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Identifying polymorphisms associated with Pseudoxanthoma Elasticum by sequencing specific regions of the ABCC6 gene

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Abstract

This paper describes research undertaken for a project run from January to April 2018 aiming at sequencing a chosen region of the ABCC6 gene in order to identify potential polymorphisms associated with Pseudoxanthoma Elasticum (PXE) and GACI. Mutations in the ATP-Binding Cassette subfamily C number 6 gene (ABCC6) gene cause PXE, a rare genetic disorder, typically inherited in an autosomal recessive manner. ABCC6 gene is a member of ATP-Binding Cassette (ABC) transporter family. ABC Transporter family proteins are ATP- dependent pumps hence they are named ATP - binding cassette (ABC) transporter family. In order to meet the aims of the project the following processes were followed: the suitable genomic area of the gene of interest for further investigation was selected; suitable primers to amplify the selected region were designed; PCR conditions using agarose gel electrophoresis; amplification of placenta DNA samples using the optimized PCR conditions; DNA sequencing of the amplified PCR products and Sequence data analysis.

Keywords: ABCC6 gene, sub-family C, ATP-binding cassette, Pseudoxanthoma Elasticum

Introduction

The project was completed in a time frame of 4 months: January to April 2018.

As a part of accomplishing this project, attending the laboratory was crucial to perform all necessary investigations/reactions including preparation of PCR reactants, programming thermocycler to perform PCR, optimisation of PCR, followed by quantification and purification of the final PCR product to be sent for sequencing.

The practical laboratory investigations/reactions were completed in a time frame of 6 days. Utilising the Laboratory for a day cost £100.00, thus for 6 days the cost was £600. Utilising the time of Laboratory technician cost £150 a day. Thus, for 6 days the cost was £900.

The project involved consultation meetings with regards to the development of the project with the Project Leader/Project Supervisor on a monthly /weekly basis. Utilising the time of the Project Leader cost £100 a day. Initially meeting were held twice a month from December to February, followed by weekly meetings in March until the final submission of the project. Thus, for an approximate number of 13 meetings, the total charge for utilising the time of Project Leader/ Supervisor equals to £1300.

Travel expenses to attend the laboratory cost and meetings with the Project Leader cost £17.45 a journey and returntrain ticket a day. Thus for 6 days of

practical work plus attending 13 meetings with the Project Leader cost £331.55.

On addition to this, there are also substantial costs paid from the university, which are associated with the cost equipment such as the thermocylcer, refrigerator, microwave, consumables which include pipette tips, DNA ladder, PCR reaction agents i.e. Taq polymerase and for employing bioinformatics agencies such as Sigma Aldrich, Eurofins Genomics to design primers and sequence the final PCR product.

The project puts emphasis on the fact that there is no effective treatment procedure in place to treat patients with PXE. Research and investigation around PXE is very limited, owing to its rare occurrence in a population. Thus there is a need for more extensive studies to take place to discover an effective and a promising method of treatment. The project also brings this to attention that unscreened liver transplants from PXE patients can result in the recipient acquiring PXE. Thus screening of transplant organs is very crucial to avoid the recipient potentially extracting PXE.

Materials and Methods

1.1 Materials

The materials, reagents and equipment employed in this experiment are shown in Table 1

Table 1. Consumables, reagents and equipment employed in this study

Consumables	Reagents	Equipment
Gloves	RNA / DNA free water	Vortex (Microcentrifuge) equipment
Microfuge tube Rack	Human Placental DNA 1µg / ml	PCR Thermocycler
Microfuge Ice Rack	Enzyme mix comprising: Taq polymerase (*Thermus aquaticus*) $MgCl_2$ (co-factor) Reaction buffer Deoxynucleotides (dNTPs) Sterile Deionised Water Red dye	Balance
Pipettes (0.1 – 10µl, 10 – 100µl, 100 - 1000µl)	Dehydrated Forward & Reverse primers	Microwave Oven
Pipette Tips ((0.1 – 10µl, 10 – 100µl, 100 - 1000µl)	TBE (0.5 x TBE)	Gel Tray/ Electrophoresis Tank
Permanent Marker	Agarose powder	Comb
Eppendorf tubes		DNA Step Ladder
PCR Tubes		Powerpack
Weighing Boat		Biodrop
100 ml measuring Cylinder		
Conical Flask		
Heavy Duty Gloves		

1.2 DNA samples

For this study, 4 different human placental DNA samples were used that had a concentration of 250ng/µl.

1.3 DNA amplification

1.3.1 Bioinformatics of ABCC6 gene

The primary stage of the project involved research of the ABCC6 gene and its sequence. The gene is located at 16: 16202789 - 16201461 on the reverse strand. The location of the potential mutation of interest reported in the literature was confirmed using the Ensemble Genome browser. It was also confirmed that the mutation is located at position 16: 16202142. Below is the selected DNA fragment of the ABCC6 gene that was used for further investigation (mutation indicated with yellow):

Strand: Reverse

Chromosomal location: Chromosome 16: 16202789 - 16201461

Gcctctgtccttggcttgcagttggccacctcctccctgtgtccccatatggtctttcctctgtttgcgccccctggtgtctccttgtgtgtctacatttcctcttcttaaaaggaccc
catcagattggattagggcccagcctaatgacctcatttttaacttcattgcctctttaaaggccctatttccaaatacagtcacattcagaggtactgggggtcggggcttcaac
atataaactttgtgaggacacaatttagcctataacaagtgggaacaaaaactccacctcttgatcttgaggagctgcagtcagattgaaaaggagtatggacagtgggggga
aataacggagaccattattgcaaaaaaaaccacactgactgatcctccacacctggactagtttgggccgactgatcctccacatctggactggtttgggcaaaggcaacac
ccttaggcacctcctctcaccagctgtaccttctccctccttcccctgcaagCCTTTTCCTGGAGTTTATTGGTGATCCCAAGCCTCCAGC
CTGGAAGGGCTACCTCCTCGCCGTGCTGATGTTCCTCTCAGCCTGCCTGCAAACGCTGTTTGAGCAGCA
GAACATGTACAGGCTCAAGGTGCTGCAGATGAGGTTGCGGTCGGCCATCACTGGCCTGGTGTACAGAA
AGgtgagccctgggggacaagggcaggtcttccagccaggaaaagcagtatcactgagcgggcagtaatgttatcagcagctgagaacgcattcagtccttgcttatta
gagtcactcaccatacattctcacttccttagttatttctctgtattatatgtatttatttttttgagatggagtcttgctctgtcacccaggctggagtgcagtggtgcaatctcggctc
actgcaacctccgcctcccgggttcaagtgattctcctgcctcagcctcctgagtaactgggactatagacacgtgtcaccacgcccagctaatttttatatttttggtagagac
aaggttttgccatgttggtcaggctggtctcgaactcctagcctcaagtgatctgtctcagctccttagttattcctgtaaggtcttgtgtgatctgtccctagggtgtctctgacct
catcccgtcacctcctcccatgctcactctcctccagccacacaggtctcctttcagttcctcaaacactccaggccc

1.3.2 Primer design

An in silico PCR output was obtained through the use of UCSC Genome Browser. This was performed to inspect the specificity of the primers.

The amplification region should include the mutation of interest, therefore, for primer design, an area of around 1000 bp flanking from the mutation was selected. The selected region was inserted into Primer 3 output software:

The following primers were generated from primer 3 output as shown in Table 2.

Table 2. Designed Primers using the Primer 3 software

Primers	Sequence Composition	Length	Tm (°C)	G/C %
Forward	tcaccagctgtaccttctcc	20	59.4	55
Reverse	ggaagacctgcccttgtc	18	58.2	61.1

The primer 3 output was sent to Eurofins Genomics in order for the respective primers to be manufactured. The primers arrived in dehydrated powder form, hence dilutions needed to be performed. Gloves were worn throughout the whole procedure to minimise the likelihood of contamination while handling the tubes and apparatus. Dilutions of the primers were made carefully by following the instructions on Eurofins Genomics Instruction sheet: 338µl for forward primer and 365µl for reverse primer to achieve a molar concentration of 100 pmol/µl. Following this a 10µM solution of the original stock solution of primers were prepared for working solution by pipetting 20µl of the original primer stock solution to 180µl of Sterile Nuclease free water.

1.4 What is Polymerase Chain Reaction (PCR) reaction

Polymerase chain reaction (PCR)was invented by Kary Mullis and Micheal Smith in the 1980s (Shampo and Kyle, 2002; Ncbi.nlm.nih.gov, 2014). PCR rooted from the natural process of generating new set of DNA to replace withering or damaged DNA through DNA Replication in living organisms (Pelt-Verkuil, Belkum and Hays, 2008). This natural technique of DNA replication is applied in laboratory practise, where temperature of varying heat is applied throughout PCR procedure to produce millions of copies of a desired segment of gene within a short number of hours (Nature.com, 2014).

PCR is comprised of three major steps: Denaturation, Annealing and Extension.

During denaturation the DNA template containing the target segment of gene is exposed to high temperature of 94°C to 96°C in order for the double strands to be separated. Following Denaturation, temperature is lowered to the annealing temperature for the primers to anneal to their complementary sites within the template. Following annealing, temperature is raised again to about 72°C, at which point the DNA polymerase binds to the primer and target DNA segment to initiate DNA amplification. This step is known as extension (Nature.com, 2014; Ncbi.nlm.nih.gov, 2014)

1.4.1 Preparation of PCR reactions

PCR reaction mixtures were formulated in nuclease free, sterile and thin membrane Eppendrof tubes.The working stock of primers, enzyme mix and the human placental DNA were all thawed in a thawing /ice-frozen PCR tube rack while being operated.

