The Effect of Apigenin on the Receptor for Advanced Glycation End Products Gene Expression in Human THP-1 Macrophages and Its Implications for Diabetic Nephropathy Related Inflammation

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Statement 1

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed:

Dated: 12th March 2018

Statement 2

This dissertation is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

Signed:

Dated: 12th March 2018

Statement 3

I hereby give consent for my dissertation, if accepted to be available for photocopying and for inter library loan, and for the title and summary to be made available to outside organizations.

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Dated: 12th March 2018
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Type 2 Diabetes Mellitus (T2DM), characterized by hyperglycaemia and insulin resistance, becomes a major cause of premature mortality worldwide. The increased formation of Advanced Glycation End-products (AGEs) during the hyperglycaemic state, and their interaction with Receptors for Advanced Glycation End-products (RAGEs) lead to activation of processes that upregulate proinflammatory cytokines, chemokines and reactive oxygen species. They are directly associated with pathogenesis of diabetes and its complications, such as diabetic nephropathy. The investigation focused on the role of apigenin in attenuation of inflammation: by binding to peroxisome proliferator-activated receptors gamma and inhibiting nuclear factor kappa B activation to alter the RAGE gene expression at mRNA level. The alternative splicing of the gene can produce different isoforms of RAGE, such as endogenous secretory RAGE (esRAGE), which acts as a decoy receptor and neutralises the harmful effects by inactivating the AGE-RAGE-NF-κB pathway.

The investigation involved differentiating human THP-1 monocytes into macrophages and treating them with 5µM and 25µM apigenin. After the treatment, RNA was extracted from the cells and cDNA was generated. The samples then proceeded to PCR and gel electrophoresis. The light sensitivity of each detected band was then measured and compared to baseline cells. EMBOSS and BLAST software were then used to identify the splice variants detected.

The results showed that apigenin treatment could potentially decrease the expression of full-length RAGE (fRAGE) cell surface receptor and increase the expression of esRAGE. Additionally, samples containing interferon gamma showed to slightly decrease the potential protective ability of apigenin against progression of an inflammatory state.

The use of apigenin in this investigation showed some beneficial results, which can be applied for treatment of diseases associated with chronic inflammation, such as diabetic nephropathy. It was observed that apigenin decreased fRAGE gene expression and upregulated the expression of esRAGE. However, the results of the study need to be further investigated.
2.0 INTRODUCTION

2.1 Inflammation

In healthy individuals, inflammation is a natural protective response of the body to infection or injury, which aims to eliminate a harmful substance or a pathogen [1]. However, the immune system can also attack its own cells, causing harmful inflammatory responses [2]. Chronic inflammation, characterised by overproduction of inflammatory cytokines and tissue destruction [3], can lead to initiation and development of many diseases, such as obesity, cancer, cardiovascular disease or type 2 diabetes mellitus [4], contributing to premature mortality and accounting for nearly 60% of all deaths worldwide [5]. Therefore, the research associated with understanding the risks, prevention and treatment of chronic inflammation is currently of a high priority amongst scientists.

2.2 Type 2 Diabetes Mellitus

According to the World Health Organisation, currently about 415 million people are suffering with diabetes mellitus worldwide and the figure is highly likely to rise to 642 million individuals by 2040 [6]. The most common form of Diabetes Mellitus (DM) is T2DM, which is characterized by hyperglycaemia, due to insulin insensitivity, caused by insulin resistance. This contributes to 85% of total DM cases [7]. The diagnostic criteria include fasting plasma glucose value ≥7.0mmol/l or plasma glucose value two hours after carbohydrate loading ≥11.1 mmol/l [6]. Most people suffering with T2DM are obese, physically inactive and lead a sedentary lifestyle, which seems to play a big part in the development of the disease [8]. However, there is also a strong correlation between genetic factors and the incidence of disease: about 25% of affected patients have a family history of T2DM [9]. Moreover, scientists found that environmental factors, such as toxic substances in water, food additives, pesticides or air pollutants are also risk factors associated with T2DM [10].
2.3 Diabetic complications

Increased concentration of plasma glucose initiates the formation of long-term vascular complications, which can be divided into macrovascular (damage of arteries) and microvascular complications (damage of small blood vessels) [11]. The macrovascular complications are associated with a damage of cardiovascular or cerebrovascular systems, whereas microvascular complications include retinopathy, nephropathy or neuropathy [11]. Diabetic nephropathy (DN) accounts for 15% of cases of end-stage renal disease (ESRD) worldwide [12]. It is clinically characterized by proteinuria and a decrease in glomerular filtration rate (GFR) [11]. The change in haemodynamic and metabolic processes leads to activation of signalling pathways, which result in increased production of proinflammatory cytokines, reactive oxygen species (ROS) and advanced glycation-end products (AGEs). These molecules are directly associated with progression of kidney disease in diabetic patients [13] [14].

