University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme
Summer Studentship
Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk within four weeks of the end of the studentship.

1. Student

   Surname: Salim Forename: Ehsan

   E-mail address: 2063847s@student.gla.ac.uk

2. Supervisor:

   Surname: Johnstone Forename: Scott

   E-mail address: Scott.Johnstone@glasgow.ac.uk

3. Research Project Report

3.1 Project Title (maximum 20 words):

Pannexin 1 as a regulator of cardiovascular disease

3.2 Project Lay Summary (copied from application):

Lipid accumulation in the walls of blood vessels results in a chronic inflammatory disease termed atherosclerosis. Inflammatory cells respond to signals released by the cells of the blood vessels in response to the lipid accumulation. Adenosine triphosphate (ATP) is increasingly recognized as a signaling molecule which promotes the recruitment of inflammatory cells. We have identified that an ATP release channel, pannexin 1, is activated in response to inflammatory signals and may therefore be important in recruiting inflammatory cells in disease. In this project, changes in the protein expression and relevance to disease will be detected in blood vessels from mice.

3.3 Start Date: 6 June 2016 Finish Date: 18 July 2016

3.4 Original project aims and objectives (100 words max):

It is hypothesized that Panx1 ATP release is critical in vascular inflammation leading to atherosclerosis. To investigate this effect, we aim to compare the levels of disease in blood vessels from wild type mice and mice with endothelial specific knockout of Panx1.

3.5 Methodology: Summarise and include reference to training received in research methods etc. (250 words max):

Samples: All tissue samples from EC-specific, tamoxifen-inducible, Panx1-knockout mice on the ApoE-/- background ($VECadER^{T2+/}Panx1^{fl/fl}/ApoE^{-/-}$) fed a high fat diet for 12 weeks with the relevant controls will be harvested in advance of the project start date and paraffin embedded for imaging or tissues stored in lysis buffer for protein analysis. Samples will be provided as a n=12 for each genotype and condition

Immunofluorescence: Using tissues in paraffin blocks Ehsan will be shown how to section the tissue blocks and will follow established protocols to de-paraffin sections, perform histochemical staining, detect proteins with primary and secondary antibodies and visualize on a Zeiss 510 confocal microscope. Images generated will be used to report on levels of disease and expression and cellular localization of the proteins.

Western Blotting: To investigate the levels of protein expression in various tissues including vascular tissues, Western blotting of tissue samples will be used to detect expression of Panx1, cytokines and other proteins under each of the conditions. Using well-established protocols for Western blotting and we will quantify the amounts of protein expressed using Licor Odyssey scanner and software.

ATP assay: To quantify the release of ATP from cells via Panx1 channels cells will be scanned with a Light Spectrometer after adding an ATP reacting reagent.

Monocyte adhesion: To quantify the adhesion of monocyte to human vascular endothelial cells due to the effects of Panx1 channels, cells will be treated and then washed before being passed through a flow cytometer to quantify the proportional presence on monocytes in the sample.

Reporting: Using an n=12 Ehsan will be shown how to quantitate the data and determine statistical significance between the samples.

3.6 Results: Summarise key findings (300 words max). Please include any relevant tables or images as an appendix to this report:

Samples: human carotid endarterectomy. Muse Brachiocephalic artery, Aortic sinus and Aortic arch. Identify plaques and regions of disease. Specific regions can be used as clear reference points for assessment and data collections during research with animal models. Also the staining of the tissue demonstrates that clear conclusions can be drawn with regards to the presence of absence of plaques. In addition to this Health and ApoE KO mice sections can be compared to give significant results (with enough samples). Further on Panx1 KO mice can also be used to compare.

Immunofluorescence: Expression levels of Panx1 channels in humans increases in disease, the same is seen in animal models. Also the analysis of mouse models with Apo-E KO shows an increased presence of plaques as well as allowing us to compare to human models. This allows us to evaluate how accurately the animal models can represent the pathology in humans. Immunofluorescence can also be used to check the effect of various drugs, gene Knock-outs and diet models on Panx1 channels expression.

Western blotting: Using this technique we were able to establish that TNF does infact upregulate Pannexin1 channels in the cells. In treating the cells with different concentrations of TNF for different durations of time we demonstrated that Pannexin1 channels increase several fold both intracellularly and on the cell membrane. We also used this technique to show that various pathway inhibitors can be used to inhibit the upregulation of Pannexin1 channels in response to TNF in the cell. This helps us to try and document the intracellular pathway of secondary messengers that is utilised in the upregulation of Panx1 channels.

ATP assay: We were able to demonstrate that SiRNA is able to down regulate Panx1 channel expression even in TNF treated cells. With this confirmed we went on to doing an ATP assay and showed that there was a significant reduction in release of ATP by cells treated with SiRNA-Panx1 which shows that Panx1 is a primary ATP channel on the cell surface.

