in humans making up 50-70% of the white cell population [4]. The samples had been collected at least 24 hrs prior to processing and sent for analysis via the postal service.

Neutrophils are short lived cells that only last for between 5-90 hours in the circulation. When activated neutrophils become phagocytic forming an essential part of the innate immune system which acts as a rapid early response to pathogens [5]. Neutrophils are recruited to the site of injury within minutes following trauma, and are the hallmark of acute inflammation. When activated they migrate to sites of infection engulfing bacteria which are destroyed in the neutrophil phagosome by reactive oxygen species generated by the NADPH oxidase. In the context of using neutrophils as an indicator of mitochondrial function it is important to consider that neutrophils in freshly isolated blood sample will be in a non-activated state. This will likely differ from neutrophils activated by pathogens where the energetic demands on the cells will be very different. Activated neutrophils will generally only be found at sites of infection [6]. Long term storage of blood or changes in temperature will result in neutrophil activation [7].

The role of mitochondria in neutrophil function remains unclear. The study by Booth/Myhill et al suggests that neutrophils have a large mitochondrial component which is important for ATP generation. In contrast to these findings, Chacko et al [8] using a very similar protocol

but using freshly isolated blood (processed within 15 minutes of collection) suggested that mitochondrial respiration is absent in the neutrophil fraction even when stimulated with FCCP to uncouple the mitochondria and ascertain the maximal rate of mitochondrial respiration.

One potentially important difference in the methodologies between these two studies in addition to the time of sample processing is that throughout the cell isolation and subsequent assay steps the cells isolated by Chacko et al are stored in RPMI-1640 media which contains high levels of glucose (11mM). The Booth/Myhill studies used a special buffered saline without any glucose. Our group has extensively investigated the effect media glucose concentration has on cellular respiration and metabolism. Our data suggests that mitochondrial respiration can be suppressed by the non-physiological glucose concentrations (> 5mM) found in most cell culture medium [9]. Under these conditions when mitochondrial respiration is attenuated ATP is generated by glycolysis and not via mitochondrial respiration. It is possible that Chacko et al may not have observed mitochondrial respiration in neutrophils due to the excess glucose in their medium. However, the Chacko studies use the mitochondrial uncoupler FCCP in their mitochondrial respiration studies which even in high glucose media will allow rates of mitochondrial respiration to be assessed. The studies by Booth et al where glucose is not present in the isolation media will favour a mitochondrial mode of ATP production if mitochondria are present. Another key difference between the two studies is that the Chako study directly determines mitochondrial function while Booth et al measure ATP levels in the presence and absence of respiratory chain inhibitors to obtain a measure of mitochondrial function. The blood processing times of the two studies; immediate in the Chako study and > 24hrs in Booth/Myhill research may also impact on neutrophil function depending upon the neutrophil activation state during storage in the heparinised blood tube.

The focus of this pilot investigation was to determine if cells isolated from whole blood can be reliably used to assess mitochondrial function as a part of a laboratory test. Control blood samples obtained from volunteers were taken and mitochondrial function assessed in the granulocyte (neutrophils, basophils and eosinophils) and peripheral blood mononuclear cells (PBMC) (lymphocytes (T-cells, B-cells and NK-cells) and monocytes). This validation is important because the studies of Booth and Myhill using results from Acumen (Dr John McLaren-Howard's laboratory) suggest that the granulocyte fraction from blood samples can be used to assay mitochondrial function and that this is impaired in ME/CFS patients. This

test is currently used by a number of private clinics at a cost of £300-400. We aimed to set up the tests required to assess mitochondrial function in blood samples from ME/CFS patients using both ATP and mitochondrial respiration as endpoints. We aimed to use cell models with known mitochondrial dysfunction and bio-energetic impairment to both validate and improve on the tests developed by Acumen. Granulocyte and PBMC mitochondrial function was assessed in control blood samples and the differences in the methodologies used by Booth et al and Chacko et al investigated. Our goal was to develop a method to assess mitochondrial function compatible with the widely used Seahorse Biosciences metabolic flux analyser and plate based fluorescent probe oxygen and pH measuring platforms. This will make the blood tests more globally accessible to a wide range of researchers. The final part of the proposal was to investigate the impact of cytokines shown to be elevated in early ME/CFS patients on mitochondrial function in human muscle and primary rodent neuronal cultures. Results and methodologies generated in this pilot will be used as part of a series grant of applications to establish whether mitochondrial dysfunction is a major factor in ME/CFS.

1. Methods

1.1 Cell Isolation – Histopaque Density Gradient

All reagent and equipment must be at room temperature. Blood was collected via syringe (i.e. not vacutainer) and transferred into lithium heparin tubes. Whole blood was spun at 700G for 10 minutes and the plasma removed and stored. Approximately 1 ml of plasma was left behind to avoid disturbing the cell pellet. Blood was made back up to the original volume with PBS and mixed by gentle inversion. A density gradient was prepared in a 15ml falcon tube by carefully and slowly layering each component on top of the others (Bottom layer 3ml Histopaque density 1.119, middle layer 3ml Histopaque density 1.077, top layer 5ml blood).Transfer tubes carefully to the centrifuge and spin at 700g for 30 minutes with the brake off. The resulting separation is depicted in Figure 1

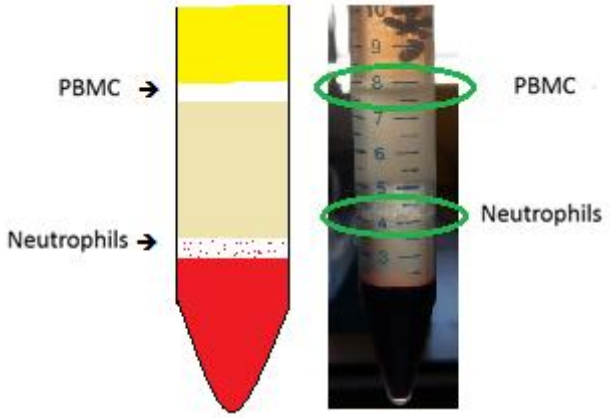

Figure 1: The PBMC layer (A) is the top band directly under the plasma/PBS layer, while the granulocyte layer (B) is located just above the red blood cell pellet.

Peripheral blood mononuclear cells (PBMC) and granulocytes layers are removed with fine tipped pastettes and placed into separate falcon tubes. Cells are washed (X2) in RPMI-1640 media containing 10% FBS and 5mM glucose. This is in line with the Chacko paper who reported a severe attenuation of oxygen consumption in the absence of cell culture media. Although Chacko used RPMI-1640 with 11mM glucose we maintained glucose levels at a more physiological 5mM. This was to prevent the potential artificial effects of high and low glucose on mitochondrial function observed in cell culture models. A glucose concentration which better reflects blood glucose levels is more likely to give a better indication of mitochondrial activity of cells in the blood stream.

1.2 Cell Isolation - Red blood Cell Lysis

Prepare 1X RBC lysis buffer (10X RBC Buffer supplied from Biolegend) in ddH2O. Add 100µl whole blood to 2ml of 1X RBC lysis buffer. Gently vortex and place in the dark for 10 – 15 mins. Centrifuge at 700G for 5 minutes. Remove supernatant and wash (X2) with PBS. Re-suspend in RPMI-1640 containing 5mM glucose for downstream use.

1.3 Cell Isolation - PALL Acrodisc

Attach a 10 mL syringe barrel to the filter inlet and mount the system over a waste collector. Add up to 10 mL blood to the syringe barrel and let blood drain through the filter via gravity. Wash the collected WBC on the device with 5 mL PBS (X2), pH 7.4 at a rate of approximately 3 drops per second. Fill a new 10 mL syringe with 5-10 mL of 1X RBC lysis buffer and attach to the filter's **outlet** using a luer connector. Place the filter with syringe over collection tube and press the plunger down washing WBCs into the tube.

1.4 Oxygen Consumption Assay

Prepare cells as outlined earlier, count and adjust density to 1 X 10^7/ml. Add 50µl to each well (500,000 cells/well). Add inhibitors as required and 15µl MitoXpress probe. Add sufficient RPMI-1640 to each well so that the final volume is 150ul. Cover with MitoXpress HS oil and read TRF overnight in a BMG plate reader Excitation TR-exl, Emission TR em^2 with integration times of 30 and 70µs. An example trace of this output is depicted in Figure 2. Lifetime (µs/hr) on the y-axis shows the oxygen being consumed i.e. increasing over time (x-axis). Calculating the slope of this curve allows the rate of oxygen consumption to be determined.

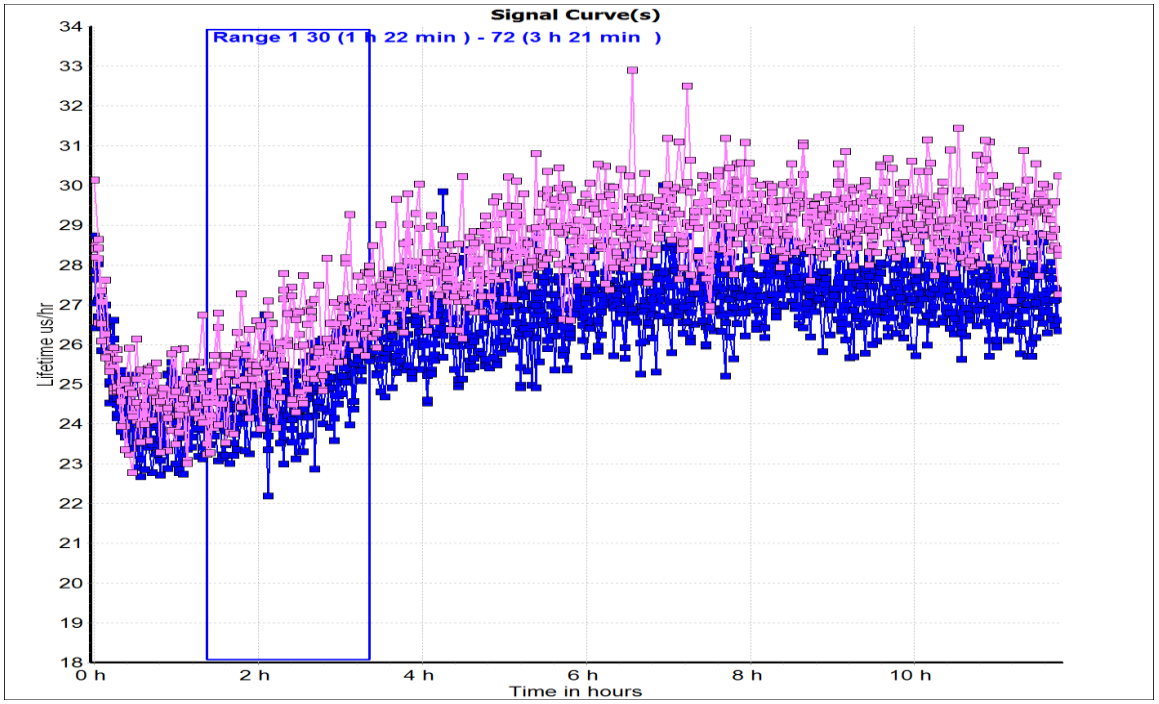

Figure 2: Representative trace of an oxygen consumption assay.

