Characterization of the promoter region of the *HAMP* gene implicated in iron metabolism and its possible association with Oesophageal Cancer in the Black South African population

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Declaration

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Summary

Oesophageal cancer (OC) is the sixth leading cause of cancer related deaths in the world with approximately 300 000 new cases reported each year. OC may be characterized into two forms with 90% of cases presenting as squamous-cell carcinoma (SCC) and the remaining 10% as adenocarcinoma (ADC). Several factors have been attributed to the development of OC, including oesphageal injury and/or irritation, chronic inflammation and excess iron associated with enhanced tumour growth.

The *HAMP* gene codes for a 25 amino-acid protein found to be primarily expressed in the liver and crucial to regulation of bodily iron status. Defects occurring in the *HAMP* gene could therefore lead to the dysregulation of the gene, resulting in an iron overload status. Iron overload is a previously described risk factor in the development of various cancers, including OC, and therefore the aim of this study was to investigate whether dysregulation of the *HAMP* gene may be involved in the cancer phenotype exhibition.

The study cohort comprised of 48 unrelated patients presenting with SCC and a control group of 51 healthy, unrelated population-matched individuals. Mutation detection techniques included polymerase chain reaction (PCR) amplification, heteroduplex single-stranded conformation polymorphism (HEX-SSCP) analysis and bi-directional semi-automated DNA sequencing analysis. Screening of the 5' regulatory region (5'UTR) of the *HAMP* gene revealed one known (-582A/G) and two novel (-188C/T and -429G/T) variants with the -429G/T variant showing statistically significant reduction in expression in patients relative to controls. Iron parameters were correlated between patient and control cohorts, as well as for variant presence and absence within individuals. Luciferase reporter constructs were used to investigate the functional implications of the presence of a variant on *HAMP* gene expression, and how these results correlated to the iron parameter statistics obtained.

Luciferase reporter assay results indicated the -188C/T and -429G/T variants to result in under-, and the -582A/G variant to result in over-expression at the basal level, relative to the respective wild-type sequence constructs. Correlation of the luciferase data with the iron parameter statistics, indicate the -429G/T variant to be coupled to significantly higher levels

of ferritin and C-reactive protein (CRP) and significantly lower levels of serum-iron and transferrin when compared to individuals without the variant. Considering only the patient group, the presence of the -188C/T and -429G/T variants were coupled to significantly lower levels of transferrin in patients with either variant, compared to patients without. The variants found within the *HAMP* promoter region are therefore able to alter gene regulation to an extent where iron parameters deviate between healthy and OC afflicted individuals, and also between patients with and without a variant. This dysregulation in iron homeostasis may play a role in the development and/ or progression of OC. Characterisation of the 5' UTR of the *HAMP* gene may contribute to linking iron regulation to the establishment of an effective screening program, facilitating the early detection of OC.

Opsomming

Slukdermkanker (SK) is die sesde grootste oorsaak van kanker-verwante sterftes in die wêreld, met sowat 300 000 nuwe gevalle wat aangemeld word elke jaar. SK kan geklassifiseer word in twee vorme, waar 90% van die gevalle plaveisel-selkarsinoom (SSC) vorm en die oorblywende 10%, adenokarsinoom (ADC). Verskeie faktore word toegeskryf aan die ontwikkeling van SK, insluitend slukderm beserings en/ of irritasie, chroniese inflammasie en oormatige ystervlakke wat geassosieer word met verhoogde gewasgroei.

Die *HAMP* geen kodeer vir 'n 25 aminosuur proteïen wat hoofsaaklik in die lewer uitgedruk word en noodsaaklik is vir die regulering van ystervlakke in die liggaam. Defekte wat in die *HAMP* geen voorkom kan dus die onreëlmatige regulering van die geen tot gevolg hê, wat lei tot yster-oorlading. Yster-oorlading is voorheen beskryf as ‘n risiko faktor in die ontwikkeling van verskillende vorme van kanker, insluitend SK en gevolglik was die doel van hierdie studie om te bepaal of die wanregulering van die *HAMP* geen betrokke mag wees by die uitdrukking van die kanker fenotipe.

Die studiepopulasie het bestaan uit 48 onverwante pasiënte met SSC en ‘n kontrole-groep van 51 gesonde, onverwante soortgelyke individue. Die mutasie opsporingstegnieke wat gebruik is, het polimerase kettingreaksie (PKR) amplifisering, heterodupleks enkelstring-konformasie polimorfisme (HEX-SSCP) analise en bidireksionele semi-outomatiese DNS volgordebepaling-analise van die geïdentifiseerde variante ingesluit. Sifting van die 5’ regulerende area (5'UTR) van die *HAMP* geen het een bekende (-582A/G) en twee nuwe (-188C/T en -429G/T) variante opgelewer, met die -429G/T variant wat statisties beduidend onderdruk is in pasiënt uitdrukkings vlakke relatief tot 'n gesonde kontole-groep. Yster-parameters van alle pasiënt en kontole individue is gekorreleerd tussen pasiënt en kontrole groepe, sowel as vir teenwoordigheid of afwesigheid van variante in elke individu. Luciferase verklikker konstrukte is gebruik om die funksionele implikasies van die teenwoordigheid van ‘n variant op *HAMP* geenuitdrukking te ondersoek, en hierdie resultate te korreleer met yster-parameter statistieke wat verkry is.

Luciferase verklikkertoetse dui aan dat die -188C/T en -429G/T variante tot verminderde, en die -582A/G variant lei tot die verhoogte uitdrukking op die basale vlak lei, relatief tot die onderskeie wilde-tipe konstukte. Korrelasie van die luciferase data met die yster-parameter statistieke, dui aan dat die -429G/T-variant gekoppel is aan aansienlik hoër vlakke van feritien en C-reaktiewe proteïen (CRP) en beduidend laer vlakke van serum-yster en transferrien in vergelyking is met individue sonder die variant. Met oorweging van slegs die pasiënt-groep, is die teenwoordigheid van die -188C/T en -429G/T variante beduidend gekoppel aan laer vlakke van transferrien in pasiënte met die variant, in vergelyking met pasiënte daarsonder. Variante binne die *HAMP* promotor is dus in staat om geenregulasie te verander tot so 'n mate dat die yster-parameters afwyk tussen gesonde en SK geaffekteerde individue, sowel as tussen pasiënte met en sonder ’n variant. Hierdie wanregulering in yster homeostase kan 'n rol speel in die ontwikkeling en/ of die progressie van SK. Karakterisering van die 5’ regulerende area van die *HAMP* geen kan grootliks bydra om ysterregulasie te verbind met die implementering van ‘n effektiewe siftingsprogram, en sodoende die vroeë opsporing van SK fasiliteer.