Monocyte adhesion flowcytometry: Using this new method of quantification of monocyte adhesion we were able to establish that when Panx1 channels are upregulated (via TNF) there is an increase in the number of Monocyte that adhere to the Human Vascular Endothelial Cells(HUVECs). Monocyte migration is a key event in the pathophysiology of Arthrosclerosis, making this a significant result as it demonstrates that blocking the production of Panx1 channels decreases the number of monocyte that adhere to HUVECs.

3.7 Discussion (500 words max):

4. Reflection by the student on the experience and value of the studentship (300 words max):

Over the short time that I was on this Studentship I have learned a vast amount about science, research, statistics and the general workings of a lab. This placement has given me a fantastic insight into what goes into the publishing of new work.
This placement has also given me the opportunity to practice and perfect essential lab techniques such as embedding and sectioning of tissue, cell treatment, preforming a western blot, cell staining, use of instruments/equipment/machines and much more.
Initially I faced many challenges as it was hard to grasp many of the methods and terminology used in the lab environment but with the support of my supervisor – Dr Scott Johnstone – and others I was able to learn and accomplish my aims.
With regards to the project that I was undertaking, at the start there was a lot of guidance to follow but as I went deeper into the topic I saw that there was still a lot more yet to be discovered. This really fascinated me as it gave me the opening to ask questions and make suggestions and become more inquisitive which is an essential part of being a scientist.
Over these 6 week I have become familiar and comfortable with the day to day working in a research environment. Most importantly I have built foundational understanding and confidence which I will carry with me for the remainder of my career to help me reach my full potential.