For each PCR reaction of a set temperature, a master mix was prepared. Preparing the master mix avoids the need to pipette miniscule amounts of reagents repeatedly, thereby reducing pipetting errors. The composition of master mix was formulated as shown in Table 3.

Table 3. Composition and quantity of PCR reaction mixture

PCR mixture components	Quantity required for single reaction	3.5 x Master Mix
Forward primer (10pmol/µl)	1µl	3.5µl
Reverse primer (10pmol/µl)	1µl	3.5µl
Nuclease free Deionised water	10µl	24.5µl
Taq polymerase enzyme mix	7µl	35µl
Total Reaction volume	**19µl**	**66.5µl**

Total volume of 66.5µl of the master mix was divided into 3 quantities; 19µl of master mix was pipetted into 3 respective reaction tubes labelled P1; Placental DNA replicate 1, P2: Placental DNA replicate 2 and C: negative PCR control (Water). P1 and P2 were replicate reaction tubes and C was the control. The purpose of the control was to check for any unspecific amplification or contamination. 1µl of placental human DNA was added to 2 replicate reaction tubes and 1µl of sterilised nuclease free water was added to control tube for each PCR reaction of a set annealing temperature. The tubes were inserted into vortex microfuge machine to mix the contents well and to get rid of any bubbles produced in the process pipetting.

1.4.2 Optimisation of PCR annealing temperature

PCR Annealing temperature X was calculated through the following formula outlined by :

$$X = Tm - 5° C$$

Tm temperature for forward & reverse primers were 59.4 ° C & 58.2 ° C respectively according to the Eurofins genomics instruction sheet. The tm temperatures were added together and then divided by 2 to obtain the Tm average;

$$\frac{59.4 + 58.2}{2} = 58.8$$ Tm Average

Hence X = 58.8 – 5

X = 53.8 ° C ≈ 54 ° C

In order to examine and assure at which annealing temperature the primers perform the best, a range of 4 temperatures were used using the formulas:

X = 58.8 – 7.8 51
X = 58.8 – 6.8 52
X = 58.8 – 5.8 53
X = 58.8 – 4.8 54

The PCR tubes were inserted into programmed thermocycler/PCR machine. Just the annealing temperature was adjusted in order for visualisation of successful banding. The setting of the programme was as follows:

PCR reactions were initially exposed to 94°C for 5 minutes to allow for sufficient denaturation of the double-stranded DNA molecules. Following this step, 30 cycles were applied, each having three different steps: the first step involved an incubation at 94°C for a 1 minute to ensure denaturation of the two DNA strands, followed by 30 seconds at the annealing temperature X, where the primers are efficiently and specifically bind to the target DNA region and lastly, by an incubation of 1 minute at 72°C to allow DNA polymerase to add nucleotides and extend the DNA strands. A final extension step of 5 minutes at 72 °C was applied in order to make sure that the enzyme has finished the elongation for all PCR products. The whole procedure took approximately 2 hours.

1.5 Agarose gel electrophoresis

Agarose gel electrophoresis (AGE) is a very simplistic and cost effective tool to visualise bands of various size under a UV imaging system. This procedure utilises electrophoresis to separate DNA fragments of differing sizes ranging from 100 bp to 25 kb (Lee *et al.*, 2012). Agarose is sequestered from seaweed plant which is comprised of recurring subunits of agarobiose which is essentially polymer of L and D – galactose. The polymers interact with each other non-covalently during gelation and arranges themselves in such a manner that the concomitant polymers act as a molecular sieve (Lee *et al*., 2012). It is owing to this sieving property the varying DNA fragment sizes are separated.

1.6 Preparation of Gel electrophoresis

While the PCR programme ran for 2 hours, gel for electrophoresis was prepared to observe/ analyse the sample of PCR tubes. Approximately 1g of agarose gel was weighed and mixed with 100ml of clean buffer TBE (Tris-Cl Boric acid and EDTA) to make up the 1% (w/v) agarose gel solution. The gel solution was made in a conical flask and 100 ml of clean buffer was measured in a 100ml measuring cylinder. The gel solution was heated in the microwave for 3 – 5 minutes. In intervals of 30 seconds the gel solution was taken out to be swirled to mix the agarose powder and was reheated until the agarose completely dissolved. The flask was placed against a dark coloured surface to make sure all the agarose granules were dissolved. Once the agarose granules dissolved, the gel solution was left to cool down until it was lukewarm. 0.5µl of gel red was added and swirled into the gel solution. This is to view the resultant amplification band under UV rays. A gel casting tray was prepared by clipping 2 rubber enclosures onto each open side of the gel tray. The casting gel tray was also fitted with a 16 well comb to make 16 hollow perforations for the PCR samples to be loaded into. The gel solution was then poured ontothe gel casting tray. The gel was left to set for an hour.

An electrophoresis tank was set up following the completion of PCR reaction. 2 rubber enclosures and the comb were removed from the gel tray which contained the set gel. The set gel was placed into the electrophoresis tray and immersed in 0.5 x TBE buffer. The buffer level above the gel was about 2 mm.

Once the arrangement of the electrophoresis tank was complete, the electrophoresis tank was oriented into the correct position with the correct anodes connected so that negatively charged DNA travel towards the positively charged anode. Care was taken to ensure not to cause movement to the electrophoresis tank loaded with samples as slight movement can cause the loaded samples to be dispersed. Hence the electrophoresis tray was set up near a power pack, which conducted the electrophoresis. Loading of the samples began with 5µl of human DNA ladder, following with 5µl of the experimental samples and control which were taken out of the PCR thermocycler after successful completion of PCR amplification. The remaining samples in the Eppendorf tubes were frozen in the refrigerator below -20°C. After loading the samples, the electrophoresis tank lid was placed and the cathode and anode were connected carefully and slowly (so to cause as little movement as possible). The electrophoresis tank was then connected to a power pack. The power pack was adjusted with a Volt of 70, current of 400 and time frame of 90 minutes. This programming of electrophoresis was maintained for all reactions.

After the completion of electrophoresis, the gel tray was taken out and placed on a sheet of drying cloth to absorb any running buffer dripping from the gel tray. The gel tray was then placed into a UV imaging system via which the amplification bands were viewed. Controlling the exposure of the view aided in revealing how thick each bands were, thereby also aided in determining which temperature the primers performed well at.

1.7 PCR product purification

Purification of PCR product is a 3 step procedure involving the addition of the correct buffer and in the right amount to purify the PCR product. This is a very critical step as adding the wrong buffer can result in impairment of the PCR product. The purpose of purification is to eliminate any un-sequenced DNA, unused primers or primer dimer compounds the maximum possible from the PCR product, which can influence the following procedures of DNA quantification and Sanger sequencing results.

11 Eppendorf tubes containing the successfully amplified PCR product obtained at temperature 52 °C were chosen for purification. Each tube originally contained 20µl of the reaction mixture. When loading the samples onto the gel for electrophoresis, 5µl of sample was pipetted into the perforations. Hence that should leave each Eppendorf tubes containing 15µl of the remaining sample. However this was not the case. The sample from each tube had to be quantified individually. The record of the volume has been shown in 6.2 of the result section.

After obtaining a total volume of PCR product in a tube, 5 times the volume of the total PCR product of Buffer PB was added to the PCR product. A quick column was set up by placing it into a 2 ml collection tube. This is to get rid of any un-sequenced DNA, unused primers or primer dimer compounds which can affect the quality of Sanger sequencing results. The PCR product and buffer Mix was transferred onto the quick column and centrifuged for 60 seconds for the elutant to collect in the collection tube. After 60 seconds of centrifuging, the collected elutant was thrown away and the quick column was replaced again for the second stage of purification.750µl of Buffer PE was pipetted into the quick column and centrifuged for 60 seconds. The elutant collected in the collection tube was thrown away and the quick column was replaced again in the collection tube. The process of centrifuging was repeated again one more time and the elutant collected in the collection tube was thrown away. The quick column was now placed in a clean 1.5 ml centrifuge tube. 50µl of Buffer EB was pipetted into the quick column and placed for centrifuge for 60 seconds. After 60 seconds, the 1.5 ml centrifuge collecting tube contained the purified PCR product collected.

1.8 Quantification of PCR products

DNA quantification was performed in order to prepare the purified PCR products for sequencing. The quantification involved diluting the purified PCR product to 5ng/µl. The 5ng/µl was required to be contained within 15µl of volume. A sophisticated equipment that was used to analyse the sample was Biodrop. The analysis involved inserting 2µl of buffer EB as blank sample into a miniscule cavity in the machine to bring the scale of the machine to 0. After the 0 reading the drop was wiped by soft piece of tissue. Following this 2 µl of the purified PCR product was inserted into the same place. A reading figure in µg/ml unit was obtained. The µg/ml unit reading is equal to the same number of ng/µl units. The amount of needed to dilute the concentration of the sample was calculated using this formula:

$C_1 \times V_1 = C_2 \times V_2$

A ng/µl x V_1 = 5 ng/µl x 15µl

$V_1 =$ //

5×15

$V_1 =$

1.9 Sanger sequencing

DNA sequencing of purified PCR products were performed by MWG Eurofins for DNA sequencing. Electropherograms were then obtained for subsequent analysis.