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List of Abbreviations and Symbols

%	percentage
<	less than
>	greater than
β	beta
β-gal	β-galactosidase
µg	micrograms
µg/dl	micrograms per deciliter
µg/ml	microgram per millilitre
µg/l	micrograms per liter
µl	microlitre
µM	micromolar
1 X	one times
10 X	ten times
3’	3- prime end
5’	5- prime end
5’-UTR	5-prime untranslated region
^{55}Fe	iron-55
^{67}Ga	gallium-67
A	adenine

ADC	adenocarcinoma
Amp	ampicillin
APS	ammonium persulphate
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BglII	*Bacillus globigii*, 2nd enzyme
BMP	bone morphogenic protein
BMP-RE	bone morphogenetic protein responsive element
bp	base pair
BSA	bovine serum albumin
C	cytosine
^{o}C	degrees Celcius
CO_2	carbon dioxide
CP	ceruloplasmin
DCT1	divalent cation transporter one
DMEM	Dulbecco's Modified Eagle's Medium
DMT1	divalent metal transporter one
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide

Ex	exonic primer
F	forward primer
FAC	ferric ammonium citrate
FBS	foetal bovine serum
FPN	ferroportin
FPN1	ferroportin-1
g	grams
G	guanine
H_3BO_3	boric acid
HAMP	hepcidin antimicrobial peptide gene
HepG2	hepatocellular carcinoma cell line
HEX-SSCP	heteroduplex single-stranded conformational polymorphism(s)
HFE	high iron gene
HJV	hemojuvelin
hr	hour
IL-6	interleukin-6
INF-γ	interferon gamma
IREs	iron responsive elements
IRP1	iron regulatory element 1
IRP2	iron regulatory element 2
Kb	kilobasepair
KCl	potassium chloride

kDa	kilodaltons
KH_2PO_4	potassium phosphate dibasic
LARII	luciferase assay reagent II
LB	Luria-Bertani
LPS	lipopolysaccharides
M	molar
mg	milligrams
mg/kg	milligrams per kilogram
mg/ml	milligrams per millilitre
$MgCl_2$	magnesium chloride
min	minutes
ml	millilitres
mM	millimolar
mRNA	messenger ribonucleic acid
Na_2HPO_4	di-sodium hydrogen phosphate
NaCl	sodium chloride
ng	nanogram(s)
NheI	*Neisseria mucosa heidelbergensis,* 1st enzyme
NO	nitrous oxide
NRAMP2	natural resistance associated macrophage protein2
ºC	degrees Celsius
OC	oesophageal cancer

P	probability
p53	tumour protein p53
P53RE	tumour protein p53 response element
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	percentage hydrogen
PLB	passive lysis buffer
pmol	picomole
PP	promoter primer
R	reverse primer
RLU	relative light units
ROS	reactive oxygen species
SCC	squamous cell carcinoma
sec	seconds
T	thymine
T1	type1
T2	type2
T_{A1}	annealing temperature1
T_{A2}	annealing temperature2
Taq	*Thermus aquaticus*
TBE	tris-borate EDTA
TEMED	N, N, N', N' - tetramethylethylenediamine

Tf	transferrin
TFR1	transferrin receptor 1
TFR2	transferrin receptor 2
T_m	melting point
TP53	tumour protein p53 gene
U	units
USF	upstream stimulatory factor
V	volts
v/v	volume per volume
w/v	weight per volume
WT	wild-type
x g	times gravity

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Chapter 1

Literature Review

1. Literature Review

1.1. A brief history of iron

Iron represents 4.7% of the earth's crust, taking the forms of magnetite, hematite and siderite, making it the fourth most abundant earthly element. It is of absolute necessity to all forms of life with the exception of a few bacterial genera which have evolved to a point where they are able to use other metals adjacent to iron in the periodic table in order to survive. The biological importance of the iron trace element remains undisputed with it being an essential cofactor or constituent of an enormous array of protein and enzymatic elements vital in cellular metabolism (Beard *et al.*, 1996).

Although ancient cultures were unaware of the valuable importance of iron they were seemingly aware of its therapeutic properties. Ancient Greeks administered iron to injured soldiers as a treatment for weakness. This weakness experienced was probably due to anemia resulting from immense blood loss of the wounded. Sixteenth century physicians prescribed iron as a medicinal treatment for some patients believed to have, what is now known to be, iron deficiency. In the early eighteenth century iron was shown to be an important component of the liver and blood and hemoglobin content was, remarkably, quite accurately estimated. The nutrition essentiality of iron was first described in 1872 and a 'mucosal block' theory was first described by Granick in 1946 (Granick, 1946; Beard *et al.*, 1996). Observations regarding the significance of iron in health and nutrition were clearly made quite some time ago; however, the actual mechanisms concerning iron metabolism at the molecular level are only now being illuminated.

1.2. Introduction to iron metabolism

Iron is an important trace element within the body as an important constituent of many enzymes required for critical cellular function, and is therefore essential to good health. Iron, however, also possesses the ability to catalyze the creation of reactive oxygen species which can prove damaging to tissues. It is for this reason that the presence of excess amounts of iron within bodily tissues be expressly limited. The double-edged sword nature iron therefore necessitate its regulation by precise systems ensuring that there are optimum amounts of iron available to bodily tissues at any particular time. The body lacks an active mechanism for iron excretion and therefore bodily iron levels are primarily regulated at absorption level in the proximal end of the small intestine (reviewed by Anderson *et al.*, 2005).

Dietary iron exists in two forms within the body, namely as inorganic iron or as iron bound to heme. The inorganic form is found to be most prevalent in the diet comprising approximately 90% of standard dietary iron intake, while the remaining 10% is iron bound heme. Heme is derived primarily from myoglobin and hemoglobin and is therefore associated with meat intake. However, although heme iron is in the minority concerning dietary intake, it is absorbed much more efficiently and may therefore contribute quite substantially to the amount of iron that enters bodily tissues (Carpenter and Mahoney, 1992). Ingested heme iron is released from proteins such as myoglobin and hemoglobin by proteolytic activity in the stomach and small intestine. This heme may then be directly taken up by intestinal enterocytes. The fact that iron remains bound to heme for uptake acclaims to the higher bioavailability of iron in this state since it is not vulnerable to the binding of a wide variety of the alternate dietary substances present (Hallberg, 1981).