1.5 ATP Assay

Equilibrate the reagents to room temperature. Seed the cells in RPMI-1640 media at 1 X 10^6/ml in a white clear bottom 96 well plate (50,000 cells/50µL). Cells are seeded in 50µL media and inhibitors or media (for the control wells) is added to appropriate wells to bring the final volume to 100µL. Mitochondrial inhibitors are added to the cells for 30 minutes before ATP is measured. A standard curve is also prepared. Following incubation with inhibitors 50µL of detergent is added to each well and the plate is placed on a shaker for 5 mins @ 700rpm (cell lysis and ATP stabilising step). After lysis 50µL of substrate is added to each well and the plate is again placed on the shaker for 5 mins @ 700rpm. The plate is dark adapted for at least 10mins to allow for any interfering light decay before measuring luminescence.

1.6 CellTox Green (cell death assay)

Prepare a cell suspension containing 1 X 10^6/ml cells. Add 2µl of CellTox™ Green Dye per 1ml of cells. Add 50µLof the CellTox™ Green Dye/cell suspension to a black clear bottomed 96 well plate. Add test compound and make up to 100µL with blank media. Measure fluorescence kinetically over time (Ex 485–500nm Em 520–530nm).

2. Results

2.1 To validate the methods of Booth et al in identifying energy defects in cells known to have mitochondrial dysfunction.

Summary points:

- To test the sensitivity of our methods at identifying mitochondrial defects we measured mitochondrial function in two cell types. One cell type (WT) had functional mitochondria and the other (Rho-0) did not.
- The assays we used were able to successfully identify the Rho-0 cells as having dysfunctional mitochondria and this demonstrated the applicability of our methods to assess white blood cells isolated from whole blood.

The first experiments conducted were proof of principle to show the applicability of measuring ATP and oxygen consumption rates to assess mitochondrial function. In this section we used wild type (WT) and Rho-0 cells. Rho-0 cells are devoid of mtDNA and will display respiratory chain dysfunction.

We cultured both cell types on standard DMEM culture media (25mM glucose). To assess the effect of glucose concentration on these cells they were plated overnight in DMEM with varying glucose concentrations and following this overnight adaptation their ATP (Figure 3) and oxygen consumption rate (OCR) (Figure 4) was measured. We investigated these parameters in both lines on 0 (Booth), 1, 2.5, 5, 11(Chacko) and 25mM (standard culture medium) glucose.

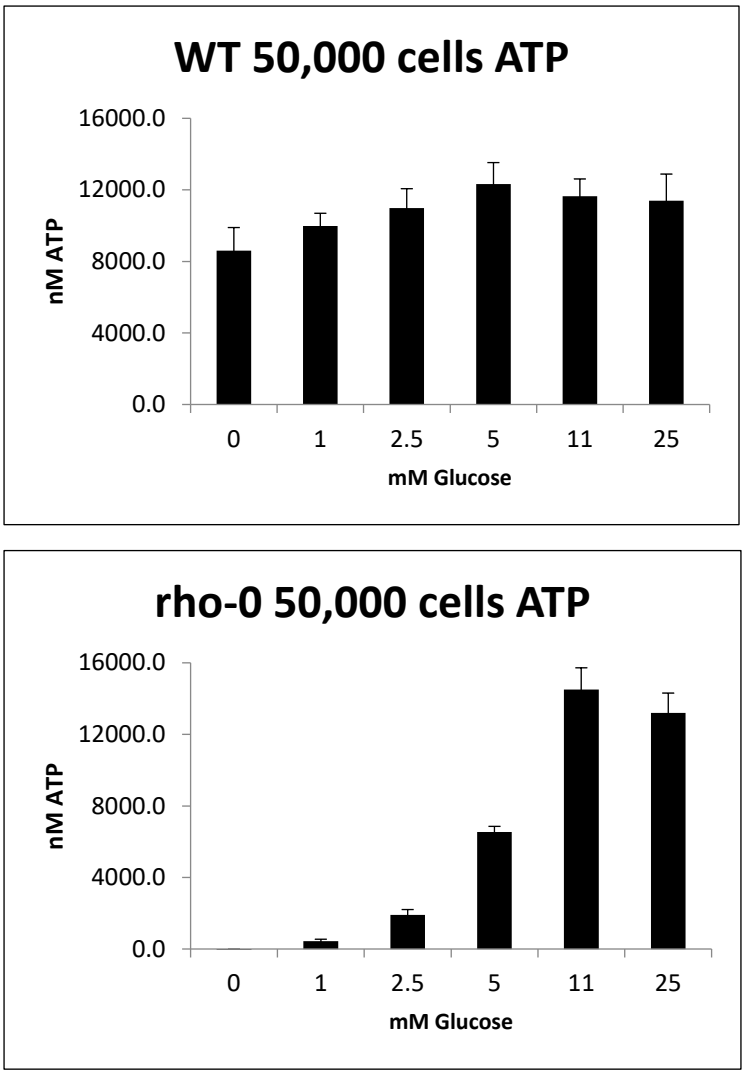

Figure 3: ATP was measured in WT (top) and Rho-0 (bottom) cells on varying concentrations of glucose. WT cells display a small decrease in ATP levels at the lower glucose concentrations while Rho-0 cells cannot be sustained on lower glucose concentrations.

Figure 3 & Figure 4 demonstrate well how cells can adapt their metabolism depending on their substrate availability, in this instance glucose concentration. Under high glucose conditions (25mM) the WT cells had low OCR but normal ATP levels suggesting a reliance on glycolysis for ATP production. As glucose was decreased and eventually removed the cells switch from using glycolysis to using their mitochondria with only a marginal loss of ATP on the 0mM glucose. In contrast, the Rho-0 cells have negligible OCR rates at all glucose concentrations tested (due to their lack of functional mitochondria).They cannot sustain themselves when glucose availability is limited as was demonstrated in their ATP profile, the cells died.

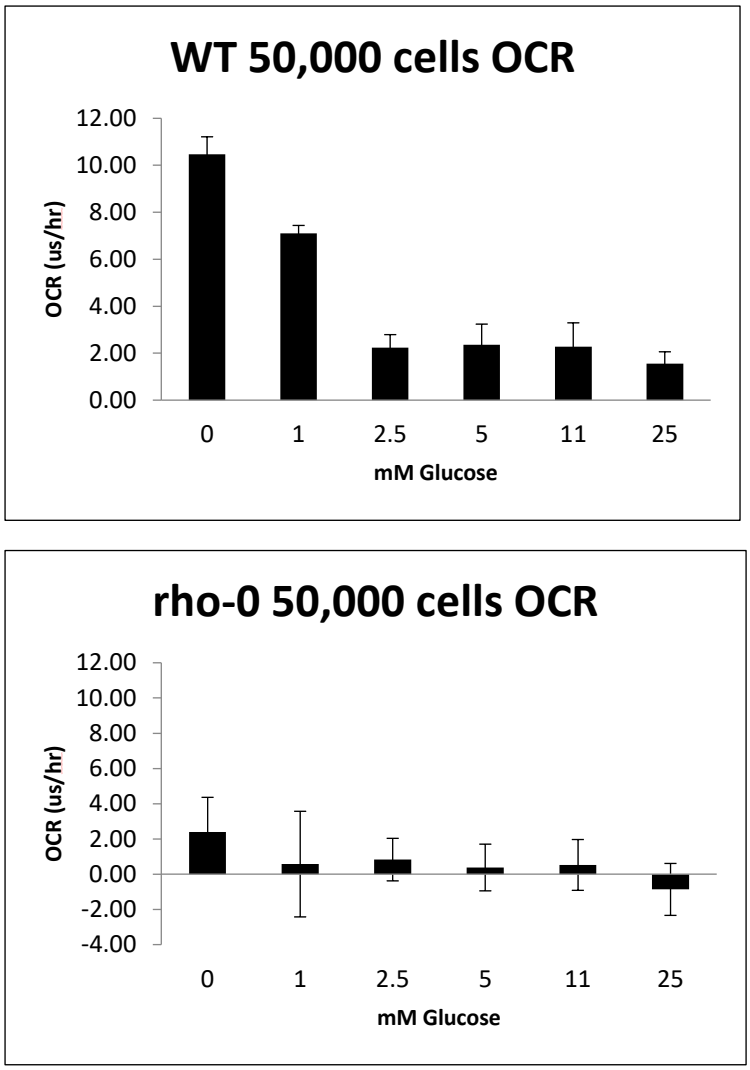

Figure 4: Oxygen consumption as a marker of mitochondrial respiration was also assessed in WT (top) and Rho-0 (bottom) cells. WT cells display low levels of OCR on higher glucose concentrations but this is significantly increased as glucose is reduced/removed. Rho-0 cells display negligible OCR at all glucose concentrations assessed.

As mentioned previously measuring the ATP generating capacity of the mitochondria is a more beneficial end point than total ATP. To do this Acumen (McLaren-Howard/Booth/Myhill) treat the cells with sodium azide, a complex IV inhibitor, and then wash it away and measure ATP. This gives an ADP to ATP conversion estimate. We next sought to optimise this part of the Acumen protocol Figure 5.

