Title of Project: **Does oxygen concentration alter platelet interaction with endothelial cell matrix?**

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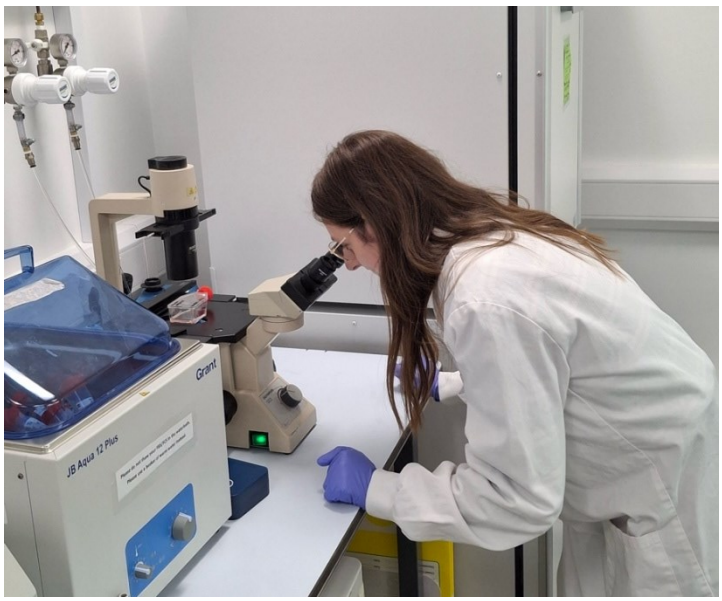
Name of Supervisor: **Dr Eamon Faulkner, Dr Sophie Leonard and Dr Simon Calaminus**

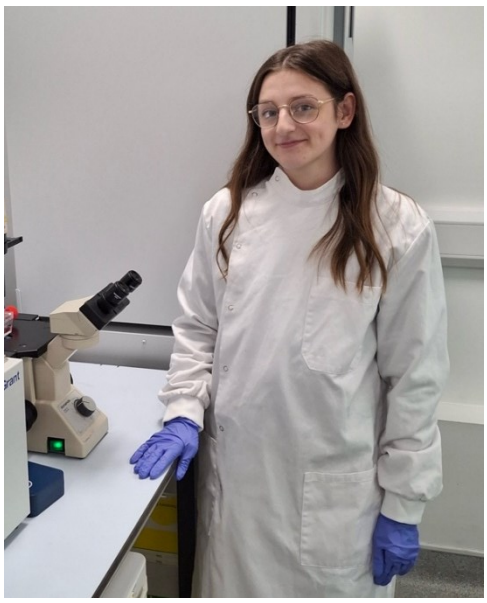
Place where project took place: **University of Hull**

Lay Summary

Blood clots are essential for stopping bleeding after injury, but when they form in the wrong place, they can cause serious conditions such as heart attacks and strokes (British heart foundation, 2020). Platelets are small blood cells that help form these clots by attaching to damaged blood vessels. However, most laboratory studies investigate platelet behaviour under standard culture conditions that do not accurately reflect the environment inside the body.

This summer project aimed to investigate how oxygen (O_2) levels can influence the way platelets interact with the extracellular matrix (ECM) produced by endothelial cells (EC), which line the inside of blood vessels. O_2 concentrations naturally vary throughout the circulation and may affect both the composition of the endothelial matrix and how platelets respond to it (Leonard, s et al, 2025). Understanding these interactions could improve laboratory models of blood clot formation and make them more representative of human physiology. By improving our understanding of how O_2 shapes platelet function and reactions with the ECM, this research could contribute to the development of better models of thrombosis and, ultimately, support future strategies to prevent and treat cardiovascular disease. Techniques such as protein analysis will also allow the identification of molecular changes that occur within the different O_2 environments in the ECM (Byron, a et al, 2013).





What was the aim of your project?

The aim is to determine how O_2 concentration alters endothelial derived ECM composition and platelet responses. HUVEC-derived matrices were compared with fibrinogen and collagen to assess changes in platelet adhesion and spreading, showing an insight into mechanisms influencing platelet behaviour and thrombus formation.

How did you address the aim?