Iron is a vital component of nearly all living organisms and is essential to numerous metabolic processes. The development of erythroid cells; DNA, RNA and protein synthesis; electron transport; cellular respiration, proliferation and differentiation all contain steps to which iron, the most abundant metal in the body, is a crucial constituent (Lieu *et al.*, 2001). Iron represents approximately 35 mg/kg body weight in adult women and 45 mg/kg body weight in adult men with the majority of iron (60 – 70%) being present in the hemoglobin of circulating erythrocytes (Bothwell *et al.*, 1995; Conrad *et al.*, 1999). A further 10% can be found in iron-containing enzymes, myoglobins and cytochromes; and the final 20 – 30% as

excess iron stored in hepatocytes and reticuloendothelial macrophages as ferritins and hemosiderins (Conrad *et al.*, 1999).

The exclusivity of iron lies in its ability to either accept or donate electrons in an oxidation-reduction reaction also known as the Fenton Reaction (Wessling-Resnick, 1999):

$$Fe^{3+} + {}^{\bullet}O_2^{-} \rightarrow Fe^{2+} + O_2; \qquad Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$

Iron may thus exist in either the insoluble ferric (Fe^{3+}) or soluble ferrous (Fe^{2+}) forms within the body. The strict control of intracellular iron levels are of the utmost importance due to the ability of iron to create reactive oxygen species (ROS), which is highly toxic to the cell. ROS, such as hydroxyl (OH^{-}) and superoxide ($^{\cdot}O_2$) molecules, possess the ability to react with almost any molecule present within living tissues, including DNA. The net effects of ROS induced DNA damage comes at great expense to bodily tissues when considering the possible impairment of cellular function which may result in inadequate or aberrant protein synthesis, impaired membrane lipid and carbohydrate formation and altered cellular proliferation (McCord, 1998). Highly refined mechanisms have been put in place to insure the storage of iron in soluble, non-toxic forms and to enable control of the body's iron homeostasis (Lieu *et al.*, 2001).

1.3. Iron regulation, uptake and storage

Iron, being involved in a number of cellular functions, needs to be effectively absorbed, transported, stored and utilised to ensure the maintenance of iron homeostasis. Considering mammalian cells, the key molecules participating in iron metabolism are regulated by the amounts of intracellular iron levels present, which are brought about by using regulatory feedback machinery involving mRNA-protein interactions within the cell cytoplasm. In an iron deficiency event, iron regulatory proteins bind to specific mRNA containing stem structures known as iron responsive elements. Iron regulatory proteins then control the expression of iron target genes by either inhibiting the translation of iron response element-

containing genes or increasing mRNA stability. The implementation of this iron regulatory feedback mechanism ensures an appropriate level of intracellular iron essential to an array of cellular processes (Lieu *et al.*, 2001).

1.3.1. Iron-mediated feedback mechanism

Both the sequences and structures of iron responsive elements (IREs) attest to their binding affinity for the aforementioned iron regulatory elements (Theil *et al.*, 1994, 1999). The stem-loop structure of IREs consists of a terminal hexa-nucleotide loop and a base-paired stem structure separated by an unpaired cytosine nucleotide residue (Haile, 1999). Variation occurring in the hexa-nucleotide structure or at the position of the unpaired cytosine nucleotide can significantly alter the binding affinity of iron regulatory proteins (IRPs) to IREs. IREs are located in the 5' untranslated region (5'UTR) of many genes as well as in the 3' untranslated region (3'UTR) of some mRNA molecules. Depending on the location of these responsive elements they may act as either enhancers or repressors of translation (Theil *et al.*, 1994). Referring to mRNA, the binding of iron regulatory proteins may prevent the binding of the translation pre-initiation complexes thus preventing protein synthesis, or, increase mRNA stability and thereby increasing protein synthesis.

1.3.2. Iron regulatory proteins (IRPs)

The regulatory proteins, iron regulatory protein 1 (IRP1) and 2 (IRP2) bind directly to iron responsive element-containing RNA. IRP1 can be found in all tissues with higher expression levels found in the liver, kidneys and intestinal tissues (Henderson *et al.*, 1993). The IRP1 shows sequence similarity to cytoplasmic aconitases (an enzyme which catalyzes the conversion of citrate to isocitrate in the citric acid cycle) and has been shown to have aconitase activity similar to that of the mitochondrial aconitase. When normal iron levels prevail, the IRP1 contains an iron-sulphur cluster bound to three cysteine residues (Emery-Goodman *et al.*, 1993; Kaptain *et al.*, 1991). This form possesses aconitase activity but lacks the ability to bind RNA effectively. Under conditions of iron deficiency the enzyme lacks the

iron-sulphur cluster needed for aconitase activity causing it to accumulate within the cell. This form, lacking aconitase activity, possesses the ability to bind to IREs with a high affinity (Rouault *et al.*, 1992).

IRP2 exhibits a 50% amino acid homology with IRP1 and binds to iron responsive elements with a similar affinity. It too is expressed in most bodily tissues, but with reduced expression levels relative to IRP1. The mechanisms of binding to IRP2 can also be compared to that of IRP1, but it is in the mechanisms responsible for the changes in iron-responsive element-regulatory protein binding activity between the two proteins that are individual of each other (Guo *et al.*, 1994; Henderson *et al.*, 1993). Then main difference between the two IRPs is that IRP2 is degraded more rapidly under conditions of high intracellular iron levels. The mechanism for this degradation remains unclear, but it is believed that an inimitable region comprising of 70 amino acids is responsible for the degradation in response to high iron levels within the cell (DeRusso *et al.*, 1995). In addition, although both IRPs bind iron responsive elements with similar affinities, they are remarkably dissimilar in the sequence regions of the IREs to which they bind. IRP2 is also missing amino acid residues required for the aconitase activity that IRP1 possesses (Phillips *et al.*, 1996; Samaniego *et al.*, 1994). Furthermore IRP2 also contains conserved cysteine residues necessary for iron-sulphur cluster formation, although, it is apparent that unlike IRP1, its RNA binding activity in not regulated by iron-sulphur clusters. The differences between IRP1 and IRP2 suggest that they perform unique functions in response to the cellular status of iron and are probably acting on different targets. There are, however, animal models in place that indicate that IRP2 is able to compensate for any loss of function occurring to IRP1 (Rouault and Klausner, 1997).