**Figure 1:** The diagram representing the differences between normal glomerulus and diabetic glomerulus. The histological changes occurring during DN include: loss of podocytes, increased mesangial extracellular matrix and mesangial cell hypertrophy. As a result, the surface area for blood filtration is reduced and the junction between cells become leaky, leading to proteinuria and developing into life-threatening condition [15] [16].
2.4 Receptor for advanced glycation end products

2.4.1 Alternative splicing of RAGE

The receptor for advanced glycation end-products is a multi-ligand receptor found on the cell surface of endothelial cells, immune cells, adipocytes, astrocytes and smooth muscle cells [17]. RAGE is composed of three parts: extracellular, transmembrane and cytosolic regions [18]. Human RAGE gene is located on chromosome 6. The alternative splicing of the gene at mRNA level can generate different variants of the gene, such as soluble forms of RAGE, which may act as decoy receptors reducing the interactions of ligands with full-length RAGE (fRAGE) [19]. The soluble forms of RAGE lack transmembrane domain, which inhibits the RAGE signalling pathway, resulting in potential decreased production of inflammatory cytokines and chemokines associated with chronic inflammation [20].

![Figure 2: The schematic diagram showing different RAGE isoforms.](image)

*Figure 2: The schematic diagram showing different RAGE isoforms. The diagram showing differences between the structure of different RAGE receptor isoforms. Full-length RAGE contains V-type domain, C1 and C2-type domain, transmembrane domain and intracellular tail [20]. N-truncated RAGE lacks V-type domain, which is necessary for ligand binding. Proteolytically cleaved fRAGE is the same RAGE isoform as fRAGE,*
however due to enzymatic cleavage, the molecule lacks transmembrane domain and intracellular domain needed for initiation of RAGE signalling pathway [20]. On the other hand, the esRAGE structure is the same as proteolytically cleaved fRAGE, however due to frameshift mutation, different variant of RAGE is produced [20]. Both structures lack transmembrane domain and intracellular domain and are classified as soluble RAGE (sRAGE) [20].

2.4.2 AGE-RAGE interactions

During the hyperglycaemic state, reducing sugars form a covalent bond with plasma proteins, resulting in a change of molecular structure and leading to the formation of AGEs. Byun et al pointed out that AGEs promote toxicity leading to overall tissue damage [21]. AGE-RAGE interactions can lead to activation of several pathways, such as nuclear factor kappa B (NF-κB), which activates the expression of proinflammatory cytokines [18]. On the other hand, NF-κB can also bind to glyoxalase and decrease its inhibitory properties on AGE production [18] as well as upregulate RAGE expression causing positive feedback loop [19].

2.5 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) are members of nuclear receptor family and include three different isoforms: PPAR-α, PPAR-δ, and PPAR-γ [22]. PPAR-γ receptors can be further divided into isoforms, depending on the cell types where they are expressed: PPAR-γ1 is expressed in all tissues, PPAR-γ2 (30 amino acids longer) is in adipose tissue, PPAR-γ3 is expressed mainly in macrophages and PPAR-γ4 is expressed in endothelial cells [23]. PPAR-γ are targeted by antidiabetic drugs to increase the insulin sensitivity in T2DM [24]. It has been proved that the interaction of PPAR-γ and its ligands take part in the regulation of inflammation. The activation of PPAR-γ pathway can suppress the release of inflammatory cytokines and chemokines, such as interferon-γ (IFN-γ) and interleukin-1β [25]. Additionally, the initiation of PPAR-γ pathway can also inhibit the activity of NF-κB, which results in decreased formation of the RAGE receptors and overall decrease of proinflammatory agents released [26].

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2.6 Apigenin as PPAR ligand

Apigenin, 4′, 5, 7-trihydroxyflavone, is a secondary plant metabolite (flavonoid), which is classified as a flavone compound [27]. It is found in various plants, such as chamomile [27], parsley, grapefruit, oranges and onion [28] as a pharmacologically active agent. In the recent years, the researchers discovered the anti-inflammatory [29], anticancer [30] and antioxidant [31] properties of the compound, therefore many in vivo and in vitro studies have been carried out to evaluate the therapeutic role of apigenin in a wide range of pathologies. The study conducted by Mahajan et al looked at the effect of apigenin in mice with myocardial injury [32]. It has been found that apigenin has a potential to bind to PPAR-γ which results in its activation and proves the cardioprotective role of the flavonoid [32]. The results of the study showed very promising and inspiring results, which should be applied and used in the investigations of other pathologies. As mentioned before, the use of activated PPAR-γ can lead to decrease of inflammation, which can be applied to diseases associated with chronic inflammation, such as DM.

![Chemical structure of apigenin](image)

**Figure 3: The diagram representing the chemical structure of apigenin.** Molecular formula of the flavonoid is C_{15}H_{10}O_{5} [33]. Apigenin belongs to flavone sub-class.
2.7 Study aims and hypothesis

1. To conduct cell viability essays using CellTitre-Blue to find two appropriate concentrations of apigenin to use in further investigations.
2. To establish any changes in RAGE expression using Polymerase Chain Reaction (PCR) and gel electrophoresis.
3. To establish any changes in splice variant expression using Polymerase Chain Reaction (PCR) and gel electrophoresis, and where possible to identify the splice variants.

This study will investigate the hypothesis that treatment with apigenin will attenuate inflammation by upregulating the soluble RAGE expression and decreasing the expression of full length RAGE in THP-1 human macrophages.
3.1 Ethical approval
The project was ethically approved on Monday 13th November 2017 by Dr Rachel Adams, Chair of BMS Ethics Panel. The reference number is 9528.