The aim of this project was addressed by investigating the impact of O_2 concentrations on endothelial ECM and the platelet responses. The process of cell culture was practiced many times before the HUVECs were cultured under the different O_2 concentrations (5% and 20%) to allow me to practice the technique and become familiar with it. The platelets used were isolated from healthy volunteers and spreading assays were performed on both matrices such as collagen and fibrinogen and HUVEC-derived ECM, this being practiced many times such as the cell culture to ensure I was familiar with the process. Platelets were then stained using FITC-phalloidin and imaged with fluorescence microscopy to allow quantification through ImageJ to assess characteristics such as surface area, actin nodules, adhesion and stress fibres. Proteomic analysis was also used to compare thrombi formed on HUVECs cultured at 5% and 20% O_2 to identify potential mechanistic differences.

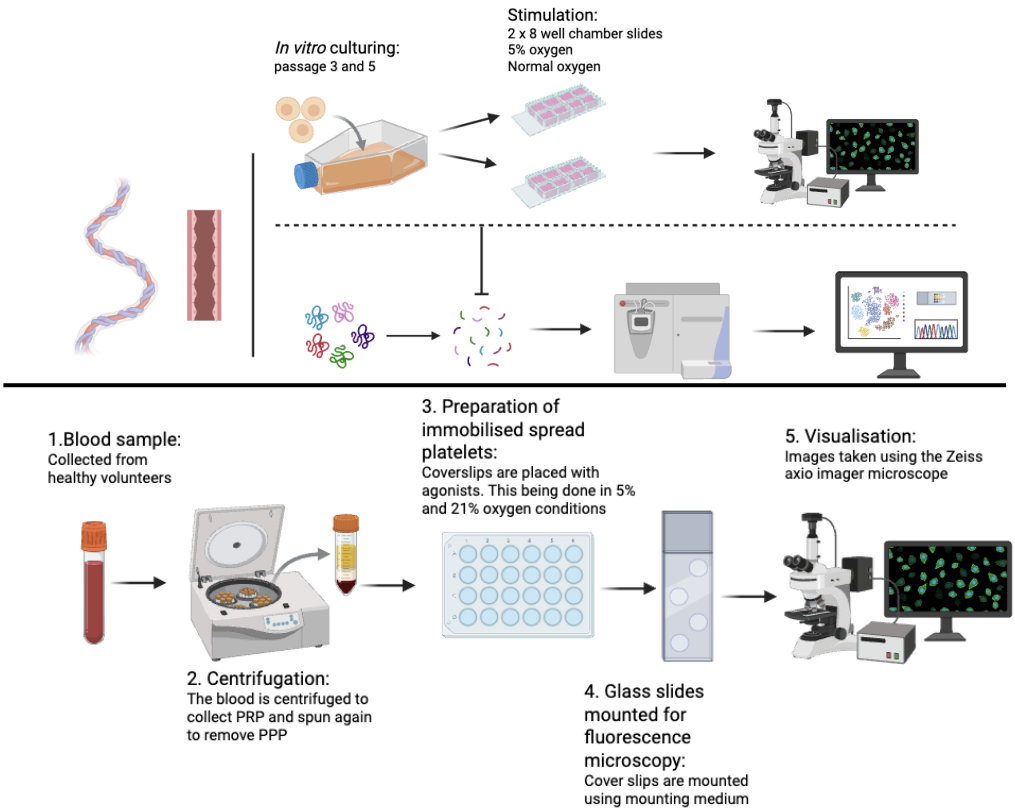


Figure 1. Schematic overview of the experimental workflow used to investigate the effects of O_2 concentration and passage number on human umbilical vein endothelial cells (HUVECs). HUVECs at passages 3 and 5 were cultured *in vitro* and seeded into 8-well chamber slides before exposure to either 5% O_2 or normal O_2 conditions. HUVEC growth, endothelial phenotype and extracellular matrix (ECM) deposition were assessed using fluorescence microscopy using endothelial junction proteins CD31 and CD144 (VE-cadherin), and the ECM component collagen IV used for characterisation. The samples from the different O_2 and passage conditions were then processed for proteomic analysis. Blood was collected from healthy volunteers into acid citrate dextrose (ACD) anticoagulant for the isolation of washed platelets. Coverslips containing the appropriate agonist or extracellular matrix substrate were incubated with washed platelets under **5% O_2 or 21% O_2 conditions**. These conditions enabled comparison of platelet behaviour under physiologically relevant O_2 concentrations and conventional atmospheric O_2 conditions. Following platelet spreading, platelets were fluorescently stained using **FITC-phalloidin** and imaged using a **Zeiss Axio Imager fluorescence microscope**.

What did you find out?