Data Presentation and Discussion

PCR Optimisation

Although the designed PCR using Primer3 software is supposed to be specific for the DNA region of interest, it was important to ensure that the amplification efficiency is high. In order to do so, an optimisation strategy was employed, where the PCR annealing

temperature was optimised. Therefore, four samples were amplified using four different temperatures in duplicate. A total of 32 different reactions were performed, out of which eight reactions were performed a day. The resultant 8 amplification bands for each reaction a day is shown by Figure 6a, b, c and d with the exception of 6c, where only one reaction failed.

As pointed in Figure 6, the unused primers are shown as very faint bands at the bottom of the gel. In general, it was believed that the amplification was very specific as there was no unspecific amplicons observed, which would otherwise be indicated by the presence of other bands in the gel. Thereby the optimisation was not very laborious because the primer design seemed to be very specific.

The amplification efficiency was very good using all temperatures with only minor differences observed. From Figure 6a & 6b it is thought that the intensity of the amplification band at temperature 52°C was the most intense than the ones at 51°C, 53°C and 54°C. Since only one temperature had to be selected, 52°C was chosen as the ideal annealing temperature.

For the selected temperature of 52°C, 4 samples were run in duplicates to confirm that the annealing temperature was the right temperature. All 8 amplification bands indicated that the PCR amplification was successful as PCR products were run into agarose gel as shown in Figure 7.

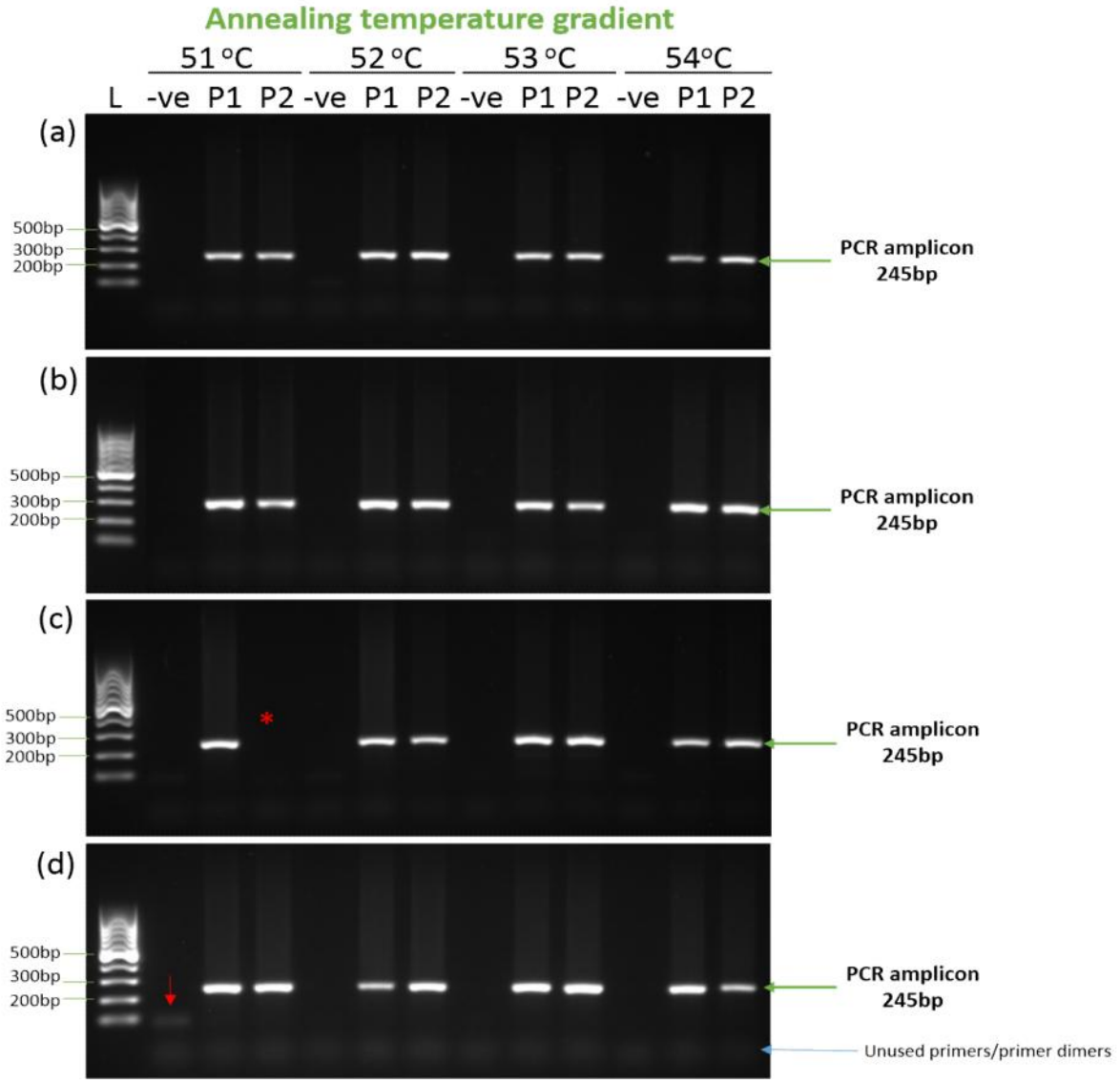

Figure 1. PCR optimisation of the ABCC6 designed fragment using an annealing temperature gradient (51 to 54 °C) in four different reactions (a, b, c and d).

As shown, the expected PCR amplicon (245 bp) was observed using all temperatures without the presence of non-specific PCR products. However, taking into account the results of all four reactions, it was concluded that the amplification efficiency was higher at 52 °C. The red asterisk indicates a failed PCR reaction, while the red arrow shows a non-specific band (~100 bp), which is suspected to be due to primer dimers. Lastly, the faint bands at the bottom of the gel image correspond to unused primers. L: DNA Ladder, P1: Placental DNA replicate 1, P2: Placental DNA replicate 2, -ve: negative PCR control (Water).

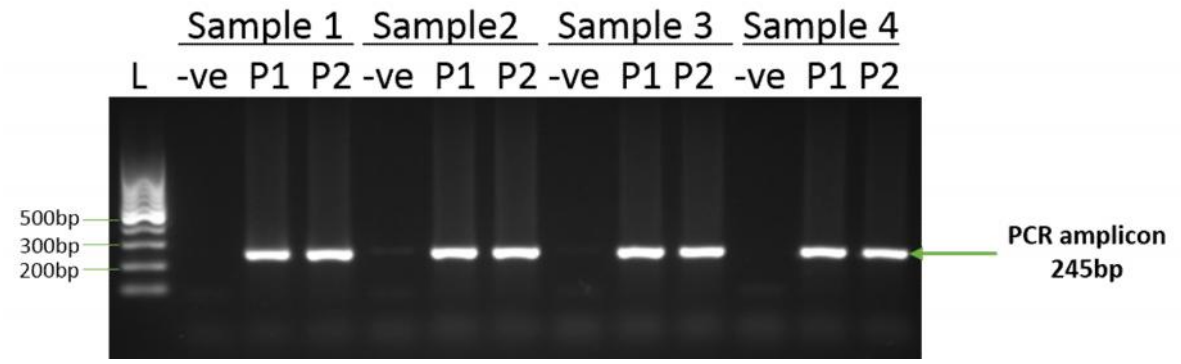

Figure 2. PCR amplification of the ABCC6 designed fragment (annealing temperature of 52 °C) in four placental DNA samples (1-4) analysed in duplicate.

As shown, the expected PCR amplicon (245 bp) was observed in all four samples .L: DNA Ladder, P1: Placental DNA replicate 1, P2: Placental DNA replicate 2, -ve: negative PCR control (Water).

Concentration of PCR product

Prior to DNA sequencing, it is important to evaluate the quality and quantity of the obtained PCR products. For this experiment, PCR amplicons from a total of 11 successful PCR reactions were combined and resulted in a final volume of 113µl (Table 3). Although the recovered amount of each PCR reaction should have been 15 µl (5 µl had been used for the visualisation of the products), for most samples this volume was smaller. It is believed that this could be due to potential pipetting errors or possible evaporation during the PCR reaction. The final volume of 113µl was used for the purification of the combined PCR product. Following purification, the final volume was 50µl of PCR product.