3.2 Cell culture
The THP-1 cell line human monocytic leukaemia cells were purchased from European Collection of Authenticated Cell Cultures (ECACC) Public Health England and were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (ThermoFisher Scientific UK) supplemented with 2µM Gibco® GlutaMAX (ThermoFisher Scientific UK), 1 mM sodium pyruvate (ThermoFisher Scientific UK), 100nM Gibco® Minimum Essential Medium Non-Essential Amino Acids (ThermoFisher Scientific UK) and 10% heat-inactivated low endotoxin SeraPro Fetal Bovine Serum (PAN-Biotech, UK). The cells were kept in suspension cultures in 75cm² flasks incubated at 37ºC in 5% CO₂ using the Heracell incubator. The cells were counted using an Improved Neubauer haemocytometer every 3-4 days. Once reaching the density of 1-1.2 x 10⁶ cells/mL, the cells were sub-cultured into new vessels at a minimum density of 3 x 10⁵ cells/mL. For the purpose of the research, the THP-1 monocytes were then differentiated into macrophages using media containing 50ng/ml Phorbol12-myristate 13-acetate (PMA) (Sigma-Aldrich) which was added for 72 hours. THP-1 cells are not fully mature monocytes, therefore the differentiated cells used instead had an increased expression of RAGE protein on the cell surface, which was more beneficial for the purposes of this experiment [34].

3.3 Cell viability
Cell viability test was performed to establish whether, and at which concentration, apigenin would have cytotoxic effect on the overall differentiated THP-1 cell population. Cell viability was established using CellTitre-Blue. The THP-1 macrophage cells were seeded into 96-well clear, sterile tissue culture treated microplate with a lid at the concentration of 50000 cells/well. Apigenin (Sigma-Aldrich) was dissolved in 99% Dimethyl Sulfoxide, DMSO (Sigma-Aldrich) to
create a 100mM stock solution. According to previously published research papers [35] [36] [37], a range of concentrations from 1.56-50µM was used to measure the cell viability in this experiment. The stock dilutions were carried out in steps to minimise the percentage error. DMSO was used in order to dilute the concentrations of apigenin. Starting from 100mM the concentration was diluted to 1000µM and then it was diluted again to 50µM. The apigenin solution containing DMSO was added directly to culture medium in row A and the doubling dilutions were performed resulting in concentrations of 25µM, 12.5µM, 6.25µM, 3.13µM and 1.56µM. There were two different time points of the incubations of the cells: half the cells were incubated for 24 hours, whereas another half was incubated for 48 hours. 20µl of CellTitre-Blue reagent (Promega, UK) was then added to each well and incubated for additional 4 hours. After the incubation period the cell fluorescence was measured using Tecan M200 Infinite multi detection reader at 560 and 590nm.

![Figure 4: The structure of the plate used in establishing the cell viability using CellTitre-Blue.](image)

The diagram above represents a 96-well plate used to evaluate the cell viability at different concentrations of apigenin. Yellow colour represents the wells used to measure the cell viability after 24 hours. Blue colour represents the wells used to measure the cell viability after 48 hours. White colour represents the wells that have not
been used. Rows A-F contained different concentrations of apigenin used which were established by doubling dilutions starting with 50µM. Row G contained DMSO only (vehicle control) whereas row H contained cells only (negative control). Vehicle control was used to find whether DMSO caused any cell toxicity that could affect the cell viability assay.

3.4 Apigenin treatment
The THP-1 macrophage cells were seeded into two 12-well plate at the concentration of 1 million cells/ml. The first plate (see Fig.5) contained cells with: 1) 25µM apigenin (1mL/well), 2) 5µM apigenin (1mL/well), 3) vehicle control (cells with DMSO) and 4) negative control (cells with media only). The structure of the second plate (see Fig.6) was the same, however each well additionally contained 10ng/ml of human recombinant interferon gamma (IFN-γ) (PeproTech). IFN-γ is a pro-inflammatory cytokine which was added to mediate and increase the inflammation processes in THP-1 macrophages [38]. Apigenin was added to cells to investigate whether it would have an effect on RAGE gene expression.

**Figure 5:** The template for apigenin treatment of THP-1 macrophages. 12-well plate containing THP-1 macrophages treated with: 25µM apigenin (A1, B1 and C1), 5µM apigenin (A2, B2 and C2), vehicle control (A3, B3 and C3). Wells A4, B4 and C4 are baseline cells with no treatment added.

**Figure 6:** The template for apigenin and IFN treatment of THP-1 macrophages. 12-well plate containing THP-1 macrophages treated with: 25µM apigenin with IFN (A1, B1 and C1), 5µM apigenin with IFN (A2, B2 and C2), vehicle control with IFN (A3, B3 and C3). Wells A4, B4 and C4 are baseline cells with no apigenin added, however they contain IFN.
3.5 RNA isolation
In order to extract the RNA from cells, the media was removed and 1ml per well of TRIzol® (ThermoFisher Scientific UK) was added to adhered cells in both plates (Fig.3, Fig.4). The homogenised sample was treated with 100µl of 99% chloroform (Sigma-Aldrich) (phase separation), 250µl of 100% isopropanol (Sigma-Aldrich) (RNA precipitation) and 1ml of 75% ethanol (ThermoFisher Scientific UK) (dehydration). The quantification of RNA was conducted using Nano Drop Software.