1.3.3. Ferritin

Free iron has the ability to form hazardous ROS and, therefore, the source of intracellular iron is ferritin. This method of storage depicts a solution to the formation of free radicals without removing the crucial supply of iron from the cell entirely. Ferritin exists in two subunits, namely, the heavy and light chains. These subunits form a protein shell that enable the storage of up to 4500 iron molecules. The execution of sequence analysis on the 5' untranslated regions of ferritin mRNAs are sufficient to allow for iron-mediated regulation of

these molecules (Aziz and Munro, 1987). It may also be noted that a *cis*-acting element, the acute box, is present downstream in the 5' untranslated region of the mRNA of the ferritin heavy and light chains. This box has the ability to regulate ferritin synthesis independently of the iron regulatory protein-mediated pathways (Rogers *et al.*, 1994). Furthermore a *trans*-acting RNA binding protein (Rouault *et al.*, 1987) has the ability to bind to the acute box in hepatic cells, where if mutations prevent this, there is interference in the formation of the pre-initiation complex and reduction in the level of ferritin synthesis. Ferritin synthesis is therefore regulated by both mRNA elements as well as RNA binding proteins, acting together or independently, in response to variating and alternate stimuli.

1.4. Dietary iron uptake

In the intestinal lumen iron is found to exist in the forms of ferrous and ferric salts. Ferric forms of iron are insoluble at pH values above 3 and therefore need to be either reduced or chelated to ensure effective and efficient absorption (Conrad *et al.*, 1999). The ferrous forms of iron remain soluble even at pH 7 and are much more easily absorbed by the body. Most dietary iron occurs in the form of ferric salts and, in order to permit efficient dietary iron absorption, it undergoes reduction mediated by the mucosal ferrireductase enzyme present in the intestines. Alternatively ferric iron uptake may occur *via* the paraferritin pathway; however, this is a much less efficient mechanism of ferric iron absorption (Conrad *et al.*, 1999).

1.4.1. Non-heme iron absorption

Absorption of both the heme and non-heme forms of iron occurs in the proximal end of the small intestine, distinctively in the crypt cells of the duodenum and jejunum. To enter the body's circulatory system, dietary iron is required to pass across the apical membrane (Fig 1.1), submit to translocation across the cytosol and finally export across the basolateral membrane of the enterocyte into circulation. The apical surfaces of the absorptive enterocytic cells contain no transferrin receptors preventing iron from entering the cell *via* the

usual transferrin-transferrin receptor pathway. Iron absorption across the apical membrane is mediated by (natural resistance-associated macrophage protein 2 (NRAMP2)), also known as divalent cation transporter 1 (DCT1) or divalent metal transporter 1 (DMT1), a divalent cation transporter (Fleming *et al.*, 1997; Gunshin *et al.*, 1997).

It is known that the *NRAMP2* gene encodes two alternate spliced forms of mRNA. The 3' untranslated region of *NRAMP2* isoform 1 contains an iron responsive element similar to that present in the mRNA of transferrin receptor 1 (*TRF1*) (Fleming *et al.,* 1998), whereas isoform 2 lacks the iron responsive element. Although *NRAMP2* is expressed in a multitude of different tissues, significantly higher expression levels are observed at the duodenum brush border; validating its role in the intestinal absorption of iron into the enterocytic cytosol (Gunshin *et al.*, 1997). The NRAMP2 protein consists of 12 transmembrane domains and is located on the plasma membrane (Su *et al.*, 1998). NRAMP2 is an acting proton-coupled divalent cation transporter capable of transporting ferrous iron as well as a broad range of alternate divalent cations (Gunshin *et al.*, 1997).

1.4.2. Heme iron absorption

Organic iron sources are absorbed much more efficiently than inorganic iron forms. Hemoglobin and myoglobin iron is initially digested in the intestinal lumen, and the resulting heme molecule may then enter the enterocytic cell as an intact metalloporphyrin entity (Majuri and Grasbeck, 1987). The heme molecule enters the cell *via* a heme receptor-mediated internalisation process (Mills and Payne, 1995), and once internalised is subsequently degraded by the heme oxygenase enzyme and iron is released as inorganic ferrous iron. As a result of the aforementioned enzymatic activity, inorganic iron now present within the enterocyte may either be stored as ferritin or be transported across the basolateral membrane and into the body circulation *via* the protein ferroportin-1 (FPN1). Any iron in the ferritin form still present at the end of the cell cycle will be lost along with the senescent cells representing an important mechanism of iron loss in humans.

FPN1 is expressed in the placenta, liver, spleen, macrophages, kidneys and intestines. The gene has an open reading frame of 562 amino acids and functional studies show that

ferroportin1 mediates iron efflux across membranes (Donovan *et al.*, 2000; McKie *et al.*, 2000). The membrane bound protein is thought to function in association with the membrane bound hephaestin and serum protein ceruloplasmin (McKie *et al.*, 2000). Ceruloplasmin and hephaestin are highly similar and function to facilitate the transport of iron into the body's circulation.