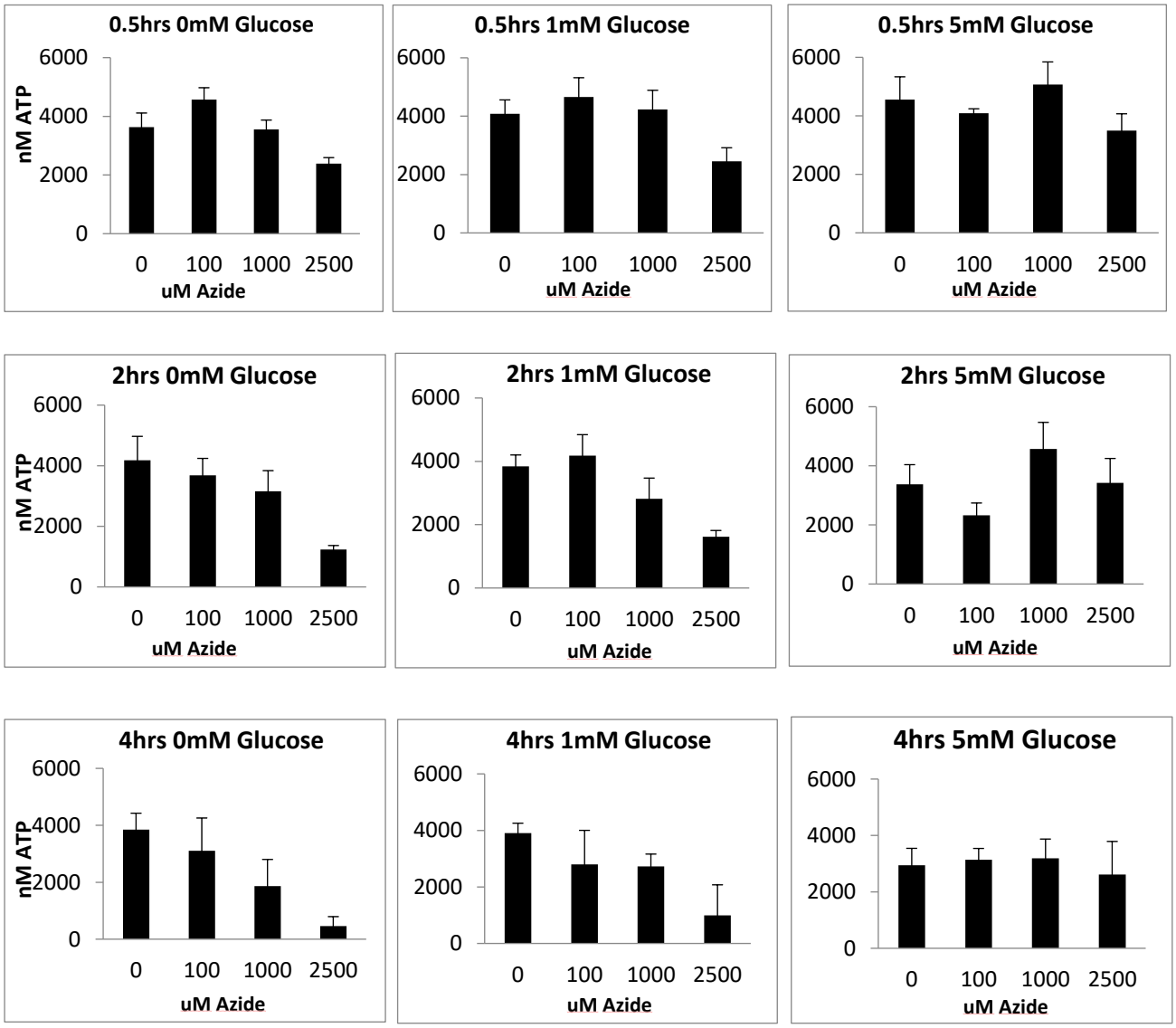

Figure 5. WT cells were adapted to different glucose concentrations overnight then they were treated with different concentrations of sodium azide over a 4hr time course. Cells needed to be on very low glucose <1mM for at least 2 hours with a high concentration of sodium azide to see a deleterious effect on ATP concentration.

The Acumen protocol uses 750µM sodium azide on the neutrophils for 30 minutes in order to knock down mitochondrial ATP production. In our WT cells we need to use almost 3X the concentration of azide for a minimum of 2 hours (without washing) to see a negative effect on ATP concentration. No deleterious effect of the azide is seen in the cells that have 5mM glucose indicating a large glycolytic component in this cell line at this glucose concentration.

2.2 Use control blood samples to carry out the protocols of Booth and Chacko to separate different white cell components. Assess mitochondrial activity in granulocytes and PBMCs.

2.2.1 Isolation

Summary points:

- We were unable to reproduce the histotopaque gradient exactly like the Acumen protocol
- When cells were isolated from fresh blood and analysed using flow cytometry we saw three distinct cell populations consistent with neutrophils, monocytes and lymphocytes (monocytes and lymphocytes make up the PBMC fraction)
- When cell fractions were isolated from blood that had been left overnight the monocyte cells appear more granular and there is no evident neutrophil population

In this section we attempted to repeat the Histopaque isolation protocol used by Acumen/Booth/Chacko to isolate granulocytes and PBMCs from whole blood. Total cellular ATP levels and whole cell mitochondrial respiration (oxygen sensing probes, Luxcel Biosciences) were determined. By running the mitochondrial oxygen consumption assay in parallel with ATP assessments we are able to determine the contribution of mitochondrial respiration to cellular ATP production.

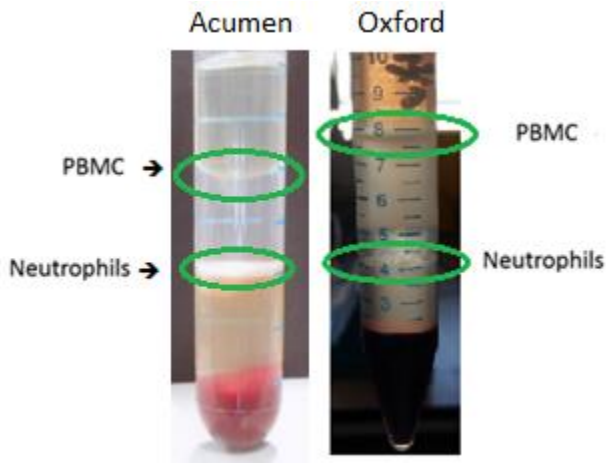

Figure 6. The left hand image is a gradient produced by Acumen depicting a large band of cells between the two Histopaque layers. This is where granulocytes are expected. By comparison, our gradient on the right hand side has a clear PBMC band (A) and a less obvious granulocyte band (B).

We were unable to replicate the Acumen methodology to consistently isolate the granulocyte cell fraction on the gradient. Figure 6 shows an image of a typical Acumen gradient (courtesy of John McLaren-Howard). In the Acumen image we see a large band of granulocyte cells and a negligible PBMC layer. Figure 6 the Oxford gradient consistently gave a strong PBMC band with a negligible granulocyte layer. Although this is not clear on the image due to image quality, cell counts of the two layers consistently supported this observation (data not shown). On the occasions where we were able to isolate a granulocyte layer it was not a well-defined band like Acumen but rather a clumpy/coagulated cell fraction that was dispersed throughout the Histopaque 1.077 layer rather that at the Histopaque interface. Despite maintaining the same isolation conditions/procedure we could not consistently isolate granulocyte cells.

Acumen analyse blood samples that are 24-48hr post phlebotomy. Following consultation with a local specialist NHS immunologist we had concerns that 24 hours was too long for neutrophils which would likely become activated [7] and that we would lose most of our granulocytes in that time period. In our initial experiments we isolated the cell fractions from fresh blood (1hr old) and blood that had been left for 24hrs (similar to Acumen samples). We also isolated total white blood cells to look at the overall population using a red blood cell lysis buffer preparation. We analysed the samples by Flow Cytometry (FACS), a useful tool that separates cells based on their size and internal composition (granularity). The results are shown in Figure 7.

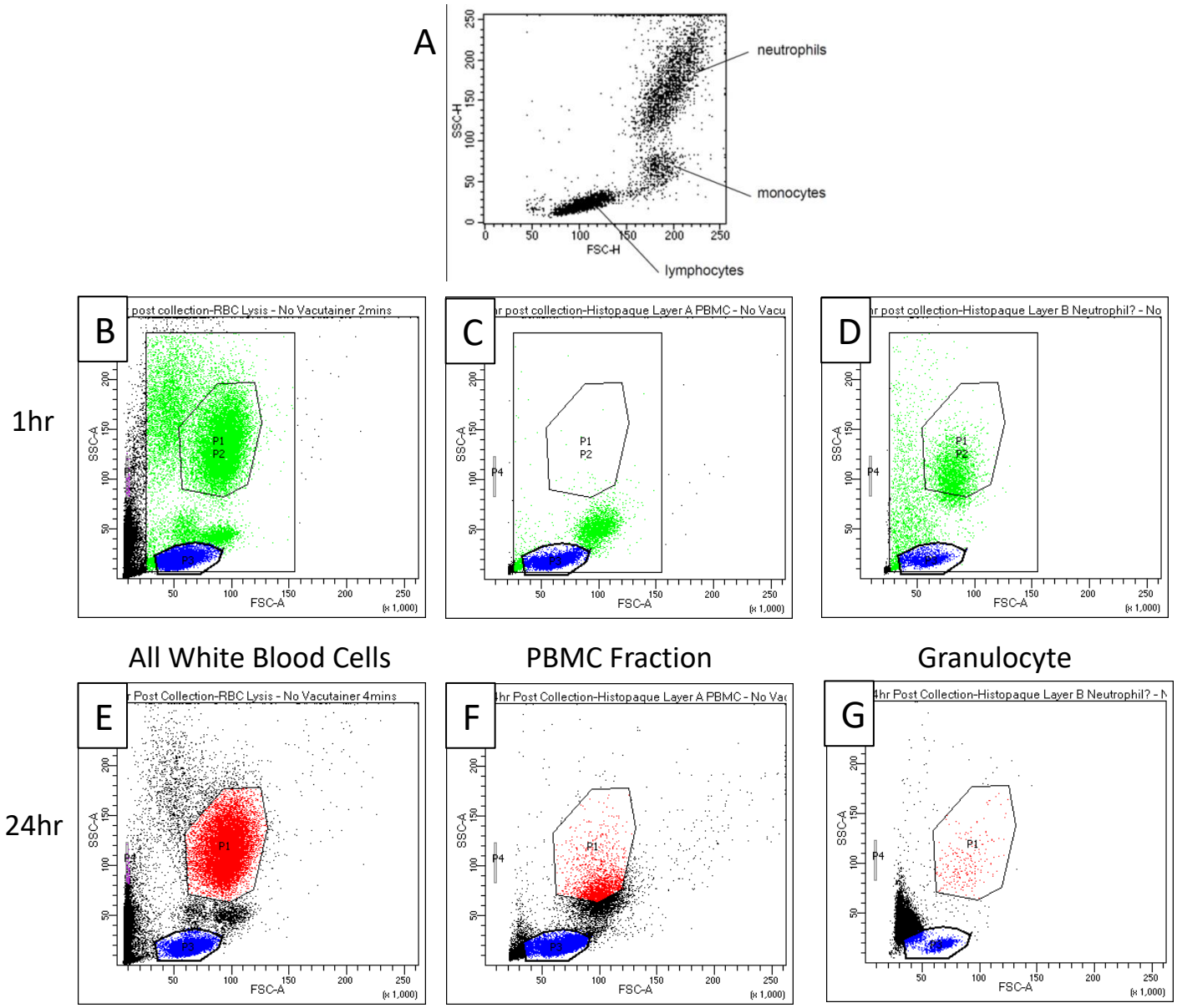

Figure 7. Cells were analysed using FACS. (A) shows a typical plot of white blood cells consisting of neutrophils, monocytes and lymphocytes. (B, C & D) were cells isolated from blood after 1 hr. (B) was whole blood that had the RBC lysed and you can see 3 distinct populations of cells. (C & D) were isolated using Histopaque. (E, F & G) were samples that were prepared from blood that was 24 hours old.