3.6 Generation of cDNA and PCR
The RNA extracted from macrophages was reverse transcribed into complementary DNA using 1µl of 10nM dNTPs (ThermoFisher Scientific UK), 1 µl of random primers diluted 1:12 (ThermoFisher Scientific UK) and various amount of pure water and RNA (the quantity of added RNA depended on the Nano Drop Software protocol, as each sample was diluted according to the lowest concentration achieved). The mix was then placed in thermocycler at 65°C for 5 minutes to make RNA linear. 4 µl of First Strand Buffer (ThermoFisher Scientific UK), 1 µl of RNase OUT (ThermoFisher Scientific UK), 2 µl of 0.1 nM MDTT (ThermoFisher Scientific UK) and 1 µl of MMLV RT (ThermoFisher Scientific UK) were then added and all the tubes were placed in thermocycler to run cycles: 10 minutes at 25°C, 50 minutes at 37°C and 15 minutes at 70°C. Created cDNA template (20µl) was then combined with 2.5µl PCR buffer (Bioline, UK), 0.75 µl of 1.5 mM Magnesium Chloride (Bioline, UK), 0.1 µl BIOTAQ™ DNA Polymerase (Bioline, UK) and 0.5 µl of 10mM dNTP mix (ThermoFisher Scientific UK) as well as RNase free water. The last components added were 0.5µl of RAGE reverse primer exon 11; 5’-TGC CTC AGG TTC CTC CGA CTG A (Sigma-Aldrich) and 0.5µl of RAGE forward primer exon 8; 5’-CCA GGG AAC CTA CAG CTG TGT G (Sigma-Aldrich), which were necessary for the DNA replication process. At this stage, PCR controls were also created to prove that no contamination was present. The controls contained all the components listed above, apart from the cDNA template. Another set of tubes was created for a housekeeping gene – GAPDH. The tubes were treated similarly, however forward and reverse RAGE primers were
replaced with forward (5'-AGA AGG CTG GGG CTC ATT TG) and reverse (5'-AGG GGC CAT CCA CAG TCT TC) GAPDH primers (Sigma-Aldrich) [39]. The primers were specifically designed to target cDNA sequence only, not the genomic DNA [39]. Housekeeping gene control was used to show that apigenin treatment would only affect RAGE gene expression.

PCR reaction was then conducted in thermocycler. For RAGE samples, 35 cycles were required and the annealing temperature of 56°C was used. On the other hand, GAPDH PCR process required only 29 cycles and the annealing temperature was 67°C. At the end of the PCR process, each tube contained 25µl of DNA.

3.7 Gel electrophoresis

In order to separate the DNA by the base pair size for visualisation, 2% agarose gel was made for the gel electrophoresis process. 1g of agarose (Bioline, UK) was mixed with 50ml of 1 x Tris-Borate-Edta (TBE) Buffer (ThermoFisher, UK) and heated up until it was boiling. 30 ml of the prepared solution was then transferred into casting tray. 12 well comb was placed to create space for samples to be loaded in when the gel became opaque (after 20-30 minutes). The gel plate was then placed in the tank containing 1 x TBE buffer (the liquid needed to cover the tank 1 cm above the gel). 5µl of gel loading buffer (Sigma-Aldrich) was added to each tube containing 25 µl of DNA sample to track their movement during gel electrophoresis. 8µl of 100bp molecular ladder (New England Biolab) was loaded into the first well as a standard against which approximate size of other molecules was identified. Other wells were filled with 8µl sample (containing the loading dye) and the process run for 70mins at 100V. Once the protocol was completed, the gel plates were removed and placed in 10µg/ml of Ethidium Bromide (Sigma-Aldrich) for 15 minutes (to visualize the staining) and then washed in deionized water for 10 minutes. Ready gel plates were then moved into GelDoc™ EZ Imager and the image was created using UV light. ImageLab converted the band intensity into light intensity value, which allowed the further analysis of data.
3.8 Statistical analysis

The two sample T-test with 95% confidence level have been applied to compare treated cells to untreated cells. The statistical data was produced using Minitab software. Standard deviation (S.D.), mean and P-value have been calculated to identify the significance of data produced. The significance of the data was considered if the p<0.05.

3.9 Identification of splice variants

BLAST and EMBOSS programmes have been used to attempt the identification of RAGE splice variants. The PCR products have been calculated by counting the number of base pairs between the areas where the primers attached to the cDNA template. The number of base pairs calculated was then compared to the fragments found on the agarose gel.
4.0 \hspace{1cm} \textbf{RESULTS} \\

4.1 Cell viability

In order to establish the cell viability, average fluorescent absorption value as well as standard deviation were calculated using the four sets of data. The absorbance of untreated cells was converted to 100% cell viability as it represented the total number of cells without apigenin or IFN-\(\gamma\) added. The treated cells samples were then compared to untreated cells and the percentage viability was calculated.