These project findings show O_2 concentration can influence both the endothelial environment and platelet responses, which supports the overall aim of investigating how O_2 affects endothelial-derived ECM composition and platelet behaviour. The results also highlighted that cell passage is an important factor when studying endothelial responses to O_2.

Proteomic analysis of HUVECs cultured under 5% and normal O_2 conditions (**Figure 2**) demonstrated variation in protein expression between the experimental groups. Principal component analysis (PCA) shown in **Figure 3** showed clear separation between passage 3 (P3) and passage 5 (P5) HUVECs, with PC1 accounting for 39% of the total variance. O_2 conditions also showed separation within each passage along PC2, which accounted for a further 19.4% of the variance. Together, PC1 and PC2 explained 58.4% of the variation within the dataset. This suggesting that both O_2 concentration and passage number influence the endothelial proteomic profile, meaning that the effect of O_2 could potentially be dependent on cell passage.

Volcano plot analysis, shown in **Figure 4,** further demonstrated differential protein abundance between the O_2 concentrations at both passages which is supportive of the observation that both O_2 concentration and passage contribute to variation within the proteomic profile.

Further investigation of proteins associated with the endothelial ECM and endothelial function identified variation in the abundance of COL4A1 and COL4A2, which form collagen IV, LAMB2, a component of laminin, and PTGIS, an enzyme involved in prostacyclin production across the experimental conditions (**Figure 5**). Variation in these proteins across the O_2 and passage conditions suggests that changes in the vascular O_2 environment may influence proteins relevant to the matrix encountered by circulating platelets.

Platelet experiments further demonstrated that O_2 concentration influenced platelet behaviour. Platelet adhesion appeared greater under 5% O_2 compared with 21% O_2 as shown within **Figure 6**, with the largest difference observed following 0.01 U/ml thrombin stimulation. However, platelet spreading, measured using surface area, showed a different pattern which is seen in **Figure 7**. Following thrombin stimulation, particularly at 0.01 U/ml, platelet surface area appeared greater under 21% O_2, whereas spreading remained lower under 5% O_2.

Overall, these findings suggest that O_2 does not produce a single uniform effect on platelet activity. Instead, physiologically relevant O_2 conditions were associated with alterations in the endothelial environment while also differentially influencing platelet adhesion and spreading. This therefore demonstrates the importance of O_2 concentration when modelling platelet–vascular interactions *in vitro* and supports the development of more physiologically representative models for investigating thrombus formation.