5. Dissemination: (note any presentations/publications submitted/planned from the work):

6. Signatures:

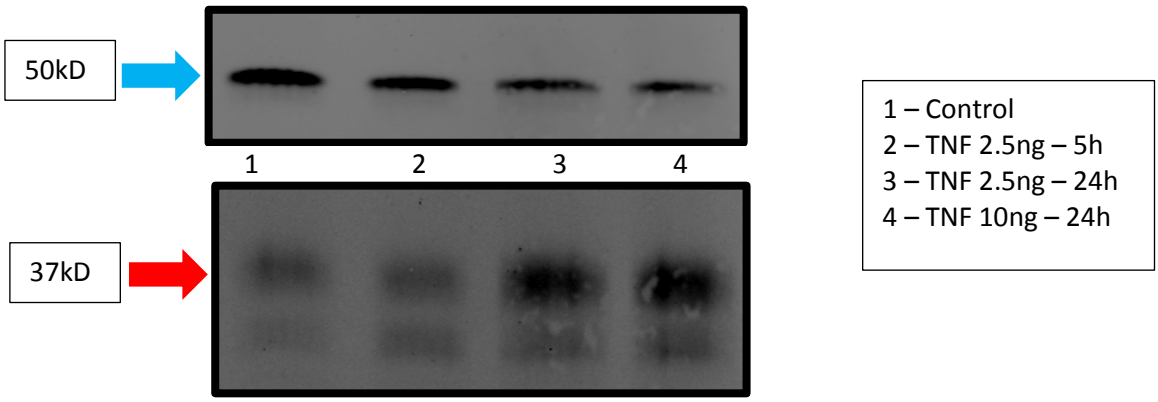
Supervisor:  Date: 19/7/2016

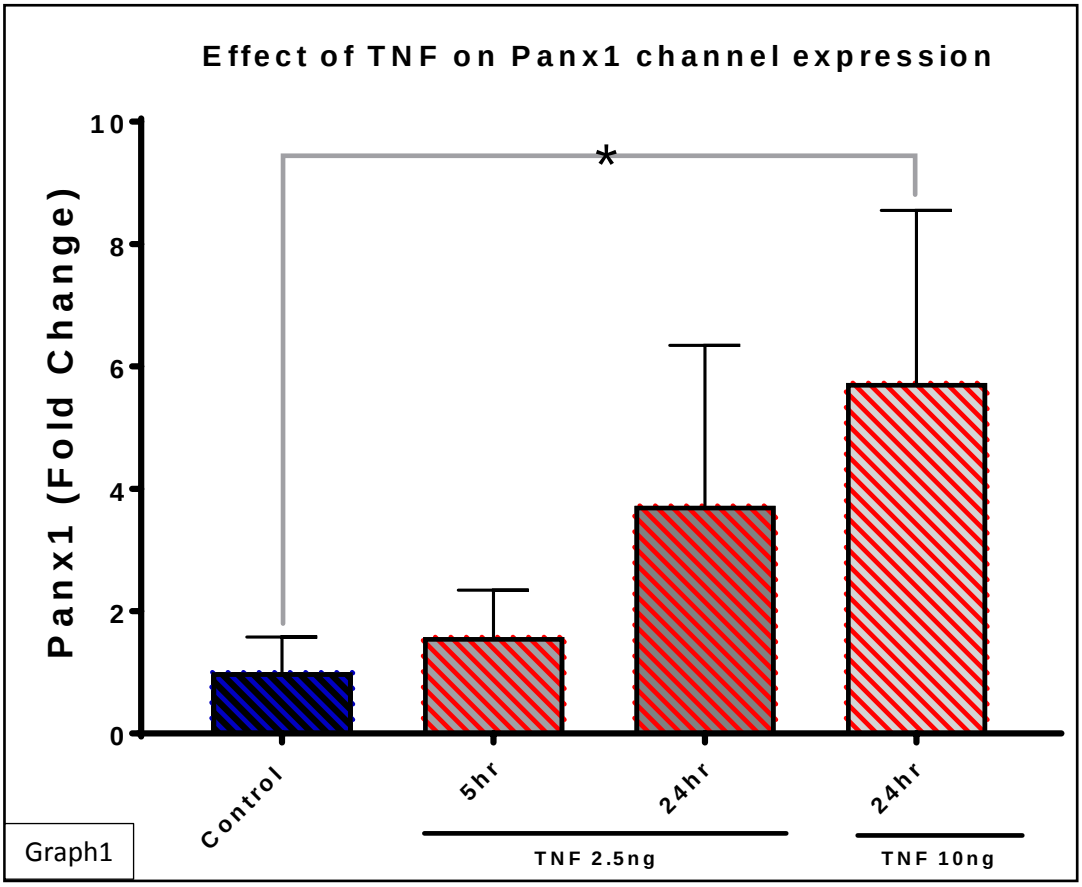
Student:  Date: 19/7/2016

Western Blot – Human Vascular Endothelial Cells – Cell culture 12 well plate
Looking at effect of TNF on the expression of Panx1 both intracellular and at cell membrane

Experiment was done to n=3

Results



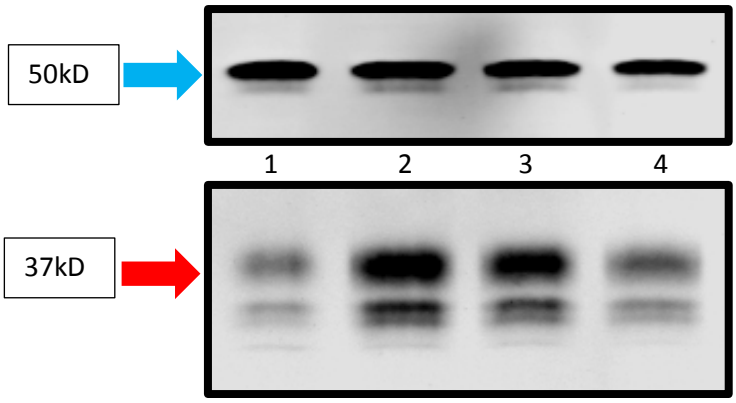


Appendix 1. Western blot of Human Vascular Endothelial cells. Treated with TNF at different concentrations and for different time durations. Experiment was preformed to n=3. The graph analyses the data to demonstrate the fold increase of Pannexin1 channels at different treatments. * represents significance.

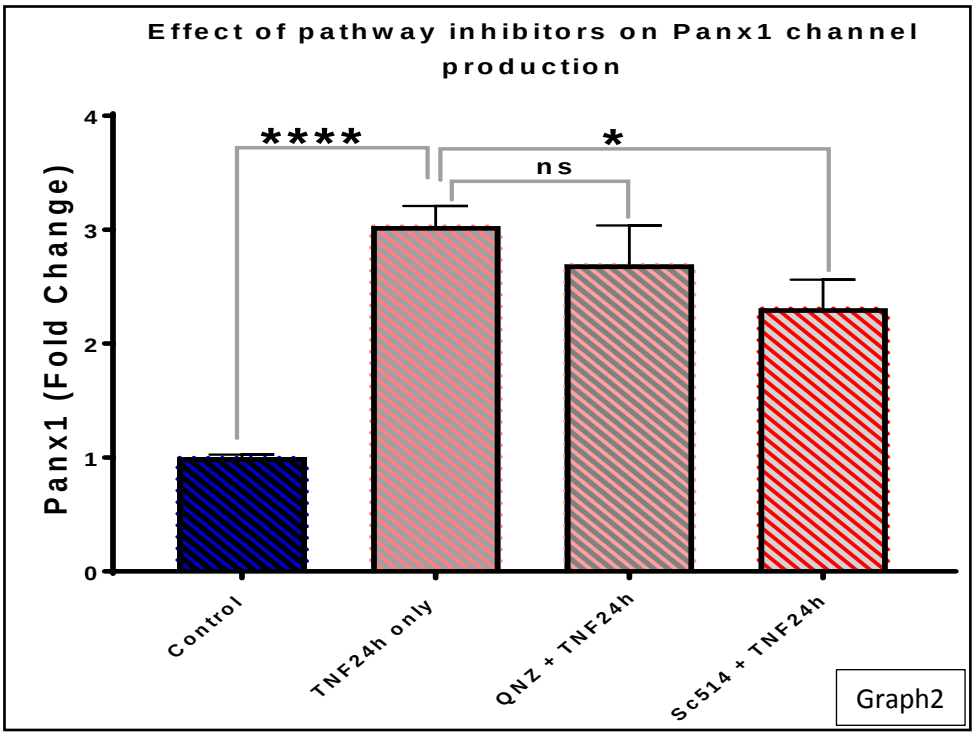
Western Blot – Human Vascular Endothelial Cells – Cell culture 12 well plate