\textbf{Figure 7: The cell viability of THP-1 macrophages treated with a range of apigenin concentrations.} The cell viability was measured at 24 and 48 hours after the treatment with various concentrations of apigenin (0\(\mu\)M - 50\(\mu\)M). The average of two time points was calculated to create the cell viability graph. The cell number of viable cells was compared to untreated cells and was converted into percentage. Vehicle control contains DMSO and cells only. In the average percentage viability of cells: 25 \(\mu\)M showed higher cell viability than 50 \(\mu\)M, therefore it was chosen to be the “higher concentration”. Error bars show \(\pm 1\) SD.
The average percentage of each viability test was based on: four repeats after 24 hours and four repeats after 48 hours treatment. The cell viability values at these time points did not differ much, therefore they were combined to show the best representation of the data. According to figure 6, there was a slight decrease in the average viable cells at 50µM apigenin (93.22%, p value= 0.706) compared with untreated cells (100%). When 25µM apigenin was used instead, there was slight decrease in cell viability observed (95.65%, p value= 0.586), compared with untreated cells, however, the overall percentage of viable cells was higher when compared with 50µM apigenin. Vehicle control used in the experiment (DMSO) showed not to have much effect on the cell viability (99.80%, p value= 0.365), therefore it was a suitable solvent used in the experiment. Although, the results gained are not significant (p value >0.05), however, the suggested data as well as research literature found [35] [36] [37] were used to establish the final concentration for the experiment.

The lower concentration used in the experiment was chosen to be 20% of the higher concentration. Therefore, the cells were treated with 5µM and 25µM of apigenin dissolved in DMSO.

4.2 Gel electrophoresis imaging

![Figure 8: The figure representing gel electrophoresis cDNA separation of sample A treated with 5µM apigenin. Detection of RAGE splice variants of sample A, using gel](image)

Ladder  C1  C2  C3  A  B  C  D  E  F  G  H
The effects of two different concentrations (5µM and 25µM) of apigenin on RAGE splicing have been investigated in this study. Figure 7 shows the RAGE gene splice variants detected by gel electrophoresis. Molecular marker was used to compare the band base pair sizes. The bands were detected at: 280bp, 320bp, 420bp, 550bp and 1100bp. The smaller gene fragments travelled greater distance due to the smaller molecular weight, compared with bigger fragments. Therefore, the fragments with greater number of base pairs are located on the top part of the gel, whereas the smaller fragments are located more towards the bottom of the agarose gel.

Cells treated with 5 µM apigenin showed stronger signal at 1100bp compared to samples in rows A (untreated cells), C (untreated cells and IFN-γ) and G (cells with 5µM apigenin and IFN-γ). However, in order to evaluate the results more accurately, light intensity of each band was measured using ImageLab software.

Throughout the experiment, GAPDH showed no splicing and remained constant, therefore it was a suitable housekeeping gene used for the experiment as it was not affected by the apigenin or IFN-γ treatment.

Additionally, the three controls used in the experiment (C1, C2 and C3) did not show any gene expression on the agarose gel, therefore no contamination was present.

Figure 8 is a representation of the gels produced during the experiment. Results of multiple gels were used to formulate the findings presented in figure 9 and figure 10.
4.3 Percentage expression of splice variants as a proportion or overall RAGE gene expression

Using the ImageLab software, the light intensity of each band detected was measured. The results were then normalised against GAPDH gene to account for the differences in the amount of cDNA present.

**Figure 9:** The effects of apigenin on the average expression of each splice variant, as a proportion of overall RAGE expression in each sample. The light intensity of eight gels produced was used to create the average data of untreated cells and cells treated with IFN. The light intensity of four gels was used to calculate the average data for cells treated with apigenin or apigenin with IFN (at both concentrations). Blue column represents 280bp splice variant. Orange column represents 320bp splice variant. Grey column represents 420bp splice variant. Yellow column represents 550bp splice variant. Green column represents 1100bp splice variant. Error bars represent ±1SD.

Overall, gel electrophoresis produced eight sets of results. To calculate the percentage expression of splice variants, the light intensities of each samples were added up (100%). Each band was then divided by the overall sample light
intensity, which represented the total amount of RAGE present. To further understand the results, statistics have been applied (two sample T-test) into all the sets of data created.

While compared to untreated cells (cells only) 20.21% (SD±1.26%), the 280bp splice variant showed significant increase in expression in the cells containing IFN-gamma but no apigenin: 29.77% (p=0.000), 5µM apigenin and IFN: 32.52% (p=0.005), 25µM apigenin and IFN: 27.49% (p=0.000). Cells containing 5µM apigenin only showed higher expression of this splice variant: 24.30%, however the results did not show any significant value (p=0.075). Cells treated with 25µM apigenin only showed decrease in expression of 280bp splice variant: 17.87%, however the result does not show significance (p=0.629)

320bp splice variant expression in the untreated cells (cells only) was 50.99% (SD±2.42%). The cells treated with IFN-γ showed higher expression of that particular splice variant compared with cells which did not contain any IFN-γ; cells only with IFN-γ – 46.18% (p=0.001), cells treated with 5µM apigenin and IFN – 46.20% (p=0.014) and cells treated with 25µM apigenin and IFN-γ – 52.37% (however, the result does not show significance as p=0.603). On the other hand, cells treated with 5µM apigenin only – 41.88% (p=0.033) and treated with 25µM apigenin only – 38.73% (however, the p=0.129, so therefore the result is not significant) show lower expression of 320bp splice variant compared to other samples as well as untreated cells (cells only): 50.99% (SD±2.42%).