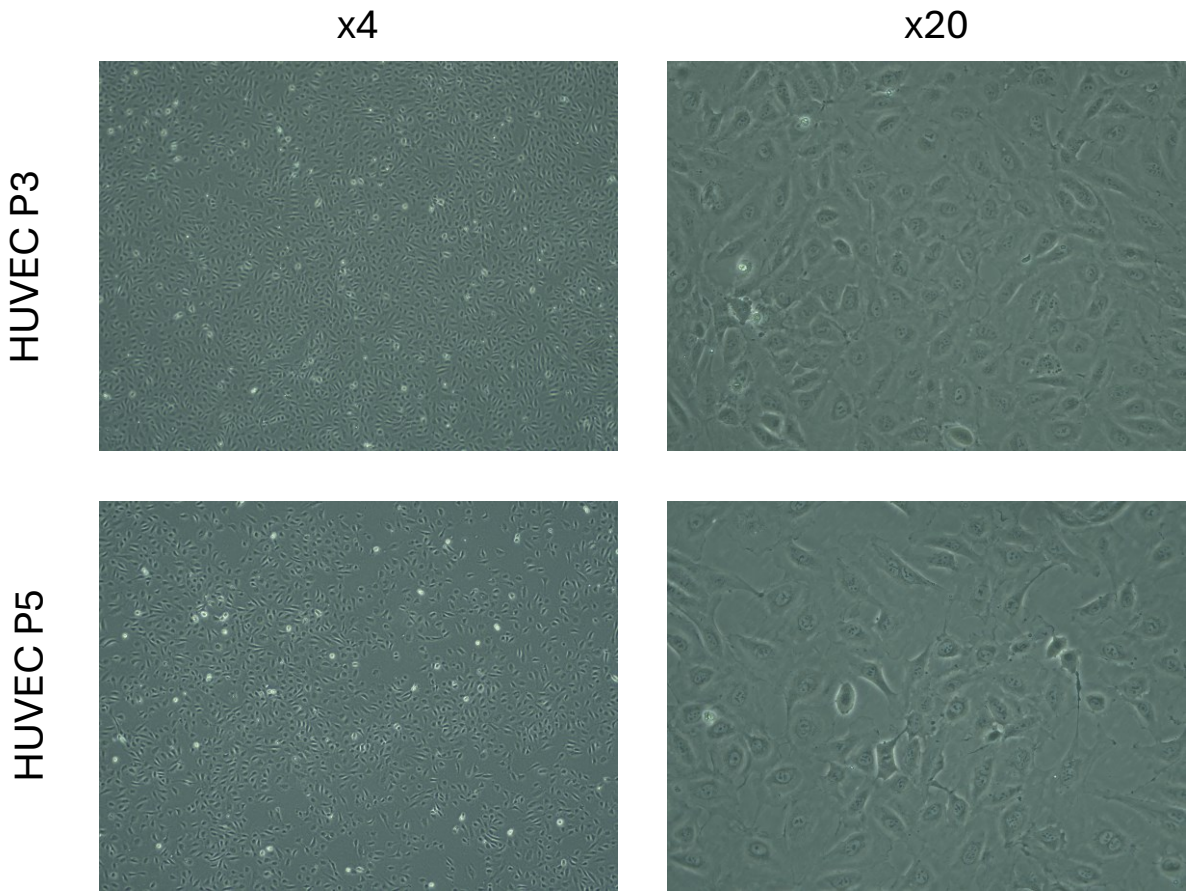


Figure 2. Images of human umbilical vein endothelial cells (HUVECs) at passages 3 and 5. HUVECs were cultured to establish endothelial cell monolayers for characterisation of endothelial-derived extracellular matrix (ECM) and platelet interaction experiments. Representative images show HUVECs at passage 3 (P3; upper panels) and passage 5 (P5; lower panels), captured at ×4 magnification (left) and ×20 magnification (right). The lower-magnification images demonstrate the overall distribution and confluency of the HUVEC cultures, while the higher-magnification images allow closer visualisation of individual cell morphology and cell–cell organisation. HUVEC culture formed part of the development of a physiologically relevant endothelial ECM model used to investigate the effects of O_2 concentration on ECM composition and subsequent platelet responses.