Table 4. PCR product pooling strategy

Series of tubes	Maximum possible volume quantified in each tube in µl
PCR amplicon 1	7.5
PCR amplicon 2	11.5
PCR amplicon 3	11
PCR amplicon 4	11
PCR amplicon 5	11
PCR amplicon 6	11
PCR amplicon 7	10
PCR amplicon 8	10
PCR amplicon 9	10
PCR amplicon 10	10
PCR amplicon 11	10
Total	**113**

It was important to obtain the exact concentration of this purified product. In order to do so, the concentration was obtained using the BioDrop as described in the methods. Using 2 µl of purified PCR sample, a concentration of 46.66µg/ml (46.66ng/µl) was obtained. The purity of the PCR sample were also assessed by the A260/A280 ratio obtained by the BioDrop. It is known that the A260/A280 ratio can act as an indication of the protein purity in the presence of nucleic acids; a pure DNA sample is expected to have a A260/A280 ratio greater or equal to 1.8. For our sample, the observed ratio was 1.81, therefore it was concluded that the combined PCR product had a good purity for downstream analysis.(Ogt.co.uk, 2018)

In order to achieve the desired final concentration of 5ng/µl in a volume of approximately 15µl, the following calculations were used:

C_1 x V_1 = C_2 x V_2, where **C_1** represents **Concentration 1,** **V_1**represents **Volume 1,** **C_2**represents **Concentration 2** and **V_2**represents **Volume 2**

46.66ng/µl x V_1 = 5 ng/µl x 15µl

$$V_1 = 5\frac{ng}{\mu l} \times 15\mu l \Big/ \frac{46.66ng}{\mu l}$$

V_1 = 1.607372482µl

The value of 1.607372482µl was rounded to 1.61µl

In order to make up total volume of 15µl. The following calculation was carried out:
15µl – 1.61 µl = 13.39 µl ~ 13.40µl

DNA Sequencing of the purified PCR product

Following Sanger sequencing performed by Eurofins Genomics, resulting electropherograms were provided. Although the sequencing was performed on both strands of the PCR product using both the forward and reverse primer, only the forward reaction was successful. A blank electropherogram was obtained by using the reverse primer, therefore only the results by using the forward primer are presented here.

Investigating the electropherogram as shown in Figure 5, when comparing the obtained sequence with the reference sequence from Ensembl, a total of two nucleotide differences were discovered. The nucleotide sequences were compared and the differences are highlighted in specific colours as shown below:

Reference sequence (Ensembl) (5'→ 3'):

TGGAGTTTATTGGTGATCCCAAGCCTCCAGCC
TGGAAGGGCTACCTCCTCGCCGTGCTGATGTT
CCTCTCAGCCTGCCTGCAAACGCTGTTTGAGC
AGCAGAACATGTACAGGCTCAAGGTGCTGCA
GATGAGGTTGCGGTCGGCCATCACTGGCCTGG
TGTACAGAAAGGTGAGCCCTGGGGGACAAGG
GCAGGTCTTCC

Obtained sequence (Sample) (5'→ 3'):

TGGAGTTTATTGGTGATCCCAAGCCTCCAGCC
TGGAAGGGCTACCTCCTCGCCGTGCTGATGTT
CCTCTCGGCCTGCCTGCAAACGCTGTTTGAGC
AGCAGAACATGTACAGGCTCAAGGTGCTGCA
GATGAGGCTGCGGTCGGCCATCACTGGCCTGG
TGTACAGAAAGGTGAGCCCTGGGGGACAAGG
GCAGGTCTTCC

Highlights:

X – Original mutation of interest
X – Previously reported mutation
X – Observed possible mutations

As shown above, in this experiment we failed to confirm the mutation under investigation. The same was observed for a previously reported disease-causing mutation in the literature (the nucleotide base highlighted in blue colour) (Nitschke *et al.*, 2012).Additionally, it should be noted that none of the two nucleotide differences observed in this study have been previously mentioned as a mutation in the literature. However, when taking a closer look at the electropherogram in Figure 9, for both positions (84 and 148) there was an overlap of two dyes (one corresponding to the expected base). This indicates that both A and G (position 84) and C and T (position 148) were added during the sequencing reaction.

The low height of the peaks together with the in-built poor quality of the called nucleotide bases flagged from the software (indicated by the red & yellow lines) raise doubts whether the two nucleotide differences are true events. However, it cannot be excluded that the peak overlap could also indicate that there is a mixture of DNA sequences in the combined

PCR sample, meaning that only some of the samples have the mutation. Although these poor sequencing resultsindicate the need to repeat the Sanger sequencing reactions, this was not possible as part of the current study. Furthermore, repeating the sequencing reaction using the reverse primer would also confirm the observations above.

To further confirm the presence of the two possible mutations, the BLAST alignment tool was employed (obtained from: http://blast.ncbi.nlm.nih.gov/Blast.cgi) as shown in Figure 10. Both nucleotide locations were the only two that did not match the reference sequence.

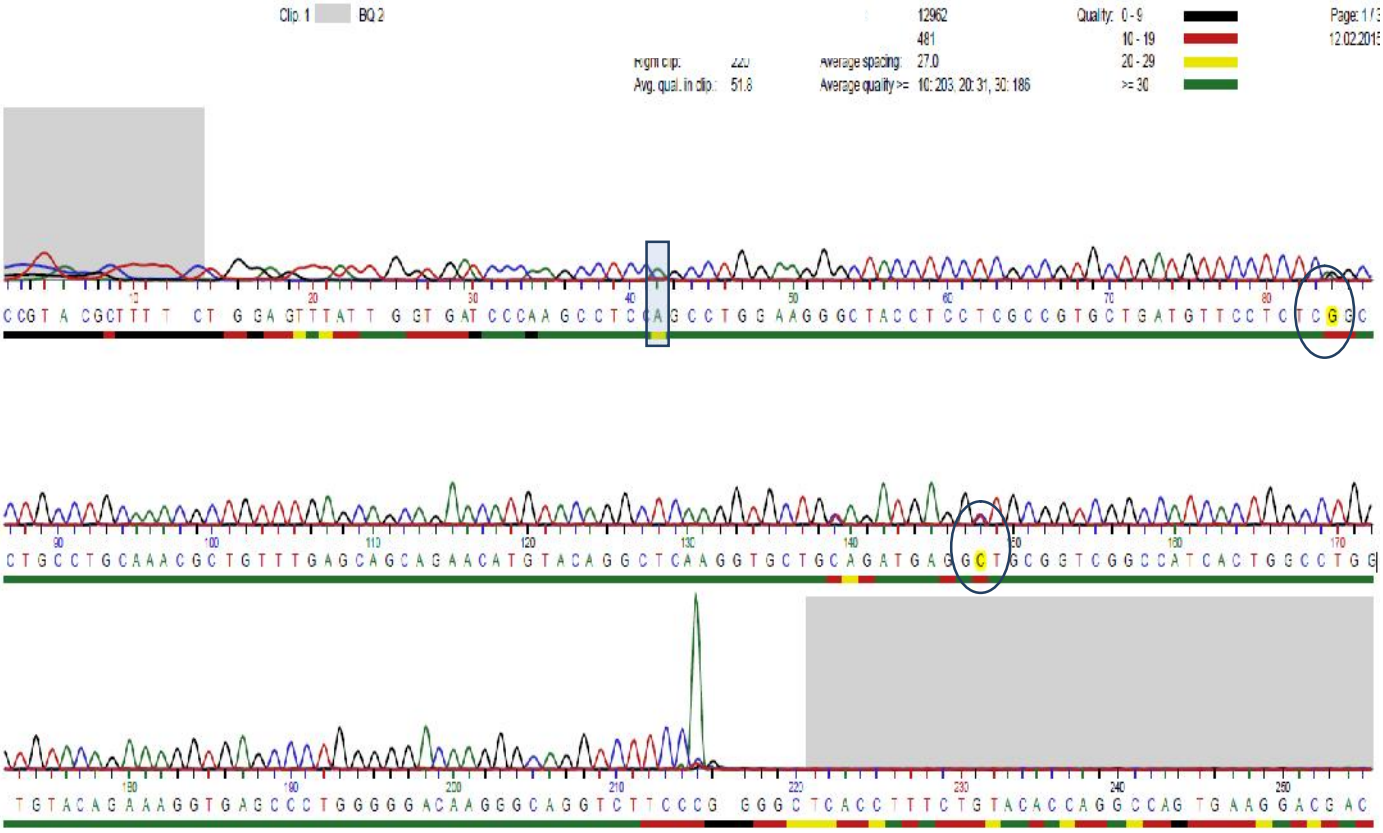

Figure 3. Electropherogram showing the obtained DNA sequence of the ABCC6 PCR amplicon (forward strand)

Sanger sequencing data where each peak corresponds to a specific nucleotide (A-green, C-blue, T-red and G-black). Coloured bars underneath the sequence correspond to the instrument's internal quality control. As shown, with the exception of the first few nucleotides, most of them have been successfully called (Quality ≥30). The transparent blue box indicates the position of the potential mutation of interest. Nucleotides encircled and highlighted in yellow (G – position – 84, C – position – 148) indicate observed mutations (DNA sequence variations) when compared to the original sequence obtained from the Ensemble genome browser.