Neutrophils, lymphocytes and monocytes can be identified using flow cytometry without any cell specific markers. Based on their relative sizes and granularity the cell populations are easily separated using forward and side scatter Figure 7A. Figure 7B shows WBC isolated after 1 hour by lysing the red blood cell population and it shows distinct neutrophil and PBMC populations. Figure 7C is the 1hour PBMC fraction from a Histopaque separation and the scatter plot shows no neutrophil like cells. Figure 7D is the 1 hour neutrophil layer from a Histopaque separation and it shows contamination with lymphocytes and other cellular debris. Figure 7E, F & G depict cells that were isolated from blood that had spent 24hrs in

heparin at room temperature. The 24hr RBC lysis sample (E) looks similar to the 1hr equivalent (B) because we can clearly see three distinct cell populations in both scatter plots. While in the Histopaque gradients the 24hr cell populations look quite different to the 1hr samples. Most notably there is a lack of neutrophil like events in (G) while (F) (PBMC fraction) gives more dispersed and granular like monocytes. Figure 7 is a key figure as it (i) highlights the loss of neutrophil cells from blood that has been left for an extended period of time in this instance 24 hours when we use the Histopaque gradient for isolation (G) and (ii) we can see in (F) that the monocyte cell population has more side scatter (as seen on the y-axis) indicating a change in granularity following the prolonged time before the cells were isolated. Overall, this figure highlights the importance of isolating the cells from fresh blood because if the blood has been left for an extended period of time then we start to lose cells when processed using Histopaque and observe changes in their morphology possibly due to activation. In light of this all further experiments were carried out on cells that had been isolated from fresh blood i.e. <1hr post sampling.

2.2.2 Cell Death

Summary points:

- Cells were not activated by plastic over the time course studied
- Activation could be induced using PMA and correlated with a sharp increase in cell death due to the oxidative burst.
- PBMC cells demonstrated a mitochondrial (Oxphos) component to their metabolism as seen by an increase in death when treated with mitochondrial inhibitors (after7-8hrs)
- Granulocyte viability were unaffected by mitochondrial inhibitors

Once the cell isolation protocol was optimised and we confirmed the time sensitive lability of the cells we assayed their mitochondrial function by measuring oxygen consumption rates (OCR) and ATP levels. We also assessed cell death over time using CellTox Green as it has been reported that monocytes in particular can become activated by plastic [10] so we wanted to be sure of the health of our cells for the during time course assays. Figure 8 shows a typical cell death plot for both PBMC and granulocyte cells. In order to assess if the plastic plates cause an activation of the cells we treated some of them with a known activator PMA. Both cell populations show significant increases in death when stimulated with PMA (turquoise and green lines). In the absence of PMA the cells do not demonstrate any cytotoxicity in the untreated wells (red lines). Interestingly in the PBMC fraction when the cells were treated

with mitochondrial inhibitors (yellow lines) there is an increase in cell death after ~8 hours. This is suggestive of a reliance on mitochondrial metabolism (Oxphos) as inhibition of the mitochondria increased death. The granulocyte viability was less affected by the mitochondrial inhibitors supporting the Chacko theory that they are glycolytic.

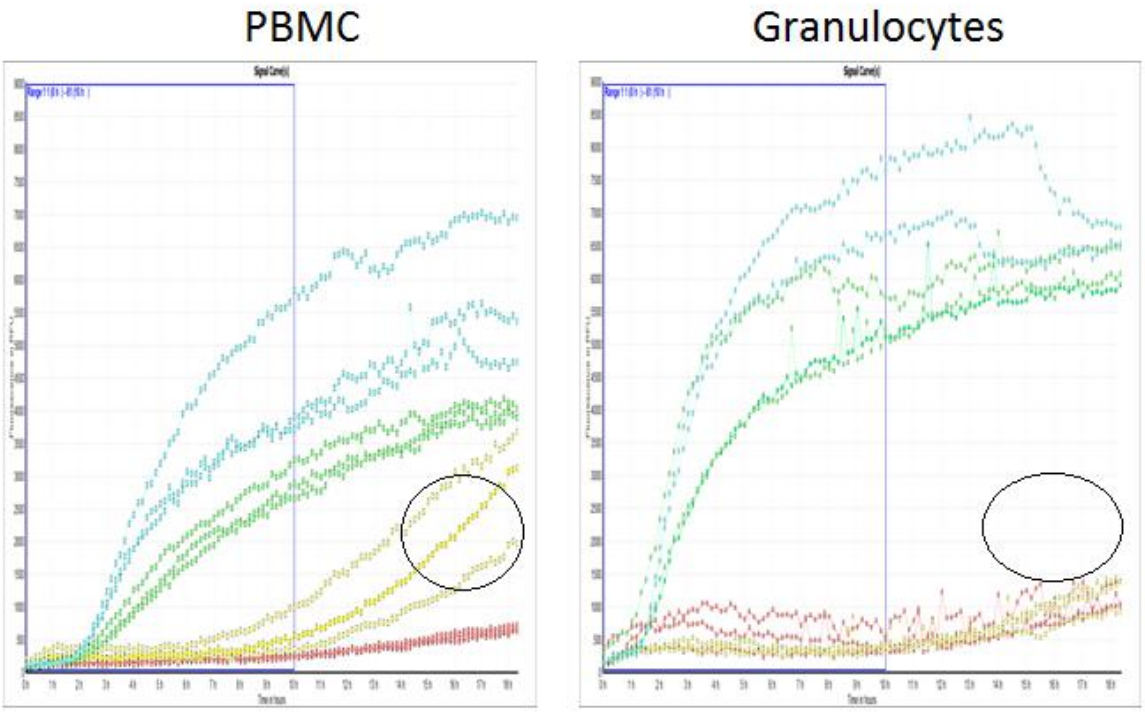

Figure 8: Cell death profiles of PBMC (left) and granulocytes (right) using CellTox Green stain. Cells were untreated (red), rotenone/antimycinA (yellow), PMA (green) or PMA & rotenone/antimycinA (turquoise) and monitored over 18hrs. PBMC cells showed increased death (circled) when treated with mitochondrial inhibitors that was absent in the granulocyte cells.

2.2.3 Oxygen consumption

Summary points:

- PBMC cells had measurable oxygen consumption rates that could be attenuated with mitochondrial inhibitors suggesting on oxidative bioenergetic phenotype
- Granulocytes did not display significant levels of oxygen consumption indicative of a more glycolytic phenotype

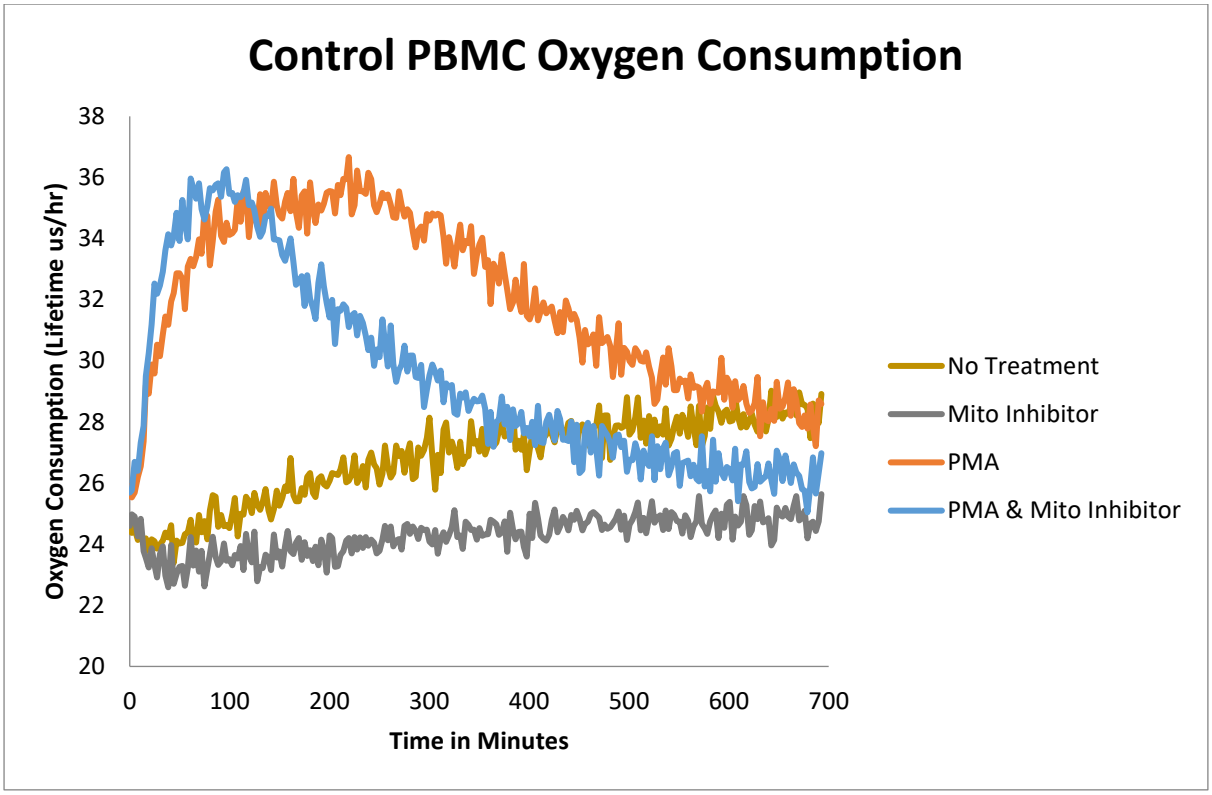

Figure 9: Representative OCR profile of PBMC cells showing inhibition when treated with rotenone/antimycinA and significant OCR rates when PMA is added to stimulate the cells.