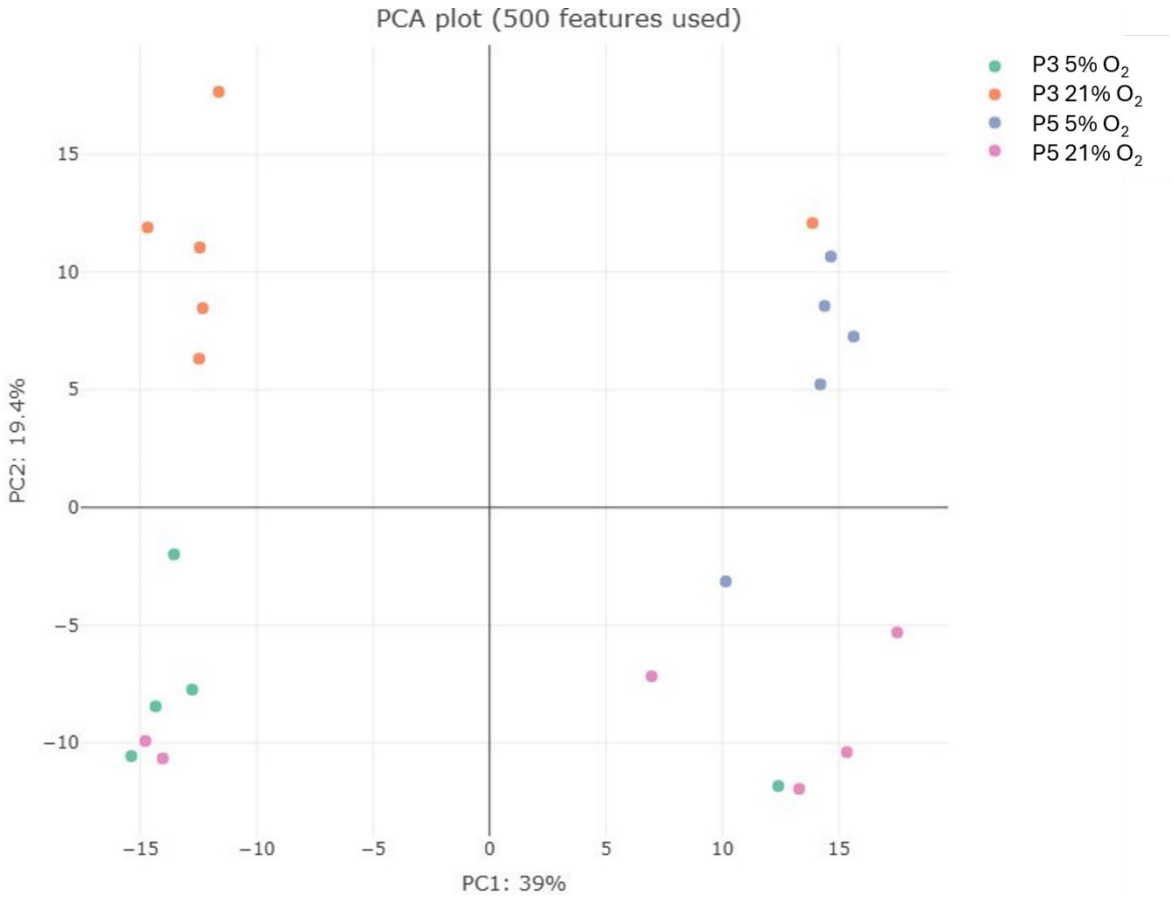


Figure 3. Principal component analysis (PCA) of proteomic profiles in P3 and P5 cells cultured under 5% and normal O_2 conditions. PCA was performed using 500 protein features to assess variation in global protein expression between experimental groups. Each point representing an individual sample coloured according to passage number and O_2 condition: P3 5% O_2 (green), P3 normal O_2 (orange), P5 5% O_2 (blue), and P5 normal O_2 (pink). Principal component 1 (PC1) accounted for 39% of the total variance and showed clear separation between P3 and P5 samples, indicating that cell passage was a major source of variation within the proteomic dataset. Principal component 2 (PC2) accounted for a further 19.4% of the variance and demonstrated separation between O_2 conditions within each passage. The differing distribution of the O_2 conditions between P3 and P5 suggests that the effect of O_2 concentration on the proteomic profile may be dependent on cell passage. Together, PC1 and PC2 accounted for 58.4% of the total variance observed within the dataset.