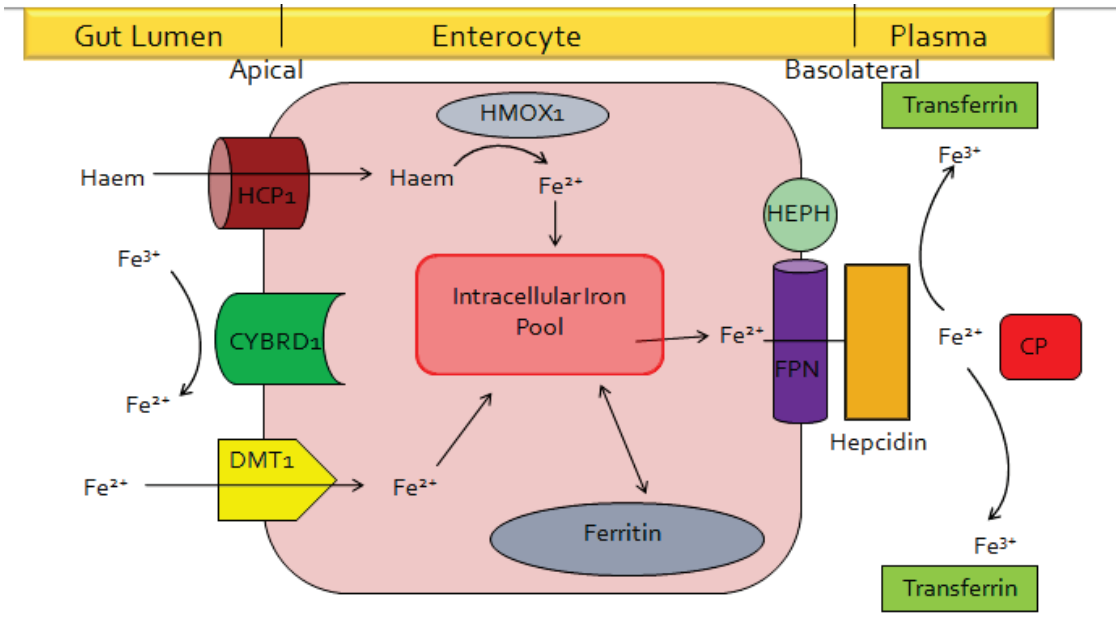

Fig 1.1 Schematic representation of the process of iron absorption in the GIT lumen. Iron enters the enterocytic cell across the apical membrane by aid of DMT1, and exits the enterocytic cells, across the basolateral membrane, by aid of the membrane-bound FPN protein. Haem may enter the enterocyte directly by means of HCP1, where it is then acted upon by HMOX1, releasing free ferrous iron into the intracellular iron pool. Membrane-bound HEPH and plasma CP are responsible for the conversion of ferrous to ferric iron, allowing it to bind to transferrin and be transported to other areas of the body. The hepcidin protein is able to bind to FPN, causing is subsequent internalization and degradation, limiting iron absorption in the intestinal lumen. (HCP1: Haem carrier protein -1, CYBRD1: cytochrome-b reductase-1, DMT1: divalent metal transporter 1, HMOX1: haemox reductase 1, FPN: ferroportin, HEPH: hephaestin, CP: ceruloplasmin, Fe^{2+}: ferrous iron, Fe^{3+}: ferric iron)

1.4.3. Regulation of the amount of dietary iron absorbed

The amount of dietary iron absorbed by the body can be regulated in three ways. 'Mucosal block' refers to a situation where the absorptive enterocytic cells are resistant to the absorption of additional iron due to the bodily intracellular quantities being met (Andrews, 1999a, b). An alternative mechanism for regulation is when the body-store iron levels are sensed and is termed 'stores regulator' (Finch, 1994). The stores regulator mechanism can influence the amount of dietary iron uptake under iron-deficient conditions, however, the exact molecular mechanisms involved in this process remains unknown. The 'erythropoietic regulator', a third mechanism, has the ability to increase the amount of iron that enters the body, independent of cellular iron levels, using a mechanism based on the amount of iron needed for erythropoiesis (Finch, 1994).

Iron Transport

1.4.4. Transferrin

Transferrin is a plasma protein responsible for the transport of iron from the site of absorption to the places of utilization and storage. The first step in transferrin mediated cellular iron uptake involves the binding of transferrin to transferrin receptor. There are two types of transferrin receptors; each has its own tissue-specific and cell-specific expression pattern. The membrane bound glycoprotein, transferrin receptor 1, is expressed in all cells, excluding mature erythrocytes. Transferrin receptor 2 is expressed solely in the liver, more particularly in the hepatocytes. Upon binding the transferrin receptor-transferrin-iron complexes are internalized and iron is released from the transferrin and enters the intracellular labile pool. Transferrin receptor-bound transferrin complexes are then recycled back to the cell surface for reuse in iron uptake (Lieu *et al.*, 2001).

The transferrin molecule comprises of two globular domains with each domain containing a high-affinity binding site for a singular iron molecule (Fe^{3+}). The transferrin molecule also possesses an affinity for the transportation of other metal ions; however, it has the highest affinity for the ferric iron. The iron-transferrin kinship is pH dependent and iron is released from the transferrin molecule when the pH falls below 6.5 (Yang *et al.*, 1984; Moos *et al.*, 2000). Alternate forms of the transferrin molecule exist; namely apotransferrin (iron-free), monoferric (one iron molecule attached), diferric (two attached iron molecules). The amount of each molecule present is dependent upon the concentration of iron and transferrin present in the blood plasma (Lieu *et al.*, 2001).

1.4.5. Transferrin receptor 1

The transferrin receptor 1 homo-dimer has a molecular weight of 90 kDa and comprises two identical subunits. Each monomer may be subdivided into three domains of which the third domain, the carboxyl-terminal ectodomain, is essential for transferrin binding (Buchegger *et al.*, 1996; Lieu *et al.*, 2001). Each homo-dimer of the transferrin receptor contains a binding site for a transferrin molecule allowing two transferrin molecules to bind to a single receptor simultaneously. The cytoplasmic portion of the transferrin receptor 1 molecule is required for endocytosis. A conserved region located within the cytoplasmic portion of the receptor is responsible for an internalization signal allowing for highly efficient endocytosis of the receptor during iron uptake (Collawn *et al.*, 1990, 1993). Phosphorylation and dephosphorylation has been suggested to be the signals for internalisation, strengthened by the fact that treatment of tissues with protein phosphatase inhibitors results in an 85% reduction in the uptake of transferrin (Beauchamp and Woodman, 1994).

1.4.5.1. Expression of transferrin receptor 1

Transferrin receptor 1 is expressed in all cells with the exception of mature erythrocytes; however, the levels of expression differ depending on the cell line. Considering rapidly dividing cells; between 10 000 and 100 000 molecules per cell can be found expressed, whereas with non-proliferating cells the expression levels are low or virtually undetectable (Ioue *et al.*, 1993). The epithelial cells of a several organs express a basal level of transferrin receptor 1; however, rapidly dividing cells, the liver, placental tissue and erythrocytes have the highest level of expression. High levels of iron are required for hemoglobin synthesis and cellular division explaining the high expression levels of transferrin receptor 1 expression (Ponka, 1999). In non-erythroid cells transferrin receptor 1 is regulated post-transcriptionally by an interaction between iron regulatory proteins and iron responsive elements occurring in the 3' untranslated region of its mRNA. The 3' region contains a string of five stem loop structures crucial for the iron mediated mRNA degradation. In the event of low iron levels each of the iron responsive elements can be bound by one regulatory protein allowing for the stabilization of the transferrin receptor 1 molecule (Hentze and Kuhn, 1996; Klausner *et al.*, 1993). Binding of the regulatory proteins blocks an endonuclease cleavage site, stabilizing the molecule and allowing for increased cell surface expression of the transferrin receptor 1 glycoprotein. The opposite is true in the event of high intracellular iron levels (Thompson *et al.*, 1999). The intracellular iron levels of erythroid cells do not have a major effect on the expression of *transferrin receptor 1* mRNA as during erythroid differentiation the transferrin receptor 1 is regulated at a transcriptional level as oppose too post-transcriptionally (Chan *et al.*, 1994). It can therefore be concluded that the expression of *transferrin receptor 1* is regulated by both transcriptional and post-transcriptional mechanisms.