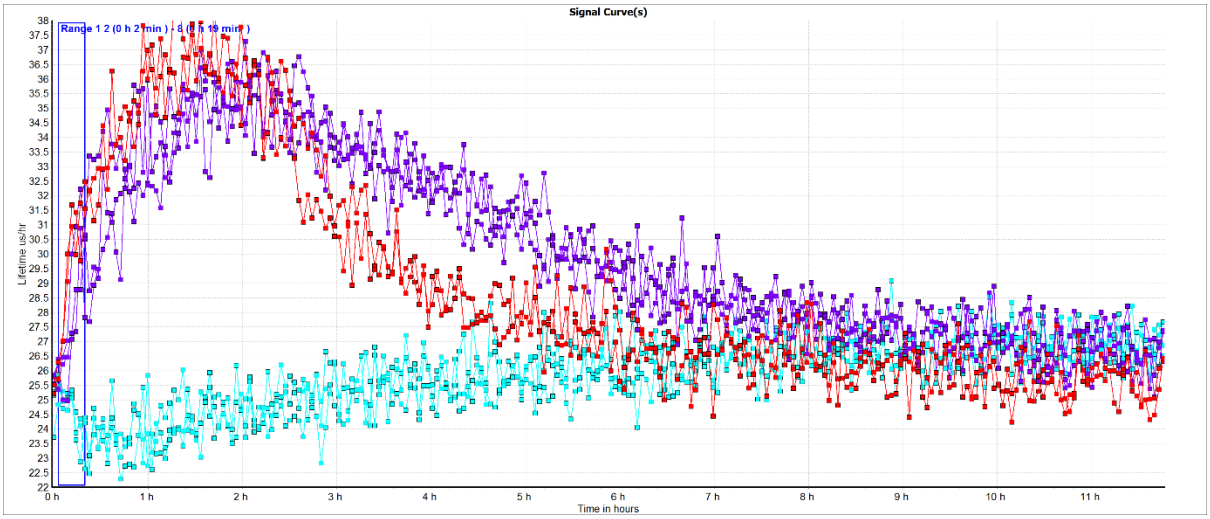

Figure 10: Representative OCR profile of granulocyte cells. Cells were treated with PMA (purple), PMA & mito-inhibitors (red) or left untreated (turquoise).

The OCR profiles of the cells highlighted some interesting information. Firstly, it required 500,000 cells/well to get a reliable OCR signal (this was 10X the cell concentration used for adherent transformed cells (Figure 4)). The PBMC cells indicated an Oxphos component when there was an increase in death when treated with mitochondrial inhibitors (Figure 8).

This Oxphos metabolism was confirmed in the OCR assay (Figure 9 yellow line). When treated with inhibitors of the electron transport chain (ETC) the OCR of PBMC cells were significantly reduced (Figure 9 grey line).

The PBMC cells also demonstrated a significant OCR rate when stimulated with PMA (Figure 9 orange line). PMA does not increase mitochondrial oxidative metabolism because when cells were treated with PMA and mitochondrial inhibitors (Figure 9 blue line) OCR rates remain unchanged. The PMA stimulated increase in OCR is due to the activation of non-mitochondrial NADPH oxidase which generates superoxide consuming O_2 in the process. The interesting finding from this data is that when stimulated with PMA after the initial OCR increase the cells (orange line) maintain oxygen consumption to a certain extent over the following hours. However, when treated with PMA and mitochondrial inhibitors (blue line) after the initial surge the cells return back to baseline much quicker. This again highlights a mitochondrial component of the cells metabolism.

Figure 10 shows a representative OCR plot using granulocytes. Some key differences between the granulocytes and PBMC are that (i) the granulocytes have a lower basal rate of oxygen consumption (turquoise line) and (ii) when stimulated with PMA they can't maintain the oxygen consumption rates beyond the initial surge (purple line) in the same way the PBMC can. This again is suggestive of a more glycolytic phenotype in the granulocyte cells as reported by Chacko et al.

As mentioned previously we intended to assess the isolated cells in both 0mM (Booth) and 11mM (Chacko) glucose concentrations. However, early OCR experiments using 0mM glucose did not work. We could not get any OCR rates the reasons for this are unknown but Chacko et al did report attenuated bioenergetics in the absence of glucose. Subsequent experiments all used plasma glucose concentrations of 5mM in the assays.

In order for a test to be clinically applicable it needs to be robust and reproducible. The Acumen test is conducted at one time point. We wanted to assess how the results obtained from individuals would vary over time. Three healthy (non ME/CFS) controls had blood taken every fortnight over the course of 8 weeks.

The OCR rates for PBMC gave quite consistent results (Table 1 & Figure 11). The 3 controls varied in age and gender but there does appear to be a level of agreement between the untreated, mitochondrial inhibitors and PMA treated ATP responses seen amongst individuals. Table 1 shows the % OCR relative to the no treatment cells. The % OCR for the

three individuals whose PBMC were treated with mitochondrial inhibitors were 21.84, 33.70 and 58.83% respectively. This clearly shows a level of reliance on the mitochondria for energy production in this cell group.

The granulocytes, as mentioned earlier, were difficult to consistently isolate using the Histopaque gradient. Their glycolytic nature made analysis of the untreated and mitochondrial inhibitor treated cells difficult as the OCR rates were very low. Table 1 shows that mitochondrial inhibitors decreased OCR in these cells to 70.92, 40.63 and 57.26 respectively in the three individuals. These results could indicate a potential mitochondrial component in these cells but it should be interpreted cautiously as the standard deviations are far greater than for the PBMC fraction. When stimulated with PMA there was a lot more variability in the granulocyte cells than in the PBMC cells.

In future studies it would be interesting to see how a ME/CFS patient population compares to a control group using the same assessments.

PBMC			Granulocyte		
Control 1	**% Untreated**	**Stdev**	**Control 1**	**% Untreated**	**Stdev**
No Treatment	100.00	0.00	**No Treatment**	100.00	0.00
Rot/AntiA	21.84	5.55	**Rot/AntiA**	70.92	25.59
PMA	494.97	291.17	**PMA**	560.92	625.88
Rot/AntiA & PMA	413.84	277.02	**Rot/AntiA & PMA**	418.47	646.29
Control 2	**% Untreated**	**Stdev**	**Control 2**	**% Untreated**	**Stdev**
No Treatment	100.00	0.00	**No Treatment**	100.00	0.00
Rot/AntiA	33.70	13.80	**Rot/AntiA**	40.63	24.85
PMA	454.34	160.25	**PMA**	320.29	253.00
Rot/AntiA & PMA	485.48	166.92	**Rot/AntiA & PMA**	291.39	412.09
Control 3	**% Untreated**	**Stdev**	**Control 3**	**% Untreated**	**Stdev**
No Treatment	100.00	0.00	**No Treatment**	100.00	0.00
Rot/AntiA	58.83	45.08	**Rot/AntiA**	57.26	N=1
PMA	614.37	172.82	**PMA**	648.19	841.74
Rot/AntiA & PMA	560.60	26.20	**Rot/AntiA & PMA**	57.26	N=1

Table 1: % OCR rates of PBMC and granulocyte cells relative to the untreated control. N=3 independent experiments for PBMC and N=2 for granulocytes.

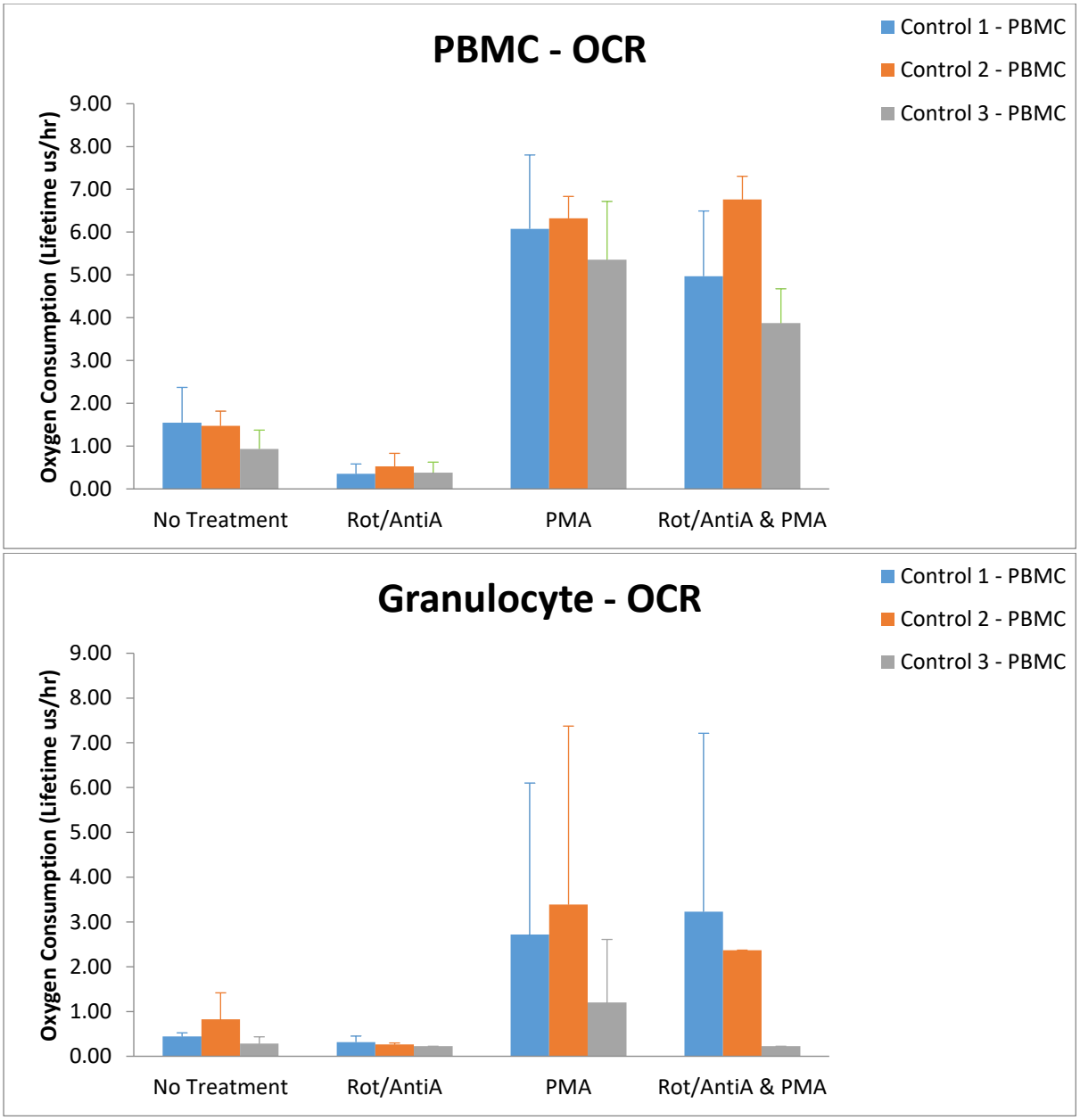

Figure 11: Oxygen consumption rates for PBMC (top) and granulocyte (bottom) cells isolated from 3 healthy individuals (non ME/CFS sufferers). Data is the mean of 3 independent blood sample isolations and the error bars represent the standard deviation. Cells were either untreated, or treated with mito inhibitors, PMA or a combination of both.