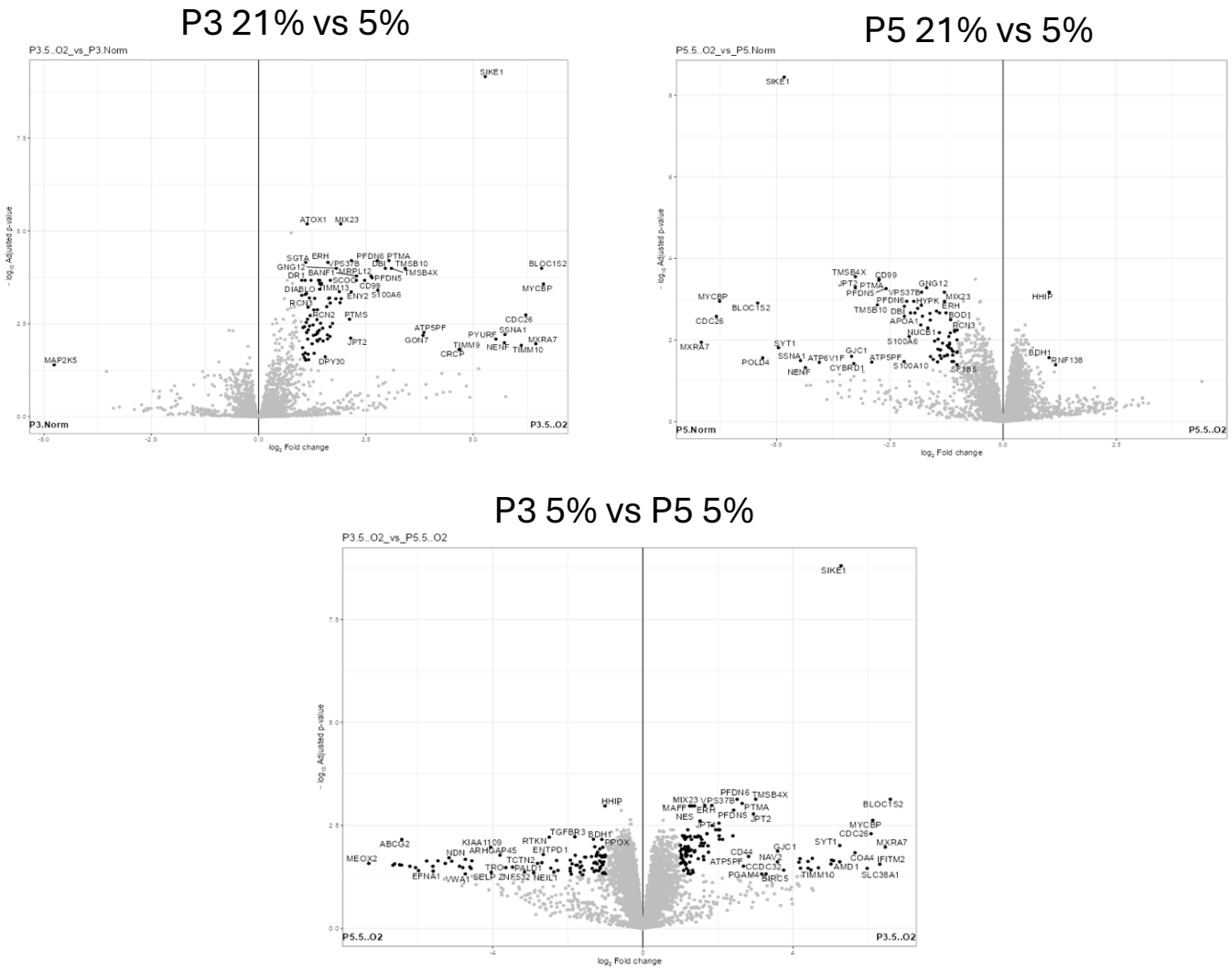


Figure 4. Differential protein abundance in HUVECs according to O_2 concentration and passage number. Volcano plots showing differential protein abundance between HUVECs cultured under physiological (5% O_2) and normal (21% O_2) O_2 conditions and between passage 3 (P3) and passage 5 (P5) cells. (A) P3 HUVECs cultured at 21% O_2 compared with P3 HUVECs cultured at 5% O_2. (B) P5 HUVECs cultured at 21% O_2 compared with P5 HUVECs cultured at 5% O_2. (C) P3 HUVECs compared with P5 HUVECs when cultured at 5% O_2. The x-axis represents $\log_2$ fold change in protein abundance, with the direction of enrichment indicated at either side of each plot, while the y-axis represents $-\log_{10}$ adjusted *p*-value, with increasing values indicating greater statistical significance. Grey points represent the wider detected proteome, while highlighted black points and labelled proteins indicate proteins identified as differentially abundant according to the thresholds applied during analysis. Together, these comparisons were used to assess the effects of O_2 concentration and HUVEC passage number on the endothelial proteomic profile.