The expression of 420bp RAGE splice variant decreases when the cells received apigenin or apigenin and IFN-γ treatment, compared with cells only: 13.37% (SD±1.12%). However, cells treated with IFN-γ only showed higher expression of the 420bp splice variant: cells only with IFN:10.10% (p=0.000), cells treated with 5µM apigenin and IFN-γ:11.65% (p=0.374, no significant changes) and cells treated with 25µM apigenin and IFN-γ – 9.18% (p=0.001). Cells treated with 5µM apigenin showed 9.59% (p=0.374, no significant changes) expression whereas the ones treated with 25µM apigenin: 6.85% (p=0.001).

The expression of 550bp splice variant is the highest in cell only: 9.35% (SD±1.12%). Cells with IFN-γ only showed 6.69% (p=0.000), whereas 5 µM apigenin with IFN-γ: 7.27% (p=0.031) and 25 µM apigenin with IFN-γ: 6.16%
5 μM apigenin treated cells showed 6.82% (p=0.065, not significant) whereas the lowest 550bp splice variant expression was showed by 25 μM apigenin: 5.39% (p=0.040).

The expression of the largest fragment (1100bp) in cells only was 6.09% (SD±1.60%). In cells treated with IFN-γ only: 7.26% (p=0.223, not significant), 5 μM apigenin with IFN-γ: 2.36% (p=0.065, not significant) and 25 μM apigenin with IFN-γ: 4.81% (p=0.111). The highest expression of 1100bp splice variant was discovered in cells treated only with apigenin: 5 μM apigenin: 17.41% (p=0.161, not significant) and even higher in 25 μM apigenin: 31.17% (p=0.111, not significant), compared to cells only: 6.09% (SD±1.60%).

The results with p value>0.05 were classified as not significant and it was concluded that with 95% confidence the results happened by a chance. Low standard deviation suggests low spread of data and therefore high reliability of the data.

4.4 The percentage change of splice variants expression of treated cells compared to baseline cells

Figure 10: The graph representing the percentage change of RAGE splice variant expression of treated cells compared to untreated cells. To clarify the changes occurring after the apigenin treatment, the expression of each splice variant of treated
cells was divided by the expression of untreated cells, then expressed by the percentage values. Blue column represents 280bp splice variant. Orange column represents 320bp splice variant. Grey column represents 420bp splice variant. Yellow column represents 550bp splice variant. Green column represents 1100bp splice variant. Error bars represent ±1SD.

As shown in the figure above, there is a high percentage increase in the expression of 1100bp splice variant in cells treated with 5µM apigenin and 25µM apigenin only compared to baseline cells. However, when the cells are treated with both IFN-γ and apigenin, the expression of 1100bp splice variant decreases. Additionally, as the expression of this splice variant increases, the expression of 280bp variant decreases.
5.1 Cell viability

The aim of the test was to establish appropriate concentrations of apigenin and use them to measure the effect of the flavonoid on the RAGE gene expression at mRNA level. As mentioned before, according to the published literature [35] [36] [37], a range of concentrations of apigenin was used to evaluate whether the flavonoid would have a toxic effect and influence the THP-1 cell viability. This study revealed that there might be a decrease in cell viability after treatment with 50µM apigenin (93.22%, p value= 0.706), as the treatment resulted in the lowest percentage of viable cells among all the concentrations (25µM, 12.5µM, 6.25µM, 3.13µM and 1.56µM).

The present investigation revealed that the use of DMSO as a vehicle control showed no overall effect on the THP-1 macrophages, therefore it was an appropriate solvent used to create the apigenin solution. This was also confirmed by Da Violante et al in the study on colon tumor cell culture [40] and by Bianchi et al in the study involving odontoblast-like cells [41].

However, no tests were conducted to assess the cell viability for cells treated with IFN-γ, therefore it remains unclear whether IFN-γ could have an impact on the cell viability. According to the published literature, IFN-γ can promote toxicity and induce cell death [42] [43]. Perhaps, this should be taken into consideration in the future experiments.

5.2 Splice variants identification

5.2.1 280bp splice variant

The aim of the present investigation was to evaluate the role of apigenin in RAGE gene splicing at mRNA level. According to the published literature, apigenin can activate PPAR-γ [44] and inhibit the activation of NF-κB, which will result in reduced number of full length RAGE proteins produced [45] [46]. The gel electrophoresis results identified five different splice variants. Using BLAST and EMBOSS software, PCR products have been compared with the agarose gel
figures produced during this investigation. However, the BLAST and EMBOSS results did not match will all the transcript splice variants identified during the experiment. Using the primer sequence and BLAST, six different splice transcripts were matching the criteria. However, according to BLAST, product length of possible RAGE splice variants could be either 259 or 304 base pairs long and for each length three possible transcript variants have been identified. Therefore, using the experiment results available, it is not possible to fully identify the splice variants and they can only be suggested.