2.2.4 ATP

Summary points:

- ATP results for the PBMCs demonstrates both inter and intra-individual variability
- This highlights how ATP levels (i) vary from person to person and (ii) are not static in an individual and are subject to variation over time
- ATP results for granulocytes show considerable variation making it extremely difficult to extract meaningful conclusions from the data set
- To assess the mitochondrial contribution to ATP levels cells were treated with mitochondrial inhibitors
- With mitochondrial inhibitors ATP levels dropped in PBMC cells by 25-37% while only dropped in granulocytes by 11-14% highlighting more of a glycolytic phenotype in granulocytes

ATP concentrations in PBMC and granulocytes isolated from the three controls were measured over the course of the 8 week study. The PBMC data shown in this section represents the mean values of four independent experiments. As mentioned earlier the granulocyte cells were difficult to consistently isolate so the ATP data for these cells is N=3.

The basal (untreated) ATP data for the PBMCs is depicted in Figure 12 (top panel). Consistent results were observed for Control 1 and Control 3 over the 4 samples. Control 2 had considerably more variation in ATP levels over the 8 weeks. This highlights how ATP levels vary between individuals and can change over time within an individual. Amongst the three controls there are apparent differences in the basal (untreated) ATP levels (Figure 12 (top panel)) with Control 3 giving the highest ATP levels. This figure (Figure 12 top panel) portrays the results expressed as raw luminescence values. Luminescence values can vary over time and are susceptible to changes in assay conditions i.e. temperature etc. To allow for run to run variability in raw data values a useful way to represent the results is to normalise each treatment to its respective untreated result for each control for each experiment. This reduces run to run variability and allows us the percentage effect of the various treatments against the untreated samples which are now set at 100% (see Figure 12 (bottom panel). Each untreated control is now expressed as 100% and the different runs can easily be compared. Figure 12 (bottom) shows that mitochondrial inhibitors reduced ATP levels to 66% (control 1), 63% (control 2) and 75% (control 3). This figure demonstrates that a 25-37% (the range of the 3 individuals) of the total ATP is dependent on mitochondrial function.

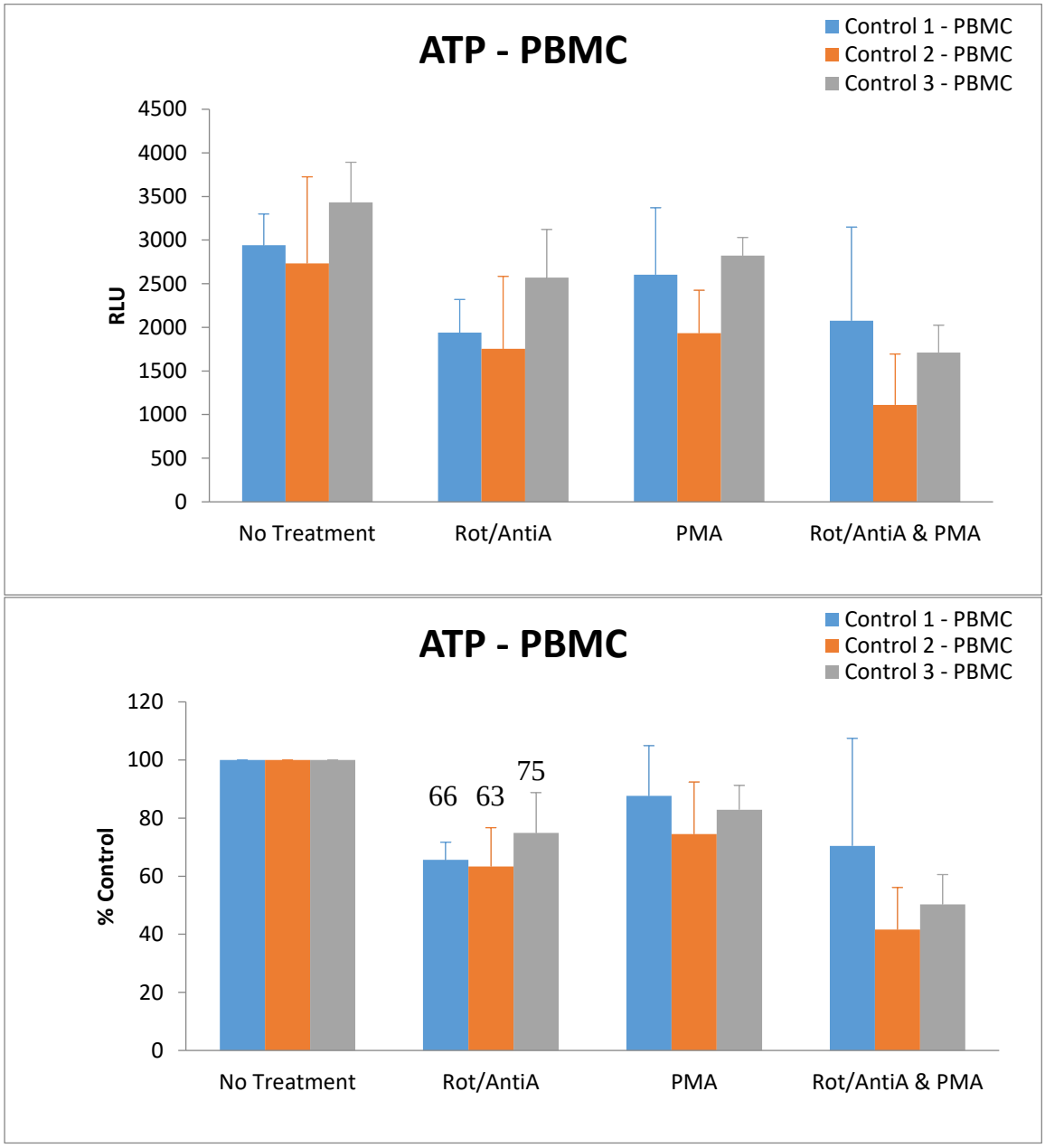

Figure 12: ATP results for PBMC cells in control cells, raw luminescence data (top) and normalised to % of untreated control (bottom). Data is the mean of 4 independent blood sample isolations and the error bars represent the standard deviation. Cells were either untreated, or treated with mito inhibitors, PMA or a combination of both. The numbers above the bars represent the mean % ATP following mitochondrial inhibition.

Analogous to Figure 12, Figure 13 comprises of a top graph (luminescence) and a bottom graph (normalised) depicting the granulocyte ATP data. What is immediately apparent in the top graph is the lower RLU values (compared to PBMC) and larger error bars. When we normalised the data (Figure 13 bottom) the results improved for controls 2&3. Control 1 still displays high levels of variation even after normalisation. In this normalised graph (Figure 13

bottom) we can see that ATP levels decrease to 86% (control 1), 89% (control 2) and 88% (control 3) when the granulocytes are treated with mitochondrial inhibitors. From this we can see that only 11-14% of the ATP in these cells comes from the mitochondria suggesting there is more of a reliance on glycolytic metabolism. This supports our OCR findings from Figure 11.