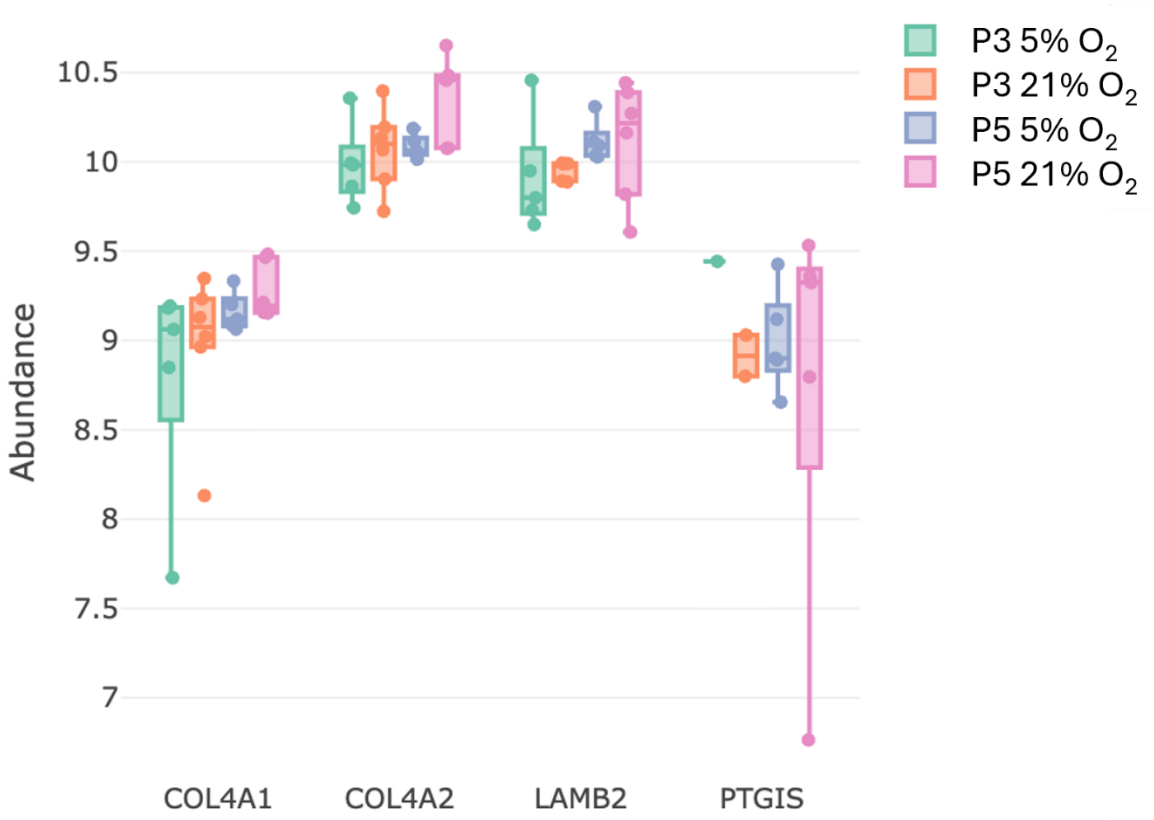


Figure 5. Abundance of selected extracellular matrix and endothelial-associated proteins in HUVECs cultured under different O_2 concentrations and passage numbers. Box and whisker plots show the relative protein abundance of COL4A1 (collagen type IV alpha 1 chain), COL4A2 (collagen type IV alpha 2 chain), LAMB2 (laminin subunit beta 2), and PTGIS (prostacyclin synthase) identified by proteomic analysis. Protein abundance is shown for passage 3 (P3) and passage 5 (P5) HUVECs cultured under 5% O_2 (P3 5% O_2 and P5 5% O_2) or normal O_2 conditions (P3 21% O_2 and P5 21% O_2). Individual points represent replicate measurements, while boxes illustrate the distribution of protein abundance within each experimental condition. The selected proteins were examined to assess whether O_2 concentration and cellular passage influence proteins associated with the endothelial extracellular matrix and endothelial function.