Looking at effect of NFkB inhibitors on the on the expression of Panx1 channels both intracellular and at the cell membrane

Results



1 – Control
2 – TNF 2.5ng – 5h
3 – TNF 2.5ng – 24h
4 – TNF 10ng – 24h



Appendix 2. Western blot of Human Vascular Endothelial cells. Treated with TNF after treatment with pathway inhibitors for 1 hour. The inhibitors used were mainly NFkB pathway inhibitors. The experiment was preformed to n=3. The graph analyses the data to demonstrate the fold decrease of Pannexin1 channels with different inhibitors. * represents significance.

ATP assay – Human Vascular Endothelial Cells – Cell culture 96 well plate

Samples for graph 1:

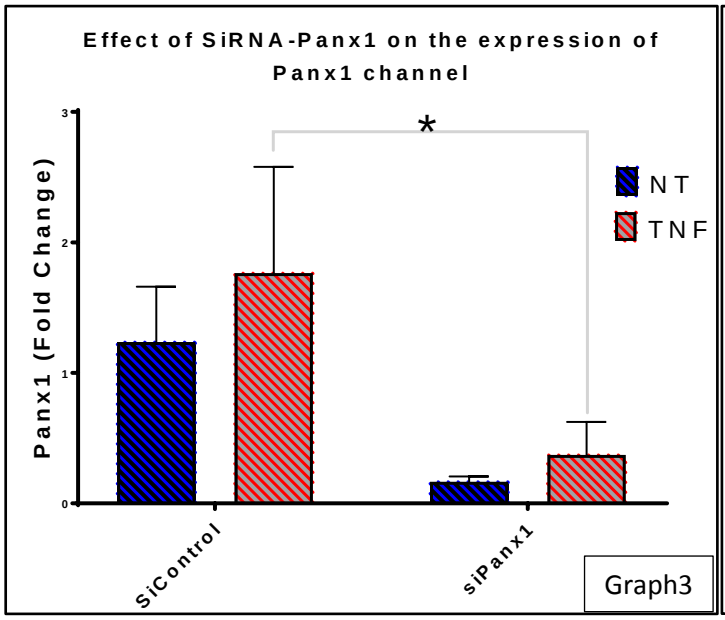
1. SiControl – Cell pretreated with SiScrambled followed by +/- TNF (NT = Not treated with TNF)
2. SiPanx1 – Cells pretreated with SiPanx1 followed by +/- TNF (NT = Not treated with TNF)

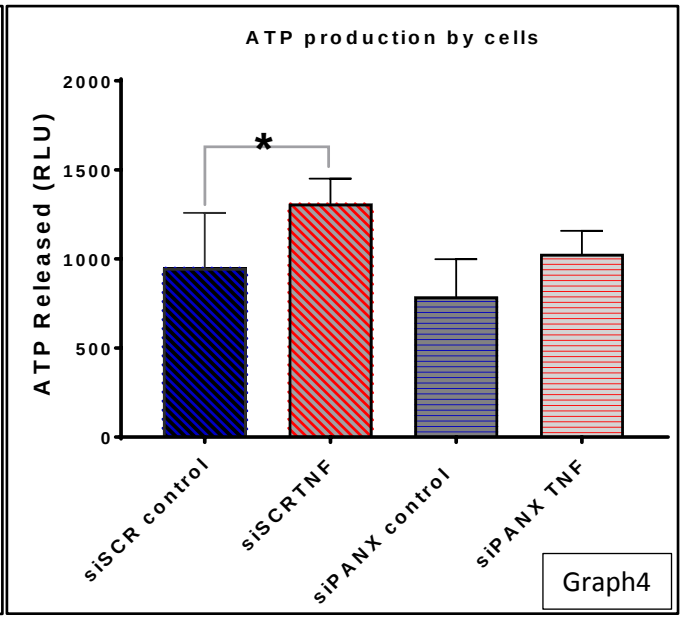
Samples for graph 4:

1. Control – Cells treated with only ARL (ATPase inhibitor)
2. TNF only – Cells treated with ARL and TNF
3. Carbenoxolone – Cells treated with ARL and Carbenoxolone
4. TNF and Carbenoxolone – Cells treated with ARL and Carbenoxolone and TNF

50 of media plus 50 Cell titer glo 2.0 promega. -> plate reader.
Experiment was done to n=6

Results





	ATP released (RLU)											
Treatments	SiScr						SiPanx1					
Control	1270	980	1340	600	860	650	980	1070	850	590	670	570
TNF only	1530	1320	1270	1390	1230	1120	1170	1190	1050	930	950	880
Cb Only	1270	1820	1200	1570	1540	1860	1870	1200	1050	1800	1150	2610
TNF + Cb	1650	1260	1560	1440	880	830	1720	1680	1440	1780	1490	1200

Appendix 3. Graph3 shows effect on expression of Panx1 channels after treatment with SiPanx1 – evidence that SiPanx1 can prevent upregulation of Panx1 channels. In Graph4 ATP assay preformed on Human Vascular endothelial cells. Cells were pre-treated with Si Scambled (SiScr) or Si Pannexin1 (SiPanx1). SiPanx1 was used to inhibit the production of Pannexin1 channels. 32 hours following SiRNA treatment cells were all treated with ARL (ATPase inhibitor) +/- Carbenoxolone +/- TNF. Media separated from the cells was reacted with ATP bioluminescence dye. The experiment was preformed to n=6. The plate was scanned using a Luminometer. Left graph represents different treatments – the left bar of each set represents the SiScr and the right bar represents the SiPanx1. * represents significance.

Monocyte adhesion flowcytometry – Human Vascular Endothelial Cells – Cell culture 12 well plate

Samples:

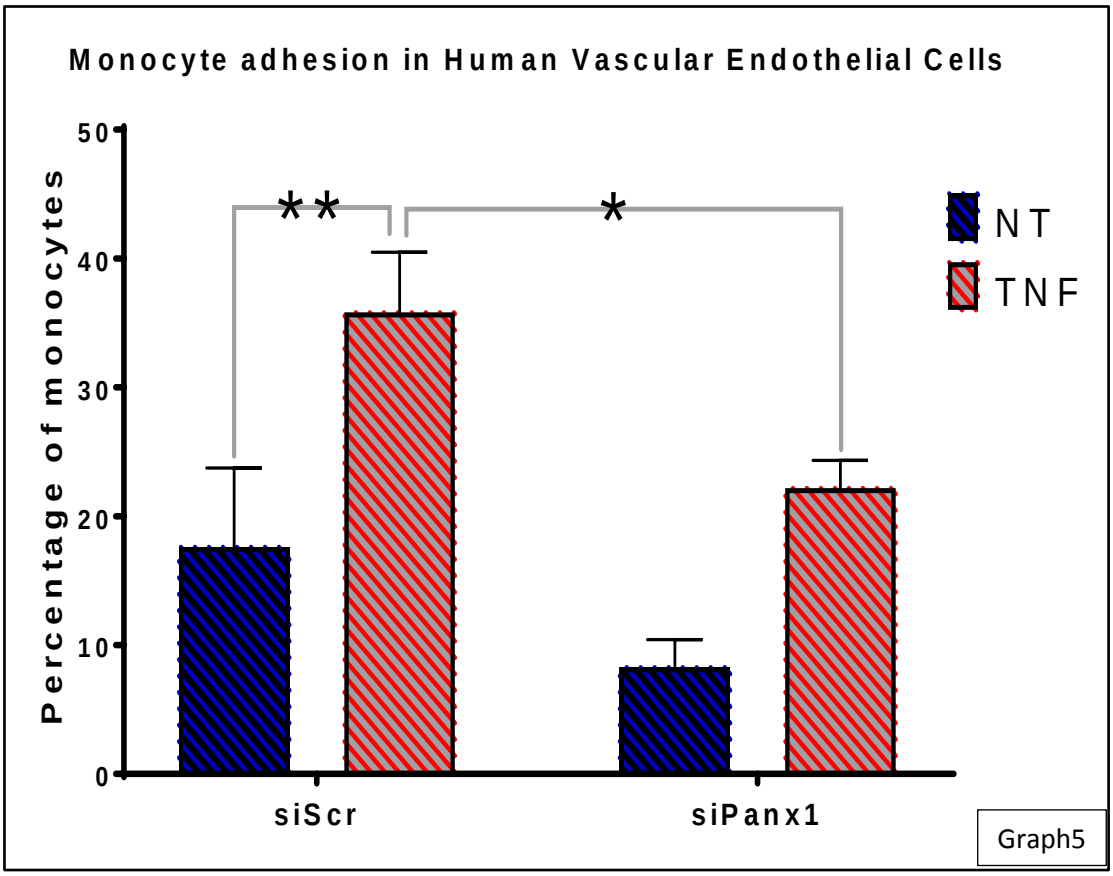
1. SiScr – Not treated (NT)
2. SiScr – TNF 2.5ng
3. SiPanx1 – Not treated
4. SiPanx1 – TNF 2.5ng

Addition of Monocytes at $1x10^5$ per well after treatment with Calcine Green.