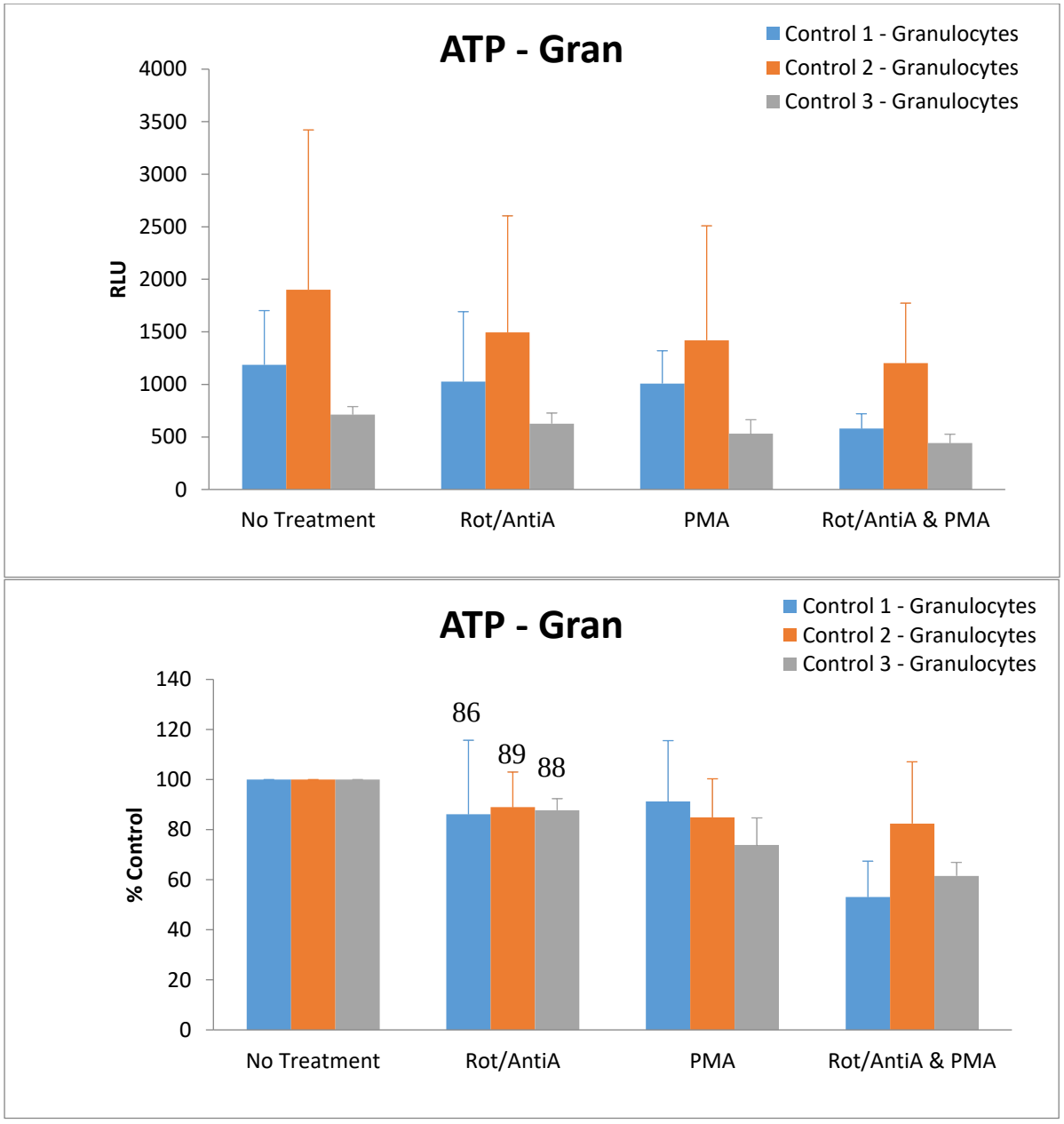

Figure 13: ATP results for granulocyte cells in control cells. Raw luminescence data (top) and normalised to % of untreated control (bottom). Data is the mean of 3 independent blood sample isolations and the error bars represent the standard deviation. Cells were either untreated, or treated with mito inhibitors, PMA or a combination of both. The numbers above the bars represent the mean % ATP following mitochondrial inhibition.

Due to run to run variation comparing or combining the raw (RLU) data from independent experiments is not the best practise, calculating the actual concentration of ATP using a standard curve is recommended. A standard curve was run each week and also a quality control (QC) sample of 50µM ATP to assess the assay performance. Each week the R^2 values were in excess of 0.99 showing excellent correlation. Weeks 1-6 also showed acceptable data for the QC samples. Assessed individually all the curves looked good, however, when all the ATP curve data was combined there were some obvious discrepancies in Weeks 1, 2, 7 & 8. In light of the lability of ATP standards and the variability we observed the best way to move forward with quantifying ATP was the eliminate the outlying Weeks 1, 2, 7, & 8 from the combined ATP curve and work out the concentrations for each week based on an amalgamated curve formula (Figure 14). Figure 15 depicts the ATP data expressed in nM for the PBMC (top) and granulocytes (bottom). The PBMC data again is quite consistent given that the data are from 3 separate individuals and 4 independent blood samples for each control.

uM	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Average	Stdev	% CV
100	1065500	454559	409350	494304	565158	576471	615199	1077000	511321	77094	15
10	180186	41483	77617	87524	48721	111111	218966	287792	81243	25835	32
1	19298	862	7826	8217	5637	9888	23733	34932	7892	1749	22
0.1	1876	342	867	1257	956	1995	2913	1022	1269	512	40
0.01	72	237	309	415	420	479	670	445	406	71	17
0.001	65	240	251	297	308	304	303	302	290	26	9
0.0001	12	288	385	466	412	398	163	278	415	36	9
0	1	524	696	827	742	651	85	366	729	75	10
QC 50uM	40.37	72.31	48.69	54.12	55.59	41.77	7.32	2.46	50	6	13

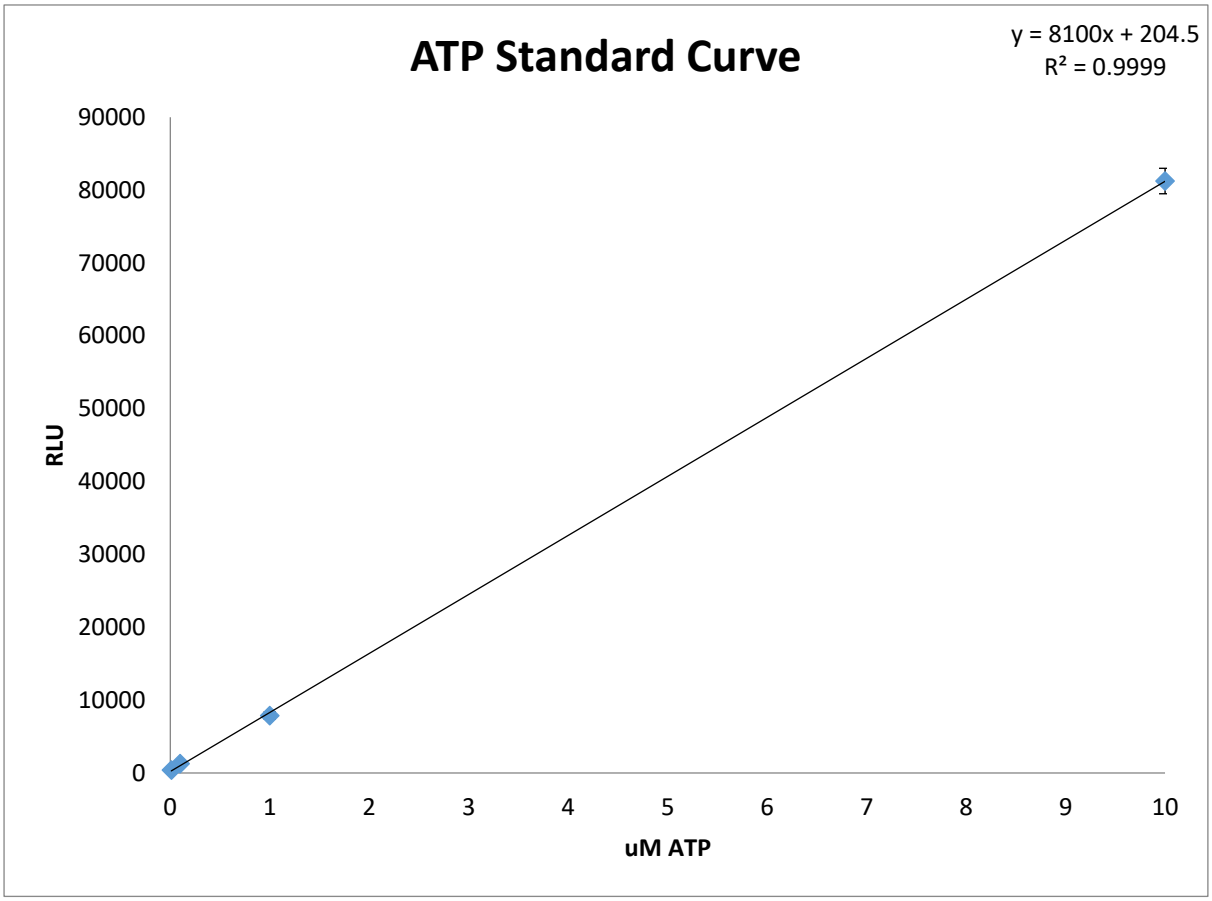

Figure 14: Combined data from each ATP standard curve over the 8 week study.

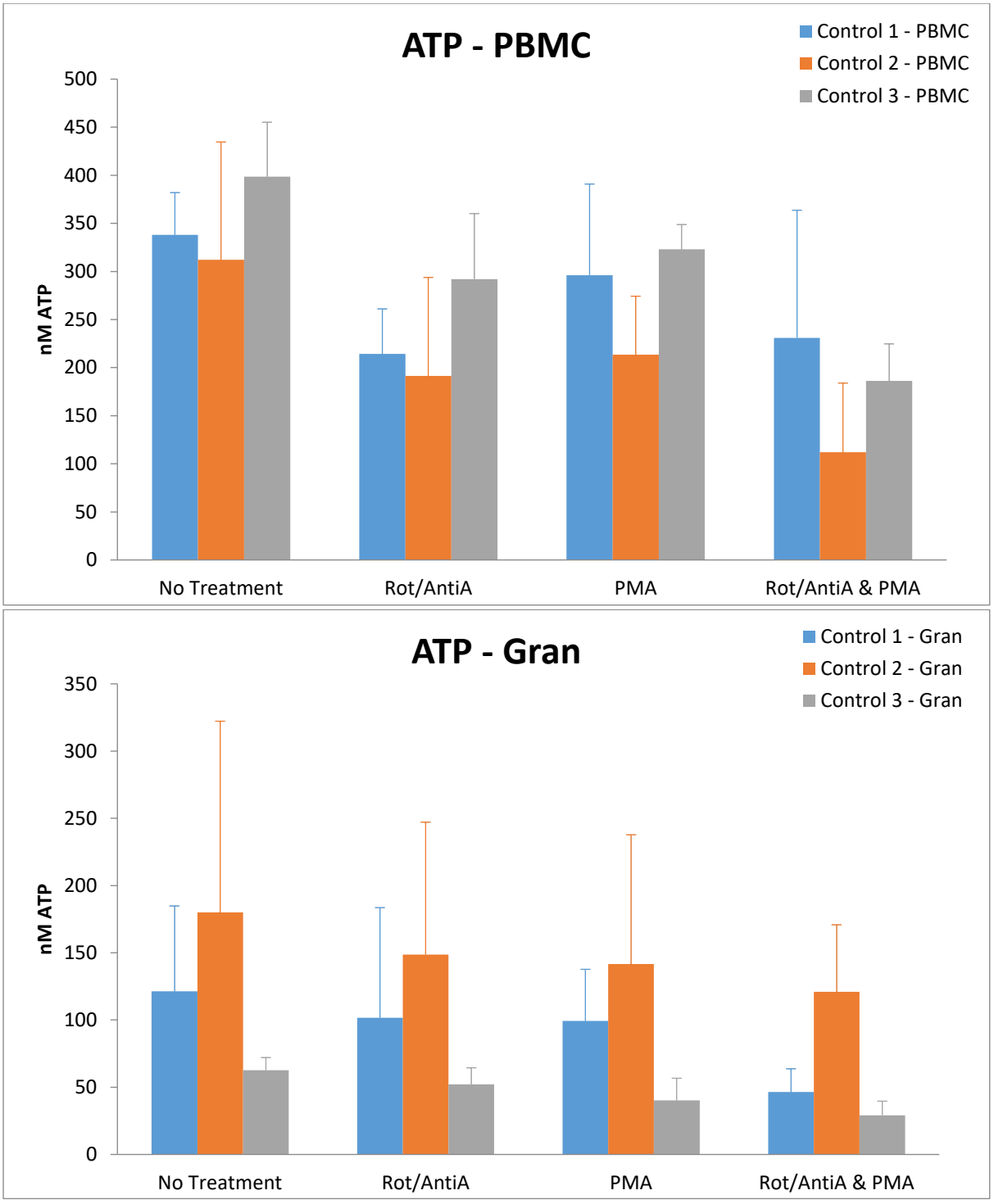

Figure 15: Mean (N=4) ATP concentrations observed in PBMC and granulocyte cells for healthy controls quantified using the combined ATP standard curve (Figure 14). Error bars represent the standard deviation of the mean.