Nonetheless, the smallest fragment detected during gel electrophoresis, which contains approximately 280bp, could be one of the three transcript variants found in the BLAST search: transcript variant 4, 5 or 6. The splice variant 6, also known as RAGE_v1, encodes for a shorter isoform of RAGE protein – isoform 6 [44]. This molecule is also known as endogenous secretary RAGE (esRAGE). Due to the lack of second last coding exon, shorter isoform with a distinct C-terminus is formed [47]. As a result, the protein does not have the transmembrane and intracellular domain which inhibits the AGE-RAGE signalling [47]. Therefore, the soluble variant of RAGE can not only inhibit the production of proinflammatory cytokines and chemokines, but also act as an antagonist for RAGE protein (isoform 1) [19]. The inhibition of both processes would eventually result in attenuation of inflammation and thus it would stop the progression of pathologies, such as diabetic complications [20]. The hypothesis of the experiment was that apigenin would attenuate the inflammation by increasing the number of soluble RAGE, therefore the 280bp fragment could be the RAGE_v1. Also, esRAGE the second most prevalent RAGE splice variant in humans, which supports the theory [48].

The expression of 280bp splice variant was higher in treated cells compared to untreated cells. Additionally, the intensity of the band was much higher when IFN-γ was present compared with samples treated with apigenin only. The results suggest that the esRAGE could have a protective effect on the cell and, therefore, in the times of increased inflammation, the esRAGE splice variant was more abundant. This was also confirmed in the study done by Piarulli et al in which they stated that esRAGE upregulation is associated with the cell’s antioxidative
defences and that the increased esRAGE production protects more against plaque formation in T2DM [49].

5.2.2 320bp splice variant

The 320bp splice variant detected could not be fully identified. However, it can potentially be transcript variant 1, also known as full length RAGE, as it is the most abundant RAGE transcript in *Homo Sapiens* [50]. As mentioned before, flRAGE and AGEs interactions play an important part in certain diseases, such as T2DM and its complications. Looking at the results of this investigation, there was a decrease in flRAGE expression when cells were treated with apigenin only, compared to the baseline. Moreover, the higher concentration of apigenin further decreased the flRAGE expression. On the other hand, cells treated with IFN-γ and apigenin still showed a decrease in flRAGE expression compared to baseline cells, however the expression was higher than in the cells in which IFN-γ was not present. This could suggest that apigenin had protective effects on the cells, however under conditions of increased inflammation, such as poorly controlled DM or (as in the case of this experiment) induced inflammation by IFN-γ, apigenin tends to have weaker protective properties. Nonetheless, the investigation supports the idea that apigenin treatment can attenuate inflammation: as the number of flRAGE decreases, and esRAGE increases, the number of proinflammatory cytokines produced and released is downregulated.

5.2.3 Other alternative splice variants

The RAGE splice variants found were not comparable to any literature in the cases of 420bp, 550bp and 1100bp. Therefore, these splice variants could not be identified in the present investigation. However, the 1100bp splice variant could have a protective effect on the cells as it greatly increases when only apigenin is present, which suggests that the properties are lost after the inflammation is induced by IFN-γ.
5.3 Implications of results into pathophysiology of Diabetic Nephropathy

Research focused on understanding the mechanism associated with production and regulation of fRAGE and eRAGE levels, would benefit the treatment of diabetic nephropathy associated with upregulation of the AGE-RAGE pathway. According to research conducted by Yamamoto et al, genetically modified mice with upregulated production of RAGE receptors quickly developed severe diabetic nephropathy [51]. Various studies have demonstrated that the formation of AGEs at an accelerated rate is associated with hyperglycaemia [52] [53].

The accumulation of AGEs is the major factor responsible for renal damage, as it causes mesangial hypertrophy, which progresses into mesangial expansion [15]. The expanding mesangial cells reduce the filtration surface area in the glomerulus and are involved in the formation of gaps between the cells. The ‘leaky’ junctions allow larger molecules to cross the barrier, thus proteinuria is present in DN patients [15]. On the other hand, it has been found that AGEs decrease the growth of pericyte cells, which are responsible for regulation of the growth of endothelial cells creating the glomerular filtration barrier [54] [55]. Therefore, the loss of endothelial cells also contributes to the development of proteinuria.

Turk et al pointed out that eRAGE levels positively correlated with HbA1c levels and negatively correlated with C-reactive protein levels associated with inflammation [56]. The upregulation of eRAGE and attenuation of fRAGE expression, caused by apigenin in the present study, can benefit DN patients. It has been shown that the use of recombinant soluble RAGE blocked the AGE-RAGE pathway [57]. Therefore, eRAGE might be used to protect against AGE-RAGE related tissue damage by eliminating the upregulated AGEs and neutralising their action on endothelial cells and preventing their accumulation.

5.4 Limitations and future recommendations

Some isoforms of RAGE might also be protein non-coding [58]. Although, the transcription takes place, the message might not be translated, therefore it remains unknown as to what proteins are produced. Protein extraction and
Western Blots can be used in the future to evaluate the RAGE protein expression in THP-1 macrophages [57].