1.4.5.2. Transferrin receptor 1 function

The cellular intake of iron from the plasma transferrin molecule is mediated by the binding of transferrin to transferrin receptor 1 on the cell surface. The number of iron molecules bound to the transferrin protein affects its affinity for transferrin receptor 1: diferric transferrin has

the highest affinity and apotransferrin the lowest (Young *et al.*, 1984). A maximum of two transferrin molecules can be bound by a single transferrin receptor 1 protein at a time and therefore four iron atoms can be mediated at a time. Tyrosine internalisation motifs located on the cytoplasmic portions of the transferrin receptor 1 molecule allows for high affinity binding to membrane bound adapter complexes. The transferrin receptor-transferrin-iron complexes interact with these adapter complexes and are then internalised by the cells via an endocytic pathway. Upon internalisation the endosome is acidified by means of an ATPase proton pump which allows for the release of the attached iron molecules. The dissociation of the now apotransferrin from transferrin receptor 1 takes place at neutral pH at the cell surface recycling both the ligand and receptor for reuse (Lieu *et al.*, 2001). Released iron passes through the endosomal membrane *via* Nramp2 and is released into the cytoplasm. The new acquired iron is either used for the synthesis of heme or is incorporated into iron containing molecules (Fleming *et al.*, 1998).

1.4.6. Transferrin receptor 2

Transferrin receptor 2 is a type II transmembrane protein sharing 66% homology with transferrin receptor 1 in its extracellular domain (Kawabata *et al.*, 1999). It contains the internalisation motif YQRV, which is similar to that of transferrin receptor 1's YTRF; however, upon sequence analysis of transferrin receptor 2 it is found that it possesses no iron responsive elements. Transferrin receptor 2 is therefore not regulated by the iron regulatory protein-mediated feedback regulatory mechanism. It is predominantly expressed in the liver, where transferrin receptor 1 levels are low. Transferrin receptor 2 also possesses the ability to bind transferrin and its binding is pH dependent. Transferrin receptor 2, however, differs from transferrin receptor 1 in its expression and regulation properties (Kawabata *et al.*, 2000). Transferrin receptor 1 plays a role in general cellular uptake of iron, whereas transferrin receptor 2 is specifically involved in iron uptake and storage in the liver due to its high expression in hepatocytes.

1.4.7. Transferrin receptors in cancer onset

The transferrin molecules supply the cells of the body with iron, therefore exhibiting a proliferative effect on these cells. In the context of this thesis the role transferrin, transferrin receptors and iron have in the growth of metastasizing tumour cells and a number of carcinoma lines can therefore be ascertained (Cavanaugh and Nicolson, 1990). Cells exhibiting a low dependence on iron express low numbers of transferrin receptors on their cell surface, and when stimulated to proliferate will sequester an increase in the amount of transferrin receptors present to facilitate iron uptake (Seiser *et al.*, 1993). This theory is supported by numerous experiments, including the finding that anti-transferrin receptor 1 antibodies can block lymphocyte activation and suppress the proliferation of tumour cells (Kemp *et al.*, 1987); transferrin receptor 1 correlated with the proliferative response in metastatic breast cancer cells (Cavanaugh *et al.*, 1999); and in a rat model expressing low amounts of rat transferrin receptor 1, the introduction and expression of human transferrin receptor 1 facilitates rat mammary adenocarcinoma cell line proliferation (Cavanaugh *et al.*, 1999).

1.4.7.1. Transferrin-associated proteins

1.4.7.1.1. *High iron gene* (*HFE)*

The HFE protein has been shown to be involved in the role of iron uptake due to its ability to associate with the transferrin receptor 1 protein. The HFE and transferrin receptor 1 molecules form a complex while within the endoplasmic reticulum, then make their way to the cell surface where they remain closely associated (Gross *et al.*, 1998). The *HFE* gene codes for a 343-amino-acid protein belonging to the major histocompatibility complex class I family. The protein is expressed in a wide variety of tissues at basal levels, with no expression occurring in brain tissue and the highest expression levels found in the liver and small intestine (Feder *et al.*, 1996). Interaction of the transferrin receptor 1 molecule with HFE results in a 5 – 10 fold reduction in the affinity of transferrin receptor 1 for transferrin

(Gross *et al.*, 1998), and as a result the amount of transferrin-bound iron by cells and the build-up of intracellular iron levels are reduced under normal HFE expression conditions (Salter-Cid *et al.*, 1999).

1.4.7.2. *Ceruloplasmin* (CP)

Ceruloplasmin is a copper-containing molecule responsible for the conversion of ferrous to ferric iron thus assisting in incorporating ferric iron into transferrin. The primary site for ceruloplasmin production is the liver and synthesis is significantly increased during periods of iron deficiency (Lieu *et al.*, 2001). Due to the ferrioxidase activity of the ceruloplasmin protein it is crucial in the mobilization of iron from tissues and its incorporation into ferric transferrin, thereby preventing potentially damaging build-up of intracellular iron levels within tissues (Gitlin, 1998).

1.5. Iron deficiency

Deficiencies of iron is the most common cause of anemia, and with iron deficiency anemia the mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentrations are low. Iron-deficient erythropoiesis is characterised by an increase in free erythrocyte protoporphyrin and microcytosis, indicated by mean corpuscular volumes of less than 83 femtoliters per cell. Major cases of anemia under these conditions are iron deficiency anemia and anemia of chronic disease; where both cases have serum iron concentrations of less than 40 μg/dl and transferrin saturations of below 15 – 20% (Cook, 1999). Serum ferritin levels are found to be below 20 μg/l in iron deficiency anemia cases, and above 100 μg/l in anemia of chronic disease instances. Serum transferrin receptor 1 levels are calculated using the transferrin receptor 1-ferritin index, where the normal range is 0.076 (±0.062 SD) and in iron deficiency cases values around 3.739 (±3.413 SD) are obtained (Punnonen *et al.*, 1997).

1.6. Iron overload

In instances of suspected iron overload the serum iron concentration, transferrin saturation and serum ferritin levels should be measured. Serum iron concentration levels above 20 μM, transferrin saturation greater than 50 – 60% and serum ferritin levels greater than 400 μg/l are typical of iron overload presenting patients (Mura *et al.*, 2000). Serum transferrin levels are considered to be early indications of hemochromatosis with transferrin saturation levels higher than 45% occurring in 98% of cases, however, serum ferritin levels are a more accurate depiction of iron accumulation. Raised serum ferritin levels are also symptomatic of conditions not associated with increased iron levels, and this need be considered in diagnosis (Lieu *et al.*, 2001).