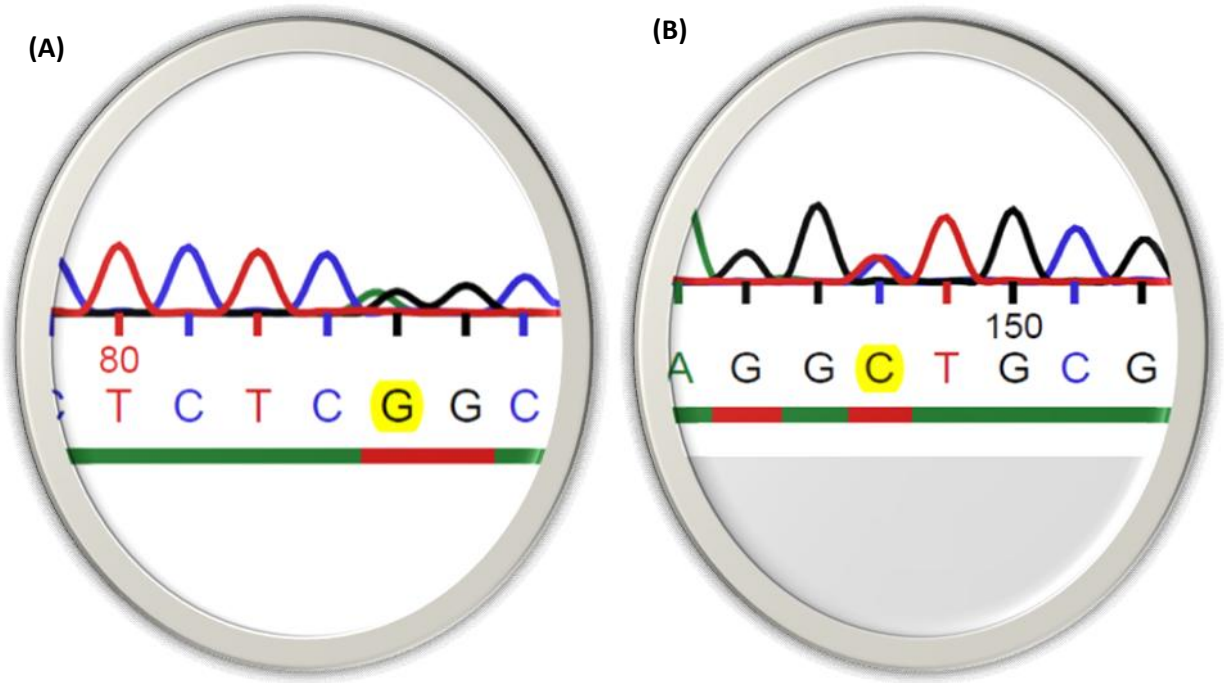

Figure 4. Observed possible mutations (A and B) following sequencing of the purified PCR sample.

(A) **Position 84**: Overlapping of green (A) and black (G) dyes (approximately the same peak height) but software has called 'G' and (B) **Position 148**: Overlapping of red (T) and blue (C) dyes (approximately the same peak height) but software has called 'C'.

Range 2: 16201970 to 16202170 GenBank Graphics Next Match Previous Match First Match

Score	Expect	Identities	Gaps	Strand
361 bits(195)	2e-97	199/201(99%)	0/201(0%)	Plus/Minus