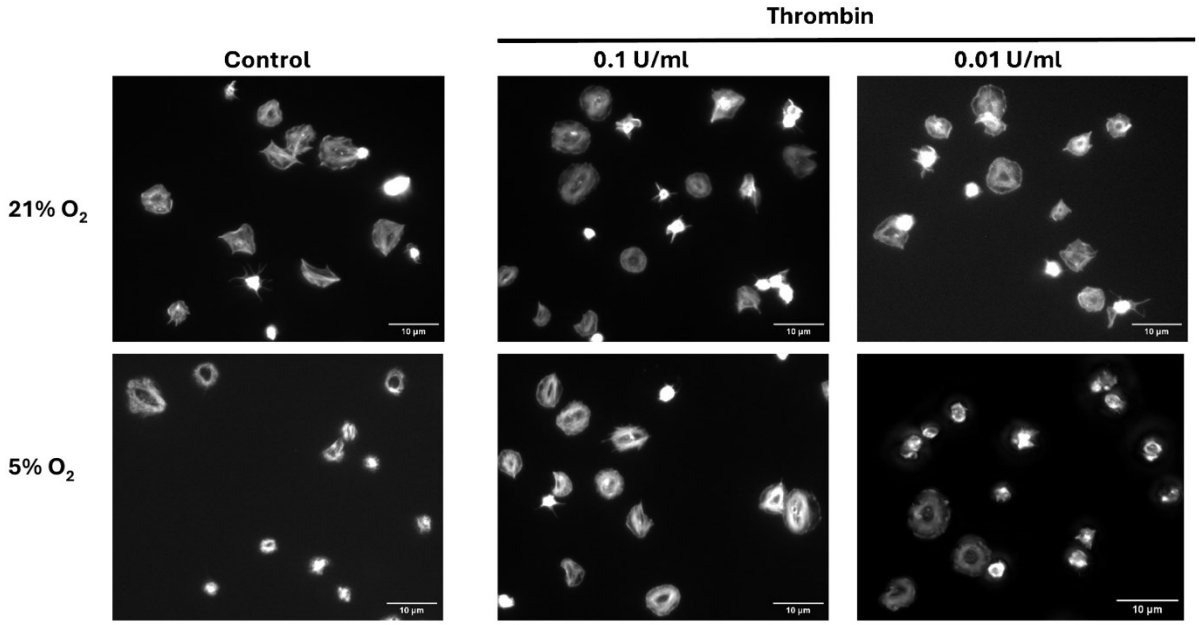


Figure 6. Representative fluorescence microscopy images of platelet adhesion and spreading under different O_2 concentrations and thrombin stimulation conditions. Platelets isolated from a healthy donor, incubated under 5% O_2 or 21% O_2 (normal atmospheric O_2 concentration) conditions and assessed under three stimulation conditions: unstimulated control, 0.1U/ml thrombin, and 0.01U/ml thrombin. Platelets were fluorescently labelled and visualised by fluorescence microscopy. Images are presented for the donor under both O_2 conditions to demonstrate the platelet responses used for subsequent quantification of platelet adhesion and platelet surface area.

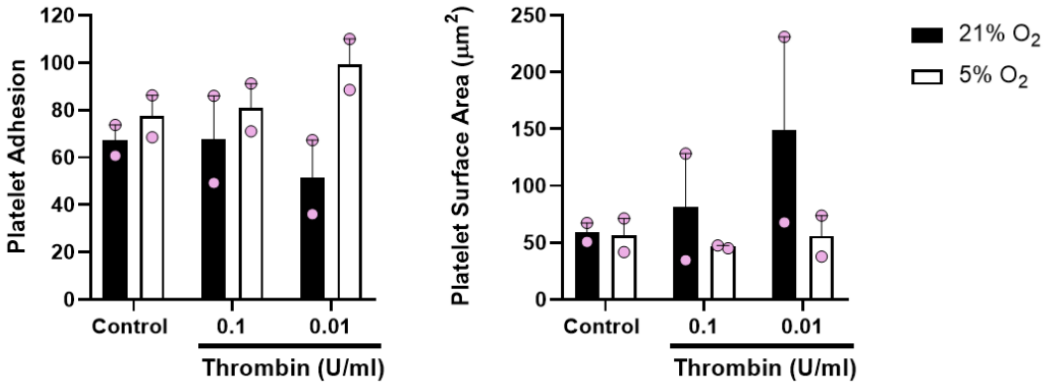


Figure 7: Effect of O_2 concentration and thrombin stimulation on platelet adhesion and spreading. Platelets were assessed under 21% O_2 (black bars) and 5% O_2 (white bars) conditions, either without thrombin stimulation or following stimulation with 0.1 U/ml or 0.01 U/ml thrombin. (Left) Platelet adhesion under each O_2 and thrombin condition. (Right) Platelet surface area (μm^2), used as a measure of platelet spreading, under the corresponding conditions. Bars represent the mean ± standard error (SE), with individual pink points representing replicate measurements.

What did you learn from participating in this project?

Participating in this project gave me a much greater understanding of platelet biology and how platelet behaviour can be influenced by the vascular environment. I particularly enjoyed developing my practical laboratory skills and seeing how different experimental techniques could be combined to

answer a clinically relevant research question. Throughout the project, I developed experience in endothelial cell culture, platelet isolation and spreading assays, immunofluorescence, fluorescence microscopy and ImageJ analysis. I also gained experience analysing complex proteomic datasets, which developed my confidence in handling and interpreting large amounts of biological data.

Most importantly, the project taught me that platelet activation is highly dependent on the environment they encounter. It improved my understanding of how factors such as O_2 concentration and the endothelial extracellular matrix can influence platelet adhesion, spreading and thrombus formation. The project also strengthened my interest in platelet and cardiovascular research and has encouraged me to continue developing my research skills.

How has this project affected your long-term goals?

This project has significantly impacted my long-term goals as it has solidified that I want to continue working within research. Before starting this project, I was interested in platelet biology and cardiovascular diseases but being directly involved within the laboratory research has increased my interest particularly in platelet interactions with endothelial cells and the vascular environment. Following from this project I plan to complete a masters by research so I can build upon the work I have already completed by further investigating platelet and endothelial interactions. This will allow me to further enhance my laboratory skills and become more independent as a researcher. My longer-term goal is to progress from the master's degree to a PhD to continue working within platelet and cardiovascular research with an ultimate plan of contributing to research that improves the understanding thrombosis and has the potential to influence future clinical practice.

References

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