2.3 Test the impact of the inflammatory cytokines found to be elevated or reduced In ME/CFS patients on mitochondrial function in control muscle.

Recent studies in ME and mitochondrial dysfunction identify the possibility of cytokines having a direct effect on mitochondrial function. In the final section of this pilot project the effect of INF γ, IL-10 IL-6 and TNFα on mitochondrial function was to be assessed. We planned to incubate primary human myoblast cells with a dose range of each cytokine to assess the impact on mitochondrial function (ATP, OCR, mitochondrial membrane potential, mitochondrial dynamics. Due to a delay in establishing the cells in culture and early experiments to optimise the assay conditions we were unable to undertake this work during the course of this project. Initial experiments though show that the myoblasts have good OCR (Figure 16 & Figure 17) and would be excellent cells to assess the impact of these cytokines on mitochondrial function. We hope to conduct this work with the arrival of a project student in the coming months.

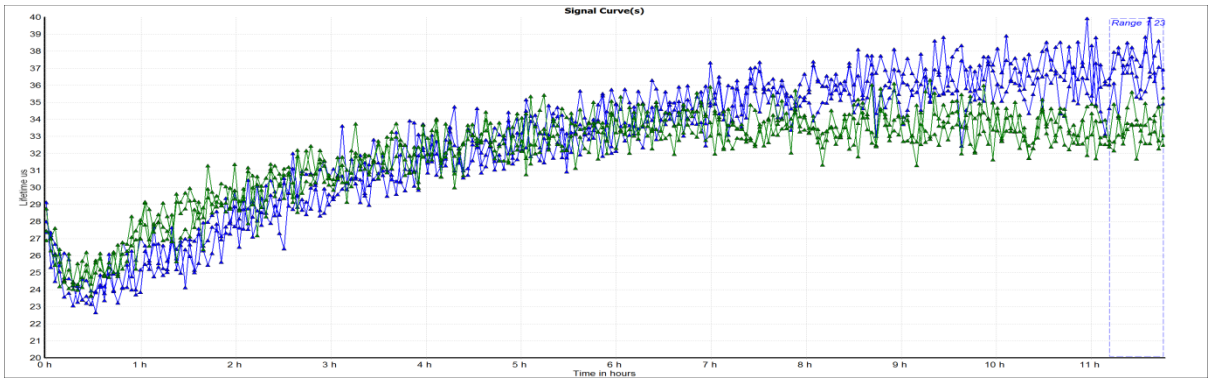

Figure 16: OCR trace of myoblasts on 0mM glucose fresh (blue) or with overnight pre-adaptation (green)

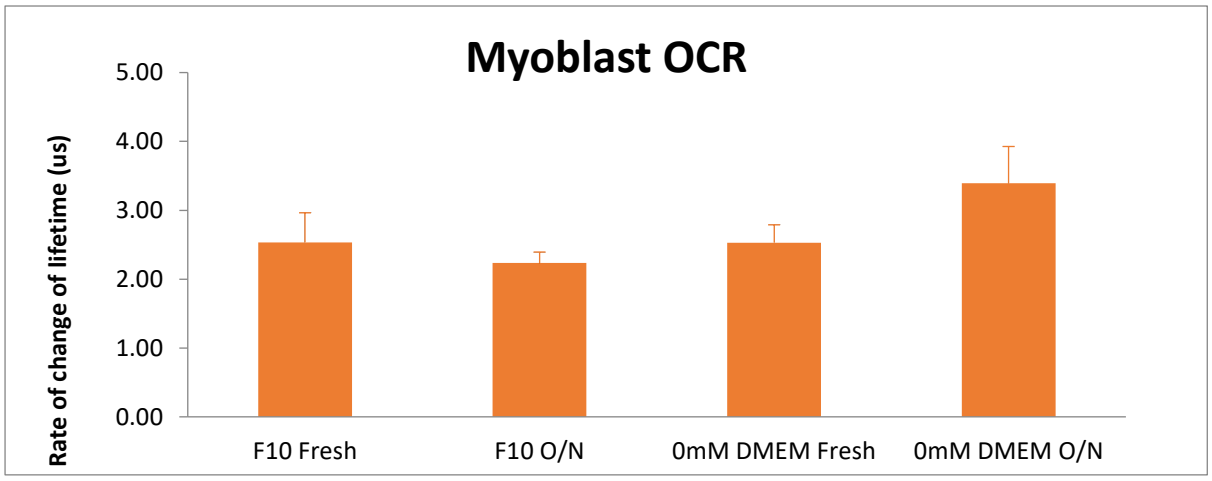

Figure 17: Myoblast OCR was assessed using F10/Hams media (growth media for cells that contains 5mM glucose) and DMEM media (containing 0mM glucose). The cells are quite oxidative and they can be pushed further by pre-adapting the cells to 0mM glucose overnight.

3. Discussion

The aim of this project was to determine the potential utility of using established protocols to assess mitochondrial function in isolated white blood cells. Approaches used by Acumen to isolate white blood cells and assess granulocyte mitochondrial function were investigated using a number of control samples.

We encountered difficulties in reproducing several aspects of these protocols. Running the Histopaque separation protocol to obtain a granulocyte fraction was inconsistent. Yields were low with clear cell banding at the gradient interface infrequently observed. Acumen provided us with an image of a typical separation showing a large granulocyte cell band and a smaller PBMC band from a 2ml sample of blood. To improve recovery we scaled up our separation volumes to allow us to use 5ml blood. However, this still did not provide us with a granulocyte fraction that compared well to the protocol run at Acumen. Why variability in obtaining a consistent granulocyte fraction remains unclear. When granulocytes were isolated in sufficient numbers to be tested in our OCR and ATP assays greater levels of variability were observed than for the PBMC fractions.

PBMC isolation using the Histopaque gradient consistently produced high yields of cells. Mitochondrial respiration in the PBMCs consistently contributed between 25-37% of cellular ATP production and showed evidence of a further increase when cells were activated. The mitochondrial contribution to cellular ATP production in granulocytes was lower at 11-14% with a much larger degree of variability within individuals in a longitudinal study and between controls. Comparing total ATP levels between the same individuals on different days was affected by reproducibility problems when running ATP standards. The contribution of the mitochondria to total ATP production on a particular sample could be determined and showed good reproducibility when PBMC's from individuals were compared over multiple samples. The same was true for mitochondrial oxygen consumption. As concentrations of ATP levels appeared to vary with different calibration curves when using a quality control sample it would be difficult with the current set up to clearly define an ATP control range. This aspect would need to be improved if a mitochondrial/ total ATP levels were to be used as a diagnostic test with ME/CFS patients compared to a control series. Granulocytes when isolated showed a much higher person to person variation in mitochondrial ATP levels and this was only slightly improved by normalising to the total ATP levels. This is likely influenced by the low levels of mitochondrial respiration and ATP

found in granulocytes banding in the Histopaque gradient. As our early studies indicated that only a subset of granulocytes form a band in the Histopaque gradient it may be possible that we are only looking at a subset of the total granulocyte fraction on the Histopaque gradient.

Since completing this project we have learned that in early 2016 Acumen no longer conduct their tests using granulocytes from a Histopaque gradient due to criticisms received regarding the use of neutrophils. A different isolation protocol using the Sigma Accuspin^{TM} 1077 system is now used by Acumen to isolate a PBMC fraction which is used for mitochondrial testing.

PBMC cells in our hands were the more robust cell population showing relatively consistent mitochondrial derived ATP and significant levels of mitochondrial respiration. In order for a test to be clinically applicable it needs to demonstrate consistent results within an individual and not vary over time. From the information we have regarding the Acumen protocol it is unclear whether they obtained their control data over time or if it was a single time point for each control. We ran an 8 week experiment where three controls were bled every fortnight so we could assess any potential individual variability. The results for this 8 week study were consistent in the context of biological variability (% CV values were predominantly <35%). However, in earlier experiments we noticed that ATP results can be greatly affected if the subject had the flu (even before symptoms materialised).

The other key question regarding the Acumen protocol is the suitability of testing samples > 24hrs after phlebotomy. Our early data using flow cytometry to look at individual white cell populations suggests that at 24hrs very few granulocytes form a layer on the Histopaque gradient. Neutrophils, monocytes and lymphocytes are present in the 24hr samples on the Histopaque gradient but have a slightly different granularity on flow cytometry. It is possible that a PBMC fraction isolated from a > 24hr post isolation blood sample could be suitable for testing for mitochondrial function. This would require further validation comparing results from PBMC's isolated 1hr and 24hr after blood isolation.

In summary the PBMC fraction appears to be the most suitable white cell type to assess mitochondrial function in freshly isolated blood samples. Mitochondrial respiration is observed and makes a significant contribution to cellular ATP levels. More straight forward approaches to generate a PBMC fraction are available and these should be explored. Comparing mitochondrial oxygen consumption between samples taken at different times is possible but will require a good standardisation to cell number and a sensitive assay to detect

oxygen consumption rates. Assessing mitochondrial ATP levels is a more sensitive assay but requires further investigations to identify a more robust approach to develop a set of consistent ATP standards. Calculating the percentage of ATP derived from mitochondrial respiration in a sample is quite straight forward and less affected by labile standards.

In the light of recent research by Fluge et al [11] which indicates that carbohydrate metabolism is potentially impaired knowing the percentage contribution of mitochondrial ATP to the total ATP pool could be very important in PBMC's which may reflect systemic effects in ME/CFS patients. From our data, using granulocytes derived from a Histopaque gradient to assess mitochondrial function is something to avoid. Evidence from other studies of freshly isolated granulocytes suggests they rely most heavily on glycolysis to generate ATP. This will likely change once granulocytes are activated by pathogens entering a more energetic phase and potentially switch to a mitochondrial mode of ATP production.

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