Experiment done to n=3

Results

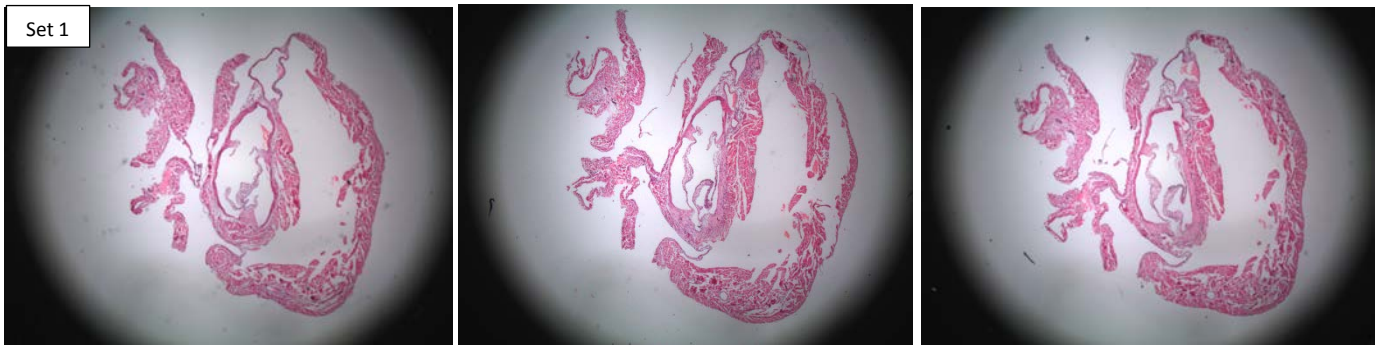
	Percentage of 10000 cells that were monocytes					
Treatments	NT			TNF		
siScr	15.1	13.1	24.6	30.8	36.5	40.1
siPanx1	6.2	8.1	10.5	24.2	19.9	22.4



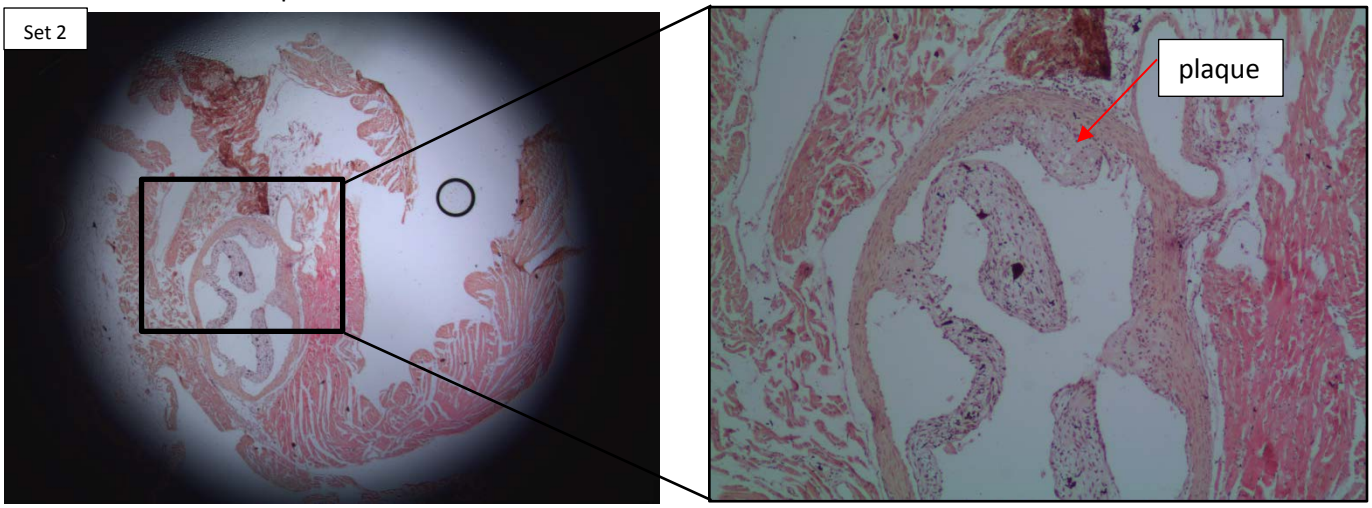
Appendix 4. Monocyte adhesion to Human Vascular Endothelial cells. Cells were pre-treated with Si Scambled (SiScr) or Si Pannexin1 (SiPanx1). SiPanx1 was used to inhibit the production of Pannexin1 channels. 32 hours following SiRNA cells were treated with TNF or left untreated. The experiment was done to n=3. Monocytes which were pre-treated with Calcine Green dye were added to the cells and left for 5 hours. The cells were washed to remove free monocytes. The content of the well was then collected. Samples were processed by a Flowcytometer until 10000 events were performed. The graph shows the difference in percentage of cells that were monocytes for each treatment. * represents significance.