Features: multidrug resistance-associated protein 6 isoform 1
multidrug resistance-associated protein 6 isoform X1

```
Query  1         TGGAGTTTATTGGTGATCCCAAGCCTCCAGCCTGGAAGGGCTACCTCCTCGCCGTGCTGA  60
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16202170  TGGAGTTTATTGGTGATCCCAAGCCTCCAGCCTGGAAGGGCTACCTCCTCGCCGTGCTGA  16202111

Query  61        TGTTCCTCTCGGCCTGCCTGCAAACGCTGTTTGAGCAGCAGAACATGTACAGGCTCAAGG  120
                 |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16202110  TGTTCCTCTCAGCCTGCCTGCAAACGCTGTTTGAGCAGCAGAACATGTACAGGCTCAAGG  16202051

Query  121       TGCTGCAGATGAGGCTGCGGTCGGCCATCACTGGCCTGGTGTACAGAAAGGTGAGCCCTG  180
                 |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16202050  TGCTGCAGATGAGGTTGCGGTCGGCCATCACTGGCCTGGTGTACAGAAAGGTGAGCCCTG  16201991

Query  181       GGGGACAAGGGCAGGTCTTCC  201
                 |||||||||||||||||||||
Sbjct  16201990  GGGGACAAGGGCAGGTCTTCC  16201970
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Figure 5. Sequence comparison using the NCBI BLAST tool.

The top strand represents the sequence obtained from the combined PCR sample, while the bottom one is the reference genomic sequence. As shown, the two observed nucleotide differences (highlighted in red boxes) were confirmed using the basic local alignment search tool.

Conclusion

The main purpose of the study was to investigate a potential previously reported mutation that is believed to be involved in the disease process of PXE and GACI. To achieve this goal, placental DNA was amplified using in-house designed primers that were able to bind to the genomic area of interest. The design of the primers was accomplished using the Primer3 software as described in the methods section. However, the proposed PCR was also optimised using an annealing temperature gradient.

There are a number of factors known to influence the quality and quantity of the resulting PCR amplicons, including annealing temperature, primer length and concentration as well as salt and $MgCl_2$ concentration. In this experiment, the annealing temperature was the prime focus during the optimisation of PCR. The annealing temperature is a very crucial aspect of PCR reaction since it influences the binding of the primers onto the DNA. It is mainly determined from the DNA sequence of the primer. Since there are three hydrogen bonds per C-G pair (while only two per A-T), DNA sequences rich in GC content require higher temperature to generate hydrogen bonds(Abstract available at/ oxford journals /Yakovchuk, Protozanova and Frank-Kamenetskii, 2006). Similarly, during denaturation, these sequences require higher energy to separate from each other (melting temperature)(Thornton and Basu, 2011).

The DNA sequence of the primer affects their performance. Firstly, in order to avoid mis-priming, the chosen primers did not include any poly-purine or poly-pyrimidine stretches(abstract available at : (Sakamoto *et al.*, 1996). Also, their GC content was relatively high, namely 55% and 61.1% for the forward and reverse primer respectively, which nevertheless fall into the optimal GC% range suggested by the software (50%). The melting temperatures (T_m) obtainedfor each individual primer (59.4°C for the reverse primer and 58.2°C for the forward primer) did not significantly differ (just by 1.2° C). Hence, using the formula described in the PCR optimisation section of the methodology, the calculated average annealing temperature was 53.8°C, which was rounded to 54° C. Thereby, as part of the optimisation of the annealing temperature, a gradient including four different temperatures (51°C, 52°C, 53°C, 54°C) were applied. As shown from the results **[Figure 6]**, although all temperatures resulted in a successful amplification, it was believed that 52°C was the optimal one, which was then used to amplify the samples **[Figure 7].**

However, the amplification of the proposed region could have been further optimised by investigating the additional factors mentioned earlier. It is known that the length of the primers is also an important factor as it affects their specificity to a particular DNA area(Stock, 2009). The longer the primer is, the better its specificity, thereby ensuring there is no non-specific binding apart from the target region. The length of the primers employed in this investigation were within the proposed optimal range (18 – 24 nucleotides) (forward – 20bp, reverse – 18bp), also recommended by Stock(2009). Although in this experiment, only the target region was amplified, the primer length could have been adjusted (longer sequence) in case of a non-specific signal. Additionally, the primer concentration could have been altered in case of a weak amplification efficiency.

Another factor that plays a crucial role in succeeding an efficient DNA amplification is the concentration of magnesium chloride ($MgCl_2$). $MgCl_2$is the main co-factor of the enzyme Taq DNA polymerase, therefore necessary for a successful amplification (Pelt-Verkuil, Belkum and Hays, 2008). It enhances the polymerising activity of Taq DNA polymerase and the rate of nucleotide incorporation, hence the greater the concentration of $MgCl_2$ the more the yield of the DNA amplification product will be. However, one should be noted that if the $MgCl_2$ concentration is too high, there is a risk of non-specific amplification (Pelt-Verkuil, Belkum and Hays, 2008).

Lastly, it is evident that the thermal cycling conditions play a significant role in the process of amplification by DNA polymerase. Apart from the annealing temperature itself, the duration of each individual step in the amplification cycle could have been changed if needed. For example, amplifying longer sequences often require a longer extension step or GC-rich sequences often require longer denaturation step (up to 96^{o}C). Finally, although the number of 30 PCR cycles was thought to be adequate considering the starting DNA material, it could be increased (up to 40) in case that a weak PCR amplicons was obtained.

Over all the results indicate the success of this investigation ,however possible procedural errors while handling the reacting components could have introduced possible degree of inaccuracies i.e.

pipetting errors. Pipetting was performed throughout the whole investigations and might have potentially influenced certain aspects of the investigation. For instance, when loading the gel and pooling strategy. As can be seen from the results, the amplicons for temperature 54 appear thin at Figure. 3a and 3c but intense at 3.c, which implies to inadequate pipetting.

Although the PCR optimisation was successful and a specific amplicons was detected, only sequencing results using the forward primer were obtained. The fact that the sequencing reaction on the other strand using the reverse primer did not work could be due to various reasons. Firstly, the PCR products were pooled and quantified using the BioDrop. It is known that this quantification method is not very specific as it can also measure unused primers and nucleotides, therefore overestimating the DNA concentration. However, since the reaction using the forward strand gave positive sequencing results, it is believed that it could more likely be due to pipetting errors. Additionally, errors while preparing the sequencing reactions cannot be excluded, however it is not possible to assess this possibility since the applied protocol is not known. For example, in order for the primer to bind and initiate the sequencing process, it is essential to apply the right temperature; therefore, the failure could have been due to unsuccessful binding of the reverse primer. From the results presented in Figure 8, it can be noted that the peak height of the added nucleotides were not very high, indicating a suboptimal sequencing reaction. Further research would involve sending the purified PCR product to a different sequencing company to repeat the sequencing reactions to further investigate the two detected single nucleotide polymorphisms. Finally it would also involve sequencing each DNA sample individually instead of pooling all the DNA samples together for sequencing to investigate which sample had the mutation

References

Bécuwe, C., Dalle, S., Ronger-Savlé, S., Skowron, F., Balme, B., Kanitakis, J. and Thomas, L. (2005). Elastosis perforans serpiginosa Associated with Pseudo-Pseudoxanthoma elasticum during Treatment of Wilson’s Disease with Penicillamine. *Dermatology*, 210(1), pp.60-63. NCBI [Online]. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15604549 [Accessed on 7 April 2018]

Belinsky, M. and Kruh, G. (1999). MOAT-E (ARA) is a full-length MRP/cMOAT subfamily transporter expressed in kidney and liver. *Br J Cancer*, [online] 80, pp.1342–1349. Available at: http://www.scopus.com/record/display.url?eid=2-s2.0-0033020785&origin=inward&txGid=03484B30321553F86AB12142C3E475EE.iqs8TDG0Wy6BURhzD3nFA%3a1 [Accessed 10 Apr. 2018].

Belinsky, M., Chen, Z., Shchaveleva, I., Zeng, H. and Kruh, G. (2002). Characterization of the Drug Resistance and Transport Properties of Multidrug Resistance Protein 6 (MRP6, ABCC6). *Cancer Research*, [online] 62, pp.6172–6177. Available at: http://cancerres.aacrjournals.org/content/62/21/6172.full.pdf+html [Accessed 21 Mar. 2018].

Bercovitch, L., Martin, L., Chassaing, N., Hefferon, T., Bessis, D., Vanakker, O. and Terry, S. (2011). Acquired pseudoxanthoma elasticum presenting after liver transplantation. *Journal of the American Academy of Dermatology*, 64(5), pp.873-878. Science Direct [Online]. Available at: http://ac.els-cdn.com/S0190962210003841/1-s2.0-S0190962210003841-main.pdf?_tid=c56317a0-e4e0-11e4-83f2-00000aacb361&acdnat=1429261656_0ee77ef0aa3b3307919a507cf9ff5830 [Accessed on 3 April 2018]

Borst, P., Evers, R., Kool, M. and Wijnholds, J. (2000). A Family of Drug Transporters: the Multidrug Resistance-Associated Proteins. *JNCI Journal of the National Cancer Institute*, 92(16), pp.1295-1302. NCBI [Online]. Abstract available at: http://www.ncbi.nlm.nih.gov/pubmed/10944550 [Accessed on 4 April 2018]

Chassaing, N., Martin, L., Calvas, P. and et al., (2005). Pseudoxanthoma elasticum: a clinical, pathophysiological and genetic update including 11 novel ABCC6 mutations. *Journal of Medical Genetics*, 42(12), pp.881-892. NCBI [Online]. Abstract available at: http://www.ncbi.nlm.nih.gov/pubmed/15894595 [Accessed on 4 April 2018]