The accumulation of iron in the liver is most commonly assessed using liver biopsy and provides information on iron content, iron distribution within tissues and an indication of cirrhosis. The hepatic iron index is used to assess the iron load of the liver, and measurements suggesting iron overload are obtained following to genetic testing which may be performed to investigate the genetic component(s) involved (Lieu *et al.*, 2001).

1.7. The *HAMP* gene

The 25 amino acid protein, hepcidin, encoded for by the *HAMP* gene is initially synthesised as an 84 amino acid peptide, which then undergoes processing steps in order to form the aforementioned iron regulatory molecule. The 25 amino acid hepcidin molecule comprises of four intra-chain disulphide bonds, eight cysteine residues and is also a member of the defensin family of molecules (Luft, 2004). These molecules are involved in innate immunity highlighting the initial role hepcidin was believed to have played. However, despite the structural similarity of the hepcidin protein to many antimicrobial peptides and its sequence conservation amongst many vertebrate species, it is doubtful that the hepcidin protein is produced in humans for its antifungal or antibacterial properties (Krause *et al.*, 2000). This theory is strengthened by the low urinary concentrations detected.

1.7.1. Foundation for hypotheses of gene function

Many animal models, using transgenic mice lines (Nicolas *et al.*, 2001), have allowed for the elucidation of the hepcidin protein in playing a vital role in the regulation of iron metabolism. Experiments by Atanasiu *et al.,* (2006) revealed that the hepcidin protein is involved in the inhibition of the intestinal absorption of iron, induction of iron sequestration in macrophages and the blockage of iron transport across the placental barrier. In essence, the hepcidin protein is up regulated in response to high bodily iron levels, preventing any further assimilation of dietary iron in the intestinal lumen. This theory is further strengthened by the fact that hepatic hepcidin levels appear lowered during processes such as erythropoiesis, allowing for increased uptake of iron in the intestinal lumen as well as the release of iron from macrophage cells. Iron is crucial to the erythropoietic process making its increased availability of the utmost importance.

In contrast to this, increased hepcidin levels are observed in cases of inflammation or iron overload. The increased level prevents any further iron assimilation at the intestinal lumen and also stimulates the sequestering of iron by macrophage cells making this crucial resource unavailable to invading pathogens. These scenarios clearly emphasize the hepcidin dual role in both regulating the homeostatic balance or iron as well as its involvement in innate immunity (Deicher and Horl, 2006).

The transcription products of the *HAMP* gene can be found in liver, muscle, intestinal, stomach, heart and lung tissues; but, its primary location is in liver tissue. Under normal circumstances it is the natural response of the body to increase the amount of hepcidin produced in response to elevated iron levels or iron overload; but, the exact mechanism allowing for this increase remains obscure. This is partly reasoned for by the fact that no iron response element binding regions have been located in the *HAMP* gene itself and therefore up and down regulation of the aforementioned gene must be taking place by methods other than direct interaction with iron regulatory binding proteins (Atanasiu *et al.,* 2006).

1.7.2. Proposed mechanisms of protein function

Ferroportin (FPN), expressed on the cell surface of hepatocytes, macrophages and intestinal enterocytes is the only known mammalian exporter of iron and a believed molecular target of the hepcidin protein (Nemeth *et al.*, 2004). Upon hepcidin binding, the FPN molecule is subsequently internalised and degraded preventing any further iron export (Figure 1.1). In the event of iron shortage hepcidin levels are reduced, FPN expressed and iron can be released from iron stores to plasma transferrin and transported to the requiring tissue sites (Atanasiu *et al.*, 2006).

An alternative mechanism of function is that the liver is able to detect the body iron status by the amount of transferrin-bound iron *via* its receptor transferrin receptor 2. Monitored increased uptake subsequently results in the up-regulation of the *HAMP* gene and increase in the amount of hepcidin protein produced. Hepcidin associates with the β2-microglobulin – HFE – transferrin receptor 1 complex, increases iron uptake in the duodenal crypt cells and increases iron retention by reticulo-endothelial macrophages. As the crypt cells then mature they are programmed to express lesser amounts of iron transport proteins and therefore allow for reduced uptake of dietary iron (Luft, 2004; Leong and Lonnerdal, 2004).

1.8. Oesophageal cancer (OC)

Worldwide, oesophageal cancer (OC) is the sixth leading cause of cancer related deaths (Pisani *et al.*, 1999). More than 90% of OC are either squamous-cell carcinomas (SCC) or adenocarcinoma (ADC), with three quarters of all ADC being found in the distal end of the oesophagus and squamous prevalence evenly spread between the middle and lower third of the oesophagus (Siewert *et al.*, 2001). The exact mechanisms of disease pathogenesis remain unclear; however, irritation of the oesophagus by oxidative damage resulting from smoking or gastroesophageal reflux causing inflammation of the oesophageal tissues, may initiate the carcinogenic process (Terry *et al.*, 2000). Once developed, the disease has the ability to spread quite rapidly and 14 – 21% of submucosal cancers, or T1 lesions, as well as 38 – 60 % of muscle-invading cancers, T2 lesions, associated with spread to the lymph nodes. At the

time of diagnosis more than 50% of patients have already radiographically detectable metastases (Siewert *et al.*, 2001; Collard *et al.*, 2001). Etiological focuses indicated that the OC risk is directly correlated to the amount of cigarettes smoked per day and the duration of smoking. Smoking brings tobacco carcinogens, in particular nitrosamines, in contact with the oesophageal tissue causing irritation (Wu *et al.*, 2001).

1.8.1. Squamous cell carcinoma (SCC)

Chronic irritation of the oesophageal mucosa is associated with increased risk for squamous-cell carcinoma development, with alcohol consumptions, smoking, and the combination of the two greatly increasing the risk for SCC development. Achalasia and oesophageal diverticuli, cases in which bacteria decomposes trapped food within the oesophagus, have also been associated with increased risk for squamous-cell carcinoma development due to the toxins and irritants released by the aforementioned bacteria present (Wu *et al.*, 2001; Sandler *et al.*, 1995; Avisar and Luketich, 2000).