H & E staining – Mouse arterial blood vessel sections – on microscope slide

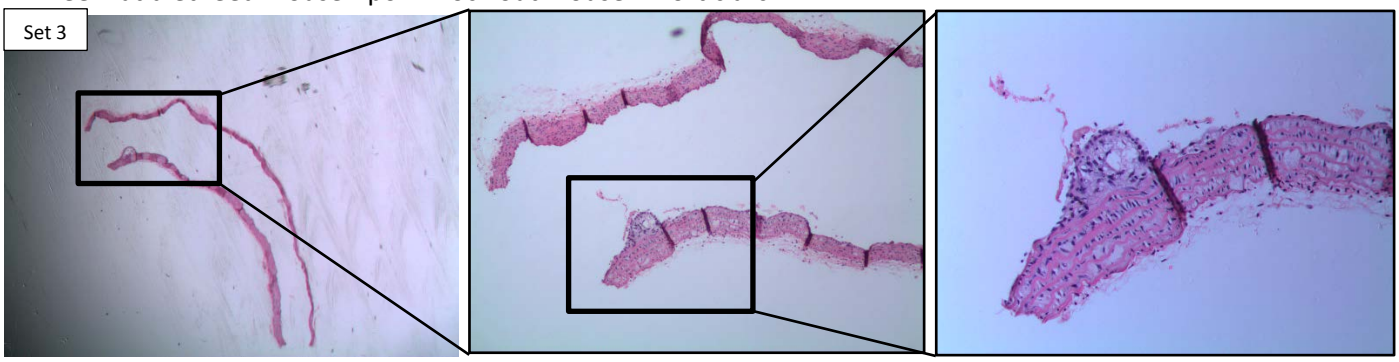
Normal Mouse – Aortic sinus



12 week fat diet feed ApoE knock out mouse – Aortic sinus



12 week fat diet feed mouse ApoE Knock out mouse – Aortic arch



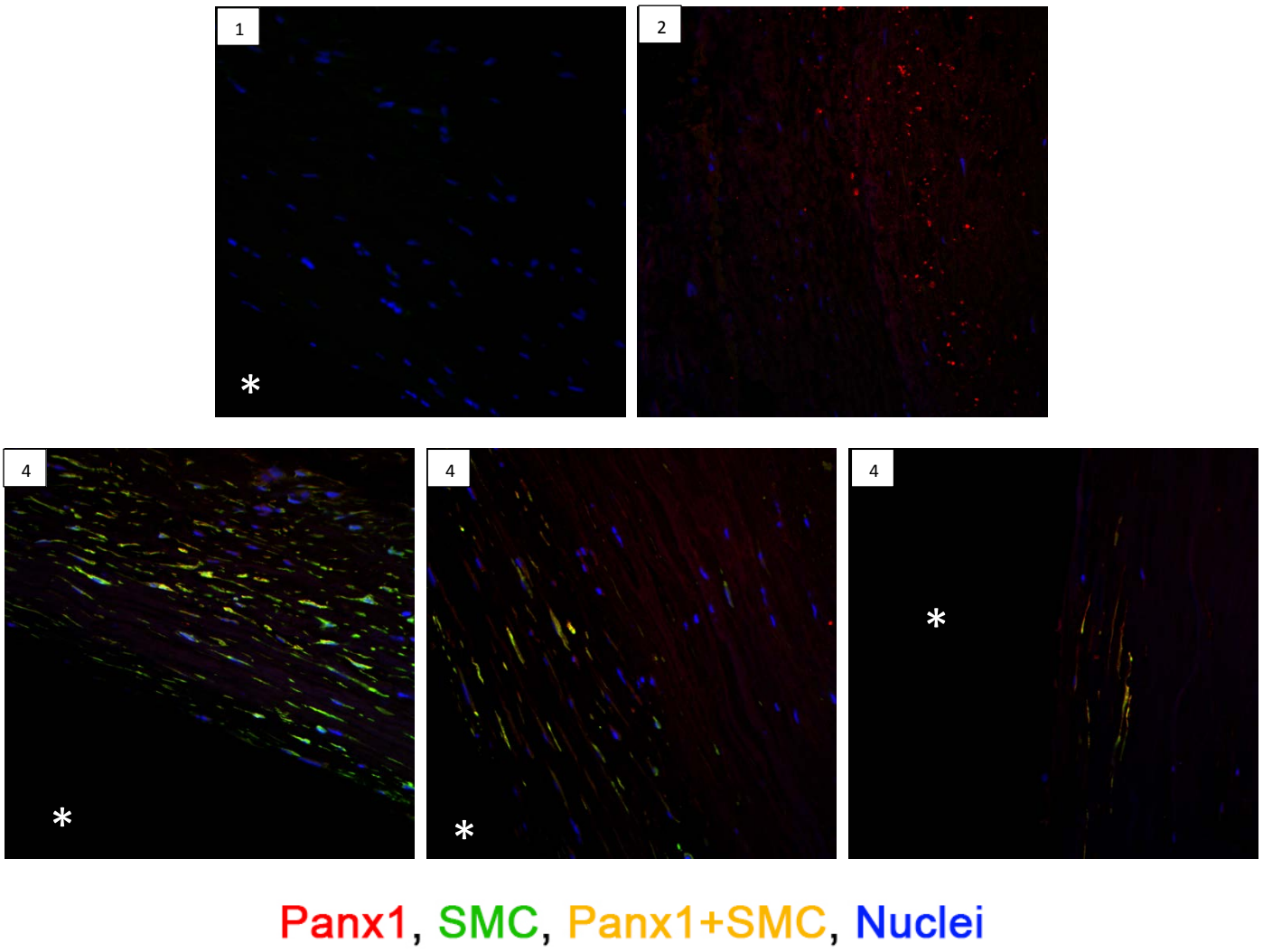
Appendix 5: H&E staining of mouse sections. Set1 shows normal mouse to demonstrate the aortic sinus location used as a reference point in animal research for plaques. Set 2 show a similar section to Set1 of a high fat diet fed mouse with a knock-out(KO) of the ApoE gene (to make it more susceptible to plaques) at the Aortic sinus reference point. Plaque is indicated by arrow. Set 3 shows Aortic arch of another ApoE KO mouse with plaque present (zoomed in), this area can also be used as a reference point for animal research.