Clinicaltrials.gov, (2018). *Magnesium Supplements In The Treatment Of Pseudoxanthoma Elasticum (PXE) - Full Text View - ClinicalTrials.gov*. [online] Available at: http://clinicaltrials.gov/show/NCT01525875 [Accessed 13 Apr. 2018].

Coatesworth, A., Darnton, S., Green, R., Cayton, R. and Antonakopoulos, G. (1998). A case of systemic pseudo-pseudoxanthoma elasticum with diverse symptomatology caused by long-term penicillamine use. *Journal of Clinical Pathology*, 51(2), pp.169-171. Abstract available at:

http://www.ncbi.nlm.nih.gov/pubmed/9602696 [Accessed on 8 April 2018]

Crespi, G., Derchi, L. and Saffioti, S. (1991). Sonographic detection of renal changes in pseudoxanthoma elasticum. *Urol Radiol*, 13(1), pp.223-225. NCBI [Online]. Abstract available at : http://www.ncbi.nlm.nih.gov/pubmed/1598746 [Accessed on 3 April 2018]

Dabisch-Ruthe, M., Kuzaj, P., Götting, C., Knabbe, C. and Hendig, D. (2014). Pyrophosphates as a major inhibitor of matrix calcification in Pseudoxanthoma elasticum. *Journal of Dermatological Science*, 75(2), pp.109-120. Science Direct [Online]. Available at: http://ac.els-cdn.com/S0923181114001121/1-s2.0-S0923181114001121-main.pdf?_tid=4dfcbbb4-e4e3-11e4-a8c0-00000aab0f02&acdnat=1429262744_c9ac4598b2263306330782ab217b8143 [Accessed on 5 April 2018]

Dean, M., Rzhetsky, A. and Allikmets, R. (2001). The Human ATP-Binding Cassette (ABC) Transporter Superfamily. *Genome Research*, 11(7), pp.1156-1166. NCBI [Online]. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2752038/ [Accessed on 28 March 2018]

Deeley, R. and Cole, S. (1997). Function, evolution and structure of multidrug resistance protein (MRP). *Seminars in Cancer Biology*, 8(3), pp.193-204. NCBI [Online]. Avaialable at: http://www.ncbi.nlm.nih.gov/pubmed/9441948 [Accessed on 7 April 2018]

Fabbri, E., Forni, G., Guerrini, G. and Borgna-Pignatti, C. (2009). Pseudoxanthoma elasticum-like syndrome and thalassemia: An update. *Dermatology Online Journal*, [online] 15(7). Available at: https://escholarship.org/uc/item/1cw031gj [Accessed 11 Apr. 2018].

Fah, L. (1991). Pseudoxanthoma elasticum—a visual diagnosis. *Schweiz Med Wochenschr*, 121, pp.660–663. NCBI [Online]. Abstract available at: http://www.ncbi.nlm.nih.gov/pubmed/2047826 [Accessed on 12 April 2018]

Finger, R., Issa, P., Ladewig, M., Götting, C., Szliska, C., Scholl, H. and Holz, F. (2009). Pseudoxanthoma Elasticum: Genetics, Clinical Manifestations and Therapeutic Approaches. *Survey of Ophthalmology*, 54(2), pp.272-285. Science Direct [Online]. Available at: http://ac.els-cdn.com/S0039625708002580/1-s2.0-S0039625708002580-main.pdf?_tid=d6b89dd2-e4e4-11e4-bbbb-00000aacb361&acdnat=1429263403_8bfb795af3189ca8d841399241e301b9 [Accessed on 11 April 2018]

Genome.gov, (2014). *All About The Human Genome Project (HGP)*. [online] Available at: http://www.genome.gov/10001772 [Accessed 10 Apr. 2018].

Gheduzzi, D., Taparelli, F., Quaglino, D., Di Rico, C., Bercovitch, L., Terry, S., Singer, D. and Pasquali-Ronchetti, I. (2001). The Placenta in Pseudoxanthoma Elasticum: Clinical, Structural and Immunochemical Study. *Placenta*, 22(6), pp.580-590. Science direct [Online]. Available at: http://ac.els-cdn.com/S0143400401906873/1-s2.0-S0143400401906873-main.pdf?_tid=1bed283c-e4e5-11e4-be04-00000aab0f27&acdnat=1429263519_ae1a88fe919f6283863bb2b534263e12 [Accessed on 13 April 2018]

Gliem, M., Zaeytijd, J., Finger, R., Holz, F., Leroy, B. and Issa, P. (2013). An update on the ocular phenotype in patients with pseudoxanthoma elasticum. *Front. Genet.*, 4. NCBI [Online]. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3617449/ [Accesed on 13 April 2018]

Hacker, S., Ramos-Caro, F., Beers, B. and Flowers, F. (1993). Juvenile Pseudoxanthoma Elasticum: Recognition and Management. *Pediatr Dermatol*, 10(1), pp.19-25. NCBI [Online]. Abstract available at: http://www.ncbi.nlm.nih.gov/pubmed/8493160 [Accessed on 7 April 2018]

Hamlin, N., Beck, K., Bacchelli, B., Cianciulli, P., Pasquali-Ronchetti, I. and Le Saux, O. (2003). Acquired Pseudoxanthoma elasticum-like syndrome in beta-thalassaemia patients. *British Journal of Haematology*, 122(5), pp.852-854. NCBI [Online]. Abstract available at: http://www.ncbi.nlm.nih.gov/pubmed/12930400 [Accessed on 13 April 2018]

Hefti, M. and Beck, A. (2014). The Human Genome Project and Personalized Medicine. *Reference Module in Biomedical Sciences*, [online] pp.3418–3422. Abstract available at: http://www.sciencedirect.com/science/article/pii/B9780123864567066041 [Accessed 10 January 2018].

Higgins, C. (1992). ABC Transporters: From Microorganisms to Man. *Annual Review of Cell Biology*, 8(1), pp.67-113.

Hoffbrand, A., Moss, P. and Pettit, J. (2006). *Essential haematology*. Malden, Mass.: Blackwell Pub.

Hu, X., Plomp, A., van Soest, S., Wijnholds, J., de Jong, P. and Bergen, A. (2003). Pseudoxanthoma elasticum: a clinical, histopathological, and molecular update. *Survey of Ophthalmology*, 48(4), pp.424-438. Avaiable at :
http://ac.els-cdn.com/S0039625703000535/1-s2.0-S0039625703000535-main.pdf?_tid=4d380a36-e4e7-11e4-a5b1-00000aacb360&acdnat=1429264461_138c94a9c905b4ec9e00cad069508a06[Accessed 15 April 2018]

Ilias, A., Urban, Z., Seidl, T., Le Saux, O., Sinko, E., Boyd, C., Sarkadi, B. and Varadi, A. (2002). Loss of ATP-dependent Transport Activity in Pseudoxanthoma Elasticum-associated Mutants of Human ABCC6 (MRP6). *Journal of Biological Chemistry*, [online] 277(19), pp.16860-16867. Available at: http://www.scopus.com/record/display.url?eid=2-s2.0-0037053355&origin=inward&txGid=D940AEC51A79DAB5AA15533940D90ABA.FZg2ODcJC9ArCe8WOZPvA%3a41 [Accessed 10 Apr. 2018].

Issa, P., Finger, R., Götting, C., Hendig, D., Holz, F. and Scholl, H. (2010). Centrifugal Fundus Abnormalities in Pseudoxanthoma Elasticum. *Ophthalmology*, 117(7), pp.1406-1414. [Online]. Abstract available at:
http://www.aaojournal.org/article/S0161-6420(09)01296-2/abstract [Accessed 11 April 2018]

Jiang, Q., Takahagi, S. and Uitto, J. (2012). Administration of Bone Marrow Derived Mesenchymal Stem Cells into the Liver: Potential to Rescue Pseudoxanthoma Elasticum in a Mouse Model (Abcc6−/−). *Journal of Biomedicine and Biotechnology*, 2012, pp.1-11. [Online]. Available at:
http://www.hindawi.com/journals/bmri/2012/818937/ [Accessed on 4 April 2018]

Kool, M., van der Linden, A., de Haas, M. and et al, (1999). Expression of human MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissues and cancer cells. *Cancer Res*, [online] 59, pp.175–182. Available at: http://www.scopus.com/record/display.url?eid=2-s2.0-0032893268&origin=inward&txGid=03484B30321553F86AB12142C3E475EE.iqs8TDG0Wy6BURhzD3nFA%3a11 [Accessed 10 Apr. 2018].

Kool, M., van der Linden, M., De Haas, M., Baas, F. and Borst, P. (1999). Expression of Human MRP6, a Homologue of the Multidrug Resistance Protein Gene MRP1, in Tissues and Cancer Cells. *Cancer Research*, [online] 59, pp.175–182. Available at: http://cancerres.aacrjournals.org/content/59/1/175.full.pdf+html [Accessed 21 Mar. 2018].

Korn, S., Selinacht, J., Huth, C. and Feller, A. (1987). Cardiovascular manifestation of pseudoxanthoma elasticum (Gronblad-Strandberg-syndrome). *Thorac Cardiovasc Surg*, 35, pp.191–194. Europe PubMed Central [Online]. Abstract available at:
http://europepmc.org/abstract/MED/2442842 [Accessed on 1st April 2018]

Le Saux, O., Bunda, S., VanWart, C., Douet, V., Got, L., Martin, L. and Hinek, A. (2006). Serum Factors from Pseudoxanthoma Elasticum Patients Alter Elastic Fiber Formation In Vitro. *J Investig Dermatol*, 126(7), pp.1497-1505. Nature [Online]. Available at:
http://www.nature.com/jid/journal/v126/n7/full/5700201a.html [Accessed on 30 March 2018]

Le Saux, O., Martin, L., Aherrahrou, Z., Leftheriotis, G., Váradi, A. and Brampton, C. (2012). The molecular and physiological roles of ABCC6: more than meets the eye. *Frontiers in Genetics*, 3. NCBI [Online]. Available at:
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3520184/ [Accessed on 27 March 2018]

Le Saux, O., Urban, Z., Tschuch, C., Csiszar, K., Bacchelli, B. and et al., (2018). Mutations in a gene encoding an ABC transporter cause pseudoxanthoma elasticum. *Nature Genetics*, [online] 25, pp.223-227. Available at: http://www.nature.com/ng/journal/v25/n2/full/ng0600_223.html#a2 [Accessed 18 Feb. 2018].

Le Saux, O., Urban, Z., Tschuch, C., Csiszar, K., Bacchelli, B., Quaglino, D. and et al.,, (2000). Mutations in a gene encoding an ABC transporter cause pseudoxanthoma elasticum. *Nat Genet*, 25(2), pp.223–227. Abstract available at:
http://www.ncbi.nlm.nih.gov/pubmed/10835642 [Accessed on 23 March 2018]

Lee, P., Costumbrado, J., Hsu, C. and Kim, Y. (2012). Agarose Gel Electrophoresis for the Separation of DNA Fragments. *Journal of Visualized Experiments*, (62). NCBI [Online]. Abstract available at:
http://www.ncbi.nlm.nih.gov/pubmed/22546956 [Accessed on 10 April 2018]

Lever, W., Elder, D. and Elenitsas, R. (1997). Congenital diseases (Genodermatoses). *Histopathology of the Skin*, chap 6J.B.Lippincott Company, Philadelphia (1997), pp.117–151.

Li, Q. and Uitto, J. (2013). Mineralization/Anti-Mineralization Networks in the Skin and Vascular Connective Tissues. *The American Journal of Pathology*, 183(1), pp.10-18. Science Direct [Online]. Available at: http://ac.els-cdn.com/S0002944013002575/1-s2.0-S0002944013002575-main.pdf?_tid=c56541f0-e4eb-11e4-9f47-00000aab0f27&acdnat=1429266380_ead74ec2396a03ec9f04d6fc729d8677 [Accessed on 15 March 2018]

Li, Q., Jiang, Q. and Uitto, J. (2014). Ectopic mineralization disorders of the extracellular matrix of connective tissue: Molecular genetics and pathomechanisms of aberrant calcification. *Matrix Biology*, 33, pp.23-28. Science Direct [Onlone]. Availbale at: http://ac.els-cdn.com/S0945053X13001005/1-s2.0-S0945053X13001005-main.pdf?_tid=0dde206e-e4ec-11e4-b09f-00000aacb35f&acdnat=1429266502_77f663409872bac6544a06c4f4ee9456 [Accessed on 16 March 2018]

Liang, S., Quirk, D. and Zhou, A. (2006). RNase L: Its biological roles and regulation. *IUBMB Life (International Union of Biochemistry and Molecular Biology: Life)*, [online] 58(9), pp.508-514. Available at: http://onlinelibrary.wiley.com/doi/10.1080/15216540600838232/pdf [Accessed 12 Apr. 2018].