1.8.2. Adenocarcinoma (ADC)

Patients suffering from chronic gastroesophageal reflux have an eight-fold increase in risk for developing adenocarcinoma (Lagergren *et al.*, 1999). Barrett's oesophagus is characterised by the replacement of stratified epithelial cells of the distal oesophagus with specialised columnar epithelium which is typically seen in the stomach or intestine. The transformation of this columnar epithelium into areas of dysplasia is a result of mutation development within this tissue, putting individuals with Barrett's oesophagus at high risk for adenocarcinoma development (Shaheen and Ransohoff, 2002). The genetic explorations characterizing the development of Barrett's oesophagus reveal chromosomal losses in chromosomes 4, 5, 9 and 18; chromosomal gains in chromosomes 8, 17 and 20; however, efforts to match these chromosomal aberrations to specific genes remain largely elusive (Walch *et al.*, 2000; Wijnhoven *et al.*, 2001).

1.9. Iron and cancer

Iron-containing enzymes are crucial for the catalization of numerous reactions key to the effective and efficient progression of DNA synthesis and energy provisions. As previously discussed, iron is transported in the serum bound to transferrin and subsequently binds to the membrane bound transferrin receptor 1 and is internalised. Cancer cells have been shown to contain higher amounts of transferrin receptor 1 than normal and therefore allow for a higher level of iron uptake (Larrick and Cresswell, 1979; Chitambar *et al.*, 1983). Radiolocalisation using ^{67}Ga, which binds to the transferrin-iron binding site and is subsequently delivered to the transferrin receptor 1, demonstrates the increased cellular iron uptake described (Chan *et al.*, 1987). Strengthening the fact that iron is an essential component of cellular proliferation is that the use of iron chelators, such as desferrioxamine, inhibit the growth of aggressive tumours both *in vivo* and *in vitro*, highlighting the dependence of tumour tissue on this trace element (Kwok and Richardson, 2002).

Taking the body's natural defense mechanisms into account, the dependence of tumour cells on iron is further demonstrated. Macrophage cells produce nitrogen monoxide (NO), when activated, comes in contact with a variety of iron containing molecules involved in energy metabolism and DNA synthesis. NO is able to inhibit the uptake of iron from transferrin due to its effect on the inhibition of ATP production as well as the mobilisation of iron from tumour cells in both the presence and absence of activated macrophages (Wardrop *et al.*, 2000; Richardson *et al.*, 1995; Watts and Richardson, 2000).

1.9.1. Neoplastic cells

The mechanisms for iron uptake, storage and utilisation appear to be similar for both normal and neoplastic cells, however, there are several molecules identified in neoplastic tissues that could play a role in iron acquisition.

1.9.1.1. Transferrin

The major iron transport protein in the blood serum is the glycoprotein, transferrin. Due to the iron-binding properties of this molecule it is an essential growth factor required for all cells in a proliferating state (Morgan, 1981; Richardson and Ponka, 1997).

The MCF-7 human breast cancer cell line has been shown to secrete a factor immunologically identical to transferrin which may act as an autocrine growth factor. This could bestow a selective advantage facilitating the growth of the rapidly dividing tumour cells in poorly vascularised areas (Vandewalle *et al.*, 1989). Other cancer cells lines have also been shown to secrete transferrin, including small cell carcinoma and T-lymphoma cells (Vostrejs *et al.*, 1988; Morrone *et al.*, 1988).

1.9.1.2. Melanotransferrin

Melanotransferrin is a homologue of the transferrin molecule, but, membrane bound. Melanotransferrin is generally not expressed, or expressed at relatively low levels within normal tissue; however, in tumour cells (especially melanoma cells) and fetal tissues, expression is observed at much higher levels (Woodbury *et al.*, 1980; Brown *et al.*, 1982). Although promising at first, recent studies performed have indicated the melanotransferrin protein in not vital for obtaining iron for proliferating neoplastic cells lines even though an N-terminal iron binding site has been identified within it (Richardson and Baker, 1990;

Richardson and Ponka, 1997). Its involvement in iron metabolism cannot be completely ruled out, but further studies are warranted to elucidate the exact function of this molecule.

1.9.1.3. Ceruloplasmin

Ceruloplasmin is a multi-copper oxidase responsible for the conversion of ferrous iron to ferric iron, enabling iron mobilization from the bodily tissues (Kaplan and O'Halloran, 1996). In a study by Mukhopadhyay *et al.* (1998) ^{55}Fe was shown to be taken up from ^{55}Fe-nitrilotriacetate, and this was mediated by ceruloplasmin. Non-physiologically relevant temperature and Fe-complexes were used and therefore this experiment was appropriately replicated (Richardson, 1999). This experiment showed ceruloplasmin to be necessary for the efflux of iron from cells, but eliminated its role in iron uptake in cancerous cell lines.

1.9.2. Ferritin and neoplastic cells

Serum ferritin levels are increased in patients suffering from several forms of neoplasia, suggesting a relationship between ferritin and cancer (Marcus and Zinberg, 1975; Hann *et al.*, 1980). An autocrine growth factor secreted by human leukemia cells has been shown to have an immunological identity with the ferritin molecule and an antibody to ferritin inhibited the growth of these cells (Kikyo *et al.*, 1994). Ferritin binding sites as well as the endocytosis of ferritin has been observed in neoplastic cells (Bretscher and Thompson, 1983). Evidence to suggest altered ferritin expression in cancer cells also exists but all in all further research needs to be performed in order to obtain a greater insight in the exact role ferritin could play in neoplastic cell proliferation.

1.10. *TP53*

p53, a tumour suppressor protein, is coded for on the p-arm of chromosome 17. Its loss of function has been associated with the development of several forms of cancer (Montesano *et al.*, 1996). Allelic loss on chromosome 17p has been specifically associated with OC. Studies show that in the case of OC, *p53* mutation in one allele (in cells pertaining two 17p alleles) is an early event in the development of oesophageal carcinogenesis. This suggests the aberrant p53 protein exerts a dominant effect that is able to abolish the effects of the remaining wild-type *p53* allele coding protein (Huang *et al.*, 1993; Maesawa *et al.*, 1994). The presence of one mutation may then favour a deletion in the other allele at a later stage (Montesano *et al.*, 1996).

1.10.1. *TP53* and carcinogenesis

The *TP53* gene codes for a 53 kDa phosphoprotein, containing sequence specific DNA binding properties, a protein with a very short half-life preventing build-up despite constant synthesis. Mutations occurring within the *TP53* gene are also amongst the most common human cancer related genetic abnormalities (Hollstein *et al.*, 1996). In the event of DNA damage the p53 protein is stabilised, extending its half-life, allowing for it to exert an effect on the many gene targets it interacts with. Some of these gene targets are responsible for cell cycle arrest and apoptosis (Kastan *et al.*, 1991; Tishler *et al.*, 1993). p53 therefore functions to prevent