Immunofluorescence – Diseased Human Carotid tissue – on microscope slides

Samples – tissue treated with different Primary antibodies and Dapi nuclear stain.

Results
The Endothelial cell antibody did not function properly but the others did

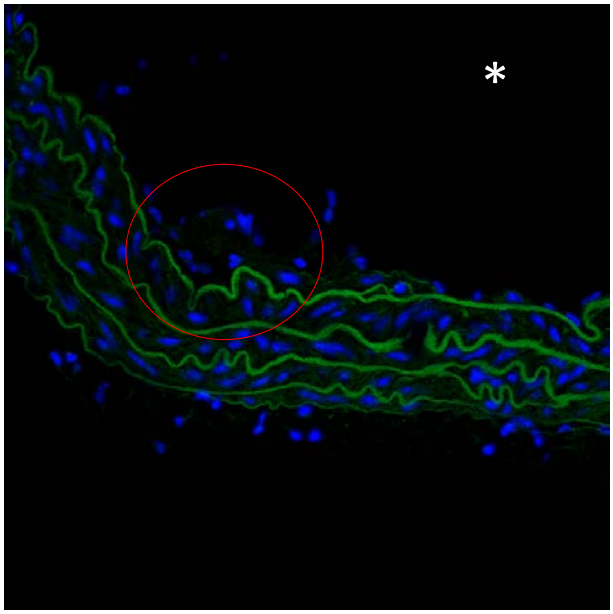
1 = No Primary antibody (only Dapi nucleus stain)
2 = Panx1 only
3 = Panx1 and Endothelial cell antibody
4 = Panx1 and Smooth Muscle cell antibody
5 = Panx1, Endothelial cells and Smooth Muscle cell antibody
* = Lumen of vessel



Appendix 6: Immunofluorescence of diseased Human Carotid Artery. Use of Primary and Secondary Antibodies specific to Panx1, Endothelial cells (EC), Smooth muscle cells/actin (SMC) and nuclei of all cells. Endothelial antibodies failed to fluoresce properly and were not visible on scan. These images demonstrate the spread and distribution of Panx1 channels into the smooth muscle layers of the blood vessel in disease. This indicates role propagation of inflammatory disease as Panx1 channels are normally considered to be mainly present in ECs

Immunofluorescence – Wild type mouse (Cre+ and Peanut Oil) – on microscope slides

Samples – tissue treated with different Primary antibodies and Dapi nuclear stain.
* = Lumen of vessel



SMA, Nuclei

Appendix 7: Immunofluorescence of mouse Carotid Artery. Panx1 stain antibodies failed to work and did not show up on scan. Red Circle highlights area of disturbed endothelial lining with cells organised in bulge – most likely a plaque. This image also highlight significant difference between human and mouse blood vessel structure – human vessel are much more complex with several more layers and different assembly.