Lorenz-Depiereux, B., Schnabel, D., Tiosano, D., Häusler, G. and Strom, T. (2010). Loss-of-Function ENPP1 Mutations Cause Both Generalized Arterial Calcification of Infancy and Autosomal-Recessive Hypophosphatemic Rickets. *The American Journal of Human Genetics*, 86(2), pp.267-272. NCBI [Online]. Abstract available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2820166/ [Accessed on 15 april 2018]

Marchionni, L. (2008). *Impact of gene expression profiling tests on breast cancer outcomes*. [Rockville, Md.: Agency for Healthcare Research and Quality. NCBI [Online . Available at: http://www.ncbi.nlm.nih.gov/books/NBK38451/ [Accessed on 18 March 2018]

Martin, L., Douet, V., VanWart, C., Heller, M. and Le Saux, O. (2011). A Mouse Model of β-Thalassemia Shows a Liver-Specific Down-Regulation of Abcc6 Expression. *The American Journal of Pathology*, 178(2), pp.774-783. NCBI [Online]. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3069908/ [Accessed on 12 March 2018]

Mendelsohn, G., Bulkley, B. and Hutchins, G. (1978). Cardiovascular manifestations of Pseudoxanthoma elasticum. *Arch Pathol Lab Med*, 102(1978), pp.298–302. NCBI [Online]. Abstract available at: http://www.ncbi.nlm.nih.gov/pubmed/580722 [Accessed on 6 February 2018]

Morita, M. and Imanaka, T. (2012). Peroxisomal ABC transporters: Structure, function and role in disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1822(9), pp.1387-1396. Science Direct [Online]. Available at: http://www.sciencedirect.com/science/article/pii/S0925443912000373 [Accessed on 20 February 2018]

Na, S., Choi, M., Kim, M., Lee, J. and Cho, S. (2010). Penicillamine-induced Elastosis Perforans Serpiginosa and Cutis Laxa in a Patient with Wilson's Disease. *Annals of Dermatology*, 22(4), p.468. NCBI [Online]. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2991731/ [Accesed on 7 February 2018]

Nature.com, (2014). *Scientists Can Make Copies of a Gene through PCR | Learn Science at Scitable*. [online] Available at: http://www.nature.com/scitable/topicpage/scientists-can-make-copies-of-a-gene-6525968 [Accessed 8 Apr. 2018].

Ncbi.nlm.nih.gov, (2014). *Polymerase Chain Reaction (PCR)*. [online] Available at: http://www.ncbi.nlm.nih.gov/probe/docs/techpcr/ [Accessed 8 Apr. 2018].

Nebert, D., Zhang, G. and Vesell, E. (2008). From Human Genetics and Genomics to Pharmacogenetics and Pharmacogenomics: Past Lessons, Future Directions. *Drug Metabolism Reviews*, 40(2), pp.187-224. NCBI [Online]. Available at : http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2752627/ [Accessed on 21 February 2018]

Nitschke, Y., Baujat, G., Botschen, U., Wittkampf, T., du Moulin, M., Stella, J., Le Merrer, M., Guest, G., Lambot, K., Tazarourte-Pinturier, M., Chassaing, N., Roche, O., Feenstra, I., Loechner, K., Deshpande, C., Garber, S., Chikarmane, R., Steinmann, B., Shahinyan, T., Martorell, L., Davies, J., Smith, W., Kahler, S., McCulloch, M., Wraige, E., Loidi, L., Höhne, W., Martin, L., Hadj-Rabia, S., Terkeltaub, R. and Rutsch, F. (2012). Generalized Arterial Calcification of Infancy and Pseudoxanthoma Elasticum Can Be Caused by

Mutations in Either ENPP1 or ABCC6. *The American Journal of Human Genetics*, 90(1), pp.25-39. Science Direct [Online]. Available at: http://ac.els-cdn.com/S0002929711004988/1-s2.0-S0002929711004988-main.pdf?_tid=8e063982-e4ee-11e4-b907-00000aacb35f&acdnat=1429267576_c2017aeed47593723d1d3e66ad2f3bdd [Accessed on 29 January 2018]

Ogt.co.uk, (2018). *Understanding and measuring variations in DNA sample quality*. [online] Available at: http://www.ogt.co.uk/resources/literature/483_understanding_and_measuring_variations_in_dna_sample_quality [Accessed 8 Apr. 2018].

Ousterout, D., Perez-Pinera, P., Thakore, P., Kabadi, A., Brown, M., Qin, X., Fedrigo, O., Mouly, V., Tremblay, J. and Gersbach, C. (2013). Reading Frame Correction by Targeted Genome Editing Restores Dystrophin Expression in Cells From Duchenne Muscular Dystrophy Patients. *Mol Ther*, [online] 21(9), pp.1718-1726. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23732986 [Accessed 9 Apr. 2018].

Paulusma, C., Bosma, P., Zaman, G., Bakker, C., Otter, M., Scheffer, G., Scheper, R., Borst, P. and Elferink, R. (1996). Congenital Jaundice in Rats with a Mutation in a Multidrug Resistance-Associated Protein Gene. *Science*, [online] 271(5252), pp.1126-1128. Available at: http://www.scopus.com/record/display.url?eid=2-s2.0-0029981430&origin=inward&txGid=D940AEC51A79DAB5AA15533940D90ABA.FZg2ODcJC9ArCe8WOZPvA%3a11 [Accessed 10 Apr. 2018].

Pelt-Verkuil, E., Belkum, A. and Hays, J. (2008). *Principles and technical aspects of PCR amplification*. [Dordrecht]: Springer. [Online]. Available at: http://link.springer.com/book/10.1007%2F978-1-4020-6241-4 [Accesed on 12 April 2018]

Poon, E., Mason, G. and Oh, C. (2002). Clinical and histological spectrum of elastotic changes induced by penicillamine. *Australasian Journal of Dermatology*, 43(2), pp.147-150. NCBI [Online]. Abstract avialble at: http://www.ncbi.nlm.nih.gov/pubmed/11982575 [Accessed on 22 February 2018]

Pope, F. (1974). Autosomal dominant pseudoxanthoma elasticum. *Journal of Medical Genetics*, 11(2), pp.152-157.

Pope, F. (1974). Two Types of Autosomal Recessive Pseudoxanthoma Elasticum. *Arch Dermatol*, 110(2), p.209.

Ringpfeil, F., Lebwohl, M., Christiano, A. and Uitto, J. (2000). Pseudoxanthoma elasticum: Mutations in the MRP6 gene encoding a transmembrane ATP-binding cassette (ABC) transporter. *Proceedings of the National Academy of Sciences*, 97(11), pp.6001-6006. NCBI [Online]. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC18548/ [Accessed on 2 February 2018]

Ringpfeil, F., Pulkkinen, L. and Uitto, J. (2001). Molecular genetics of pseudoxanthoma. *EXPERIMENTAL DERMATOLOGY*, 10, pp.221–228. Wiley online Library [Online]. Available at: http://onlinelibrary.wiley.com/doi/10.1034/j.1600-0625.2001.100401.x/full [Accesed on 2 February 2018]

Sakamoto, N., Akasaka, K., Yamamoto, T. and Shimada, H. (1996). A Triplex DMA Structure of the Polypyrimidine: Polypurine Stretch in the 5′ Flanking Region of the Sea Urchin Arylsulfatase Gene. *Zoological Science*, 13(1), pp.105-109. NCBI [Online]. Abstract available at: http://www.ncbi.nlm.nih.gov/pubmed/8688804 [Accessed on 9 April 2018]

Schachner, L. and Young, D. (1974). Pseudoxanthoma Elasticum With Severe Cardiovascular Disease in a Child. *Archives of Pediatrics & Adolescent Medicine*, 127(4), p.571. NCBI [Online] . Available at: http://www.ncbi.nlm.nih.gov/pubmed/4544975 [Accessed on 23 March 2018]

Scheffer, G., Hu, X., Pijnenborg, A., Wijnholds, J., Bergen, A. and Scheper, R. (2002). MRP6 (ABCC6) Detection in Normal Human Tissues and Tumors. *Lab Invest*, [online] 82(4), pp.515-518. Available at: http://www.readcube.com/articles/10.1038/labinvest.3780444 [Accessed 12 Apr. 2018].

Schneider, E. and Hunke, S. (1998). ATP-binding-cassette (ABC) transport systems: Functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiology Reviews*, 22(1), pp.1-20. Oxford Journals [Online]. Available at: http://femsre.oxfordjournals.org/content/22/1/1.long [Accessed on 25 March 2018]

Shampo, M. and Kyle, R. (2002). Kary B. Mullis—Nobel Laureate for Procedure to Replicate DNA. *Mayo Clinic Proceedings*, 77(7), p.606. NCBI [Online]. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/12108595 [Accessed on 10 February 2018]

Sherer, D., Bercovitch, L. and Lebwohl, M. (2001). Pseudoxanthoma elasticum: Significance of limited phenotypic expression in parents of affected offspring. *Journal of the American Academy of Dermatology*, 44(3), pp.534-537. NCBI [Online]. Abstract available at: http://www.ncbi.nlm.nih.gov/pubmed/11209132 [Accesed on 7 February 2018]

Sun, N., Liang, J., Abil, Z. and Zhao, H. (2012). Optimized TAL effector nucleases (TALENs) for use in treatment of sickle cell disease. *Molecular BioSystems*, [online] 8(4), p.1255. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22301904 [Accessed 9 Apr. 2018].

Terry, S. (2013). Disease advocacy organizations catalyze translational research. *Front. Genet.*, 4. Frontier [Online]. Available at: http://journal.frontiersin.org/article/10.3389/fgene.2013.00101/full [Accessed on 28 January 2018]

Thomas, P., Cote, G., Wohllk, N., Haddad, B., Mathew, P., Rabl, W., Aguilar-Bryan, L., Gagel, R. and Bryan, J. (1995). Mutations in the sulfonylurea receptor gene in familial persistent hyperinsulinemic hypoglycemia of infancy. *Science*, [online] 268(5209), pp.426-429. Available at: http://www.scopus.com/record/display.url?eid=2-s2.0-0029021696&origin=inward&txGid=D940AEC51A79DAB5AA15533940D90ABA.FZg2ODcJC9ArCe8WOZPvA%3a1 [Accessed 10 Apr. 2018].

Thornton, B. and Basu, C. (2011). Real-time PCR (qPCR) primer design using free online software. *Biochemistry and Molecular Biology Education*, 39(2), pp.145-154. Springer [Online]. Available at: http://onlinelibrary.wiley.com/doi/10.1002/bmb.20461/abstract

Uitto, J., Boyd, C., Lebwohl, M., Moshell, A., Rosenbloom, J. and Terry, S. (1998). International Centennial Meeting on Pseudoxanthoma Elasticum: Progress in PXE Research. *J Investig Dermatol*, 110(5), pp.840-842.

Uitto, J., Pulkkinen, L. and Ringpfeil, F. (2001). Molecular genetics of pseudoxanthoma elasticum: a metabolic disorder at the environment–genome interface?. *Trends in Molecular Medicine*, 7(1), pp.13-17.

Uitto, J., Váradi, A., Bercovitch, L., Terry, P. and Terry, S. (2013). Pseudoxanthoma Elasticum: Progress in Research Toward Treatment: Summary of the 2012 PXE International Research Meeting. *J Investig Dermatol*, 133(6), pp.1444-1449.

Vasiliou, V., Vasiliou, K. and Nebert, D. (2008). Human ATP-binding cassette (ABC) transporter family. *Hum Genomics*, 3(3), p.281. NCBI [Online]. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2752038/ [Accessed on 9 February]

Wilson, D. (1990). *Clinical Methods : The History, Physical and Laboratory examinations*. 3rd ed. Boston: Emory University School of Medicine, Atlanta, Georgia, pp.439-442.

Yakovchuk, P., Protozanova, E. and Frank-Kamenetskii*, M. (2006). Base-stacking and base-pairing contributions into thermal stability of the DNA double helix. *Nucleic Acids Research*, [online] 34(2), pp.564-574. Available at: http://nar.oxfordjournals.org/content/34/2/564.full.pdf+html [Accessed 8 Apr. 2018].

Zebardast, N. and Adelman, R. (2012). Intravitreal Ranibizumab for Treatment of Choroidal Neovascularization Secondary to Angioid Streaks in Pseudoxanthoma Elasticum: Five-year Follow-up. *Seminars in Ophthalmology*, 27(3-4), pp.61-64.

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