The splice variants detected in the present investigation could not be fully identified, because they were either not present in the BLAST and EMBOSS search and literature, or there were multiple possibilities that could match the base pair number. Perhaps, designing specific primers or extracting and sequencing the splice variants could generate superior data, as some splice variants could not be identified in the investigation.

As mentioned before, there is no cell viability for IFN-γ. Also, it remains unclear whether IFN-γ by itself could affect the gene splicing.

The THP-1 macrophages are derived from a child case of acute monocytic leukaemia [59]; thus, they are not an accurate representation of cells present in healthy individuals [60].

5.5 Conclusion

The findings of this investigation suggest that apigenin could influence the RAGE gene expression at mRNA level, by binding to PPAR-γ and inactivating NF-κB. The flavonoid could potentially upregulate esRAGE production, resulting in the inactivation of the AGE-RAGE pathway. By binding to AGEs, esRAGE would decrease the accumulation of AGEs associated with renal damage in DN. Moreover, as the production of cytokines would be downregulated, the treatment with apigenin could lead to attenuation of inflammation. The data also suggests that flRAGE could be downregulated, resulting in the decreased formation of RAGE and ligand complexes. Nonetheless, the splicing mechanism of the RAGE gene is not fully understood yet. This discovery could be a phenomenon leading to pharmacologically targeting and upregulating esRAGE expression, which would prevent the development of DN. However, some data is statistically insignificant, therefore further research is needed.


7.0 APPENDICES

7.1 Ethical approval letter

Kolsut, Djeana
BSc (Hons) Biomedical Sciences
Cardiff School of Health Sciences

Dear Applicant

Re: Application for Ethical Approval: The effect of flavonoids on RAGE and sRAGE mRNA expression in the macrophage model

Project Reference Number : 9528

Your ethics application, as shown above, was considered by the Biomedical Sciences Ethics Panel on 08-11-17.

I am pleased to inform you that your application for ethical approval was APPROVED.

Minor issues may still need addressing before you commence any work – if so these will be listed below.

1. Include URN in application

Where changes to the information sheet, consent form and/or procedures are deemed necessary you must submit revised versions to the relevant ethics inbox. If you are a student – your supervisor must do this on your behalf.

Note: Failure to comply with any issues listed above will nullify this approval.

Standard Conditions of Approval

1. Your Ethics Application has been given a Project Reference number as above. This MUST be quoted on all documentation relating to the project (e.g. consent forms, information sheets), together with the full project title.

2. All documents must also have the approved University Logo and the Version number in addition to the reference and project title as above.

3. A full Risk Assessment must be undertaken for this proposal, as appropriate, and be made available to the Committee if requested.

4. Any changes in connection to the proposal as approved, must be referred to the Panel/Committee for consideration without delay quoting your Project Reference Number. Changes to the proposed project may have ethical implications so must be approved.

5. Any untoward incident which occurs in connection with this proposal must be reported back to the Panel without delay.
6. If your project involves the use of human samples, your approval is given on the condition that you or your supervisor notify the HTA Designated Individual of your intention to work with such material by completing the form entitled “Notification of Intention to Work with Human Samples.” The form must be submitted to the PD (Sean Duggan), BEFORE any activity on this project is undertaken.

This approval expires on **08.11.17**. It is your responsibility to reapply/request extension if necessary.

Yours sincerely

Dr Rachel Adams  
Chair of BMS Ethics Panel  
Cardiff School of Health Sciences  

Tel: 029 20416855  
E-mail: radams@cardiffnet.ac.uk  
Cc:  

PLEASE RETAIN THIS LETTER FOR REFERENCE
7.2 The fluorescence absorbances of the cell viability test

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7.3 RNA quantification using Nano Drop software

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7.4 Gel electrophoresis figures

*Sample A with 5µM apigenin*

*Sample A with 25µM apigenin*
Sample B with 5µM and 25µM apigenin

Sample C with 5µM and 25µM apigenin
### 7.5 Identification of splice variants using EMBOSS

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| 1                  | 303                               | • Also known as RAGE  
• Encodes for isoform 1 |
| 2                  | 281                               | • RAGE_v5  
• Longer isoform containing an additional protein segment |
| 3                  | 285                               | • RAGE_v4  
• Shorter isoform missing two internal protein segments |
| 4                  | 236                               | • RAGE_v6  
• Shorter isoform (4) with a distinct C-terminus |
| 5                  | 258                               | • RAGE_v9  
• Shorter isoform (5) with a distinct C-terminus |
| 6                  | 236                               | • RAGE_v1  
• Shorter isoform (6, also known as esRAGE) with a distinct C-terminus  
• Lacks the transmembrane and intracellular domains |
| 7                  | 755                               | • RAGE_v8  
• Shorter isoform (8) with a distinct C-terminus |
| 8                  | 335                               | • RAGE_v10  
• Shorter isoform (6, also known as esRAGE) |
| 9                  | 189                               | • RAGE_v16  
• Shorter isoform (7, also known as hRAGEsec) with a distinct C-terminus |
### 7.6 Statistical analysis of data

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**Significant (green) = p<0.05  Not significant (red) = p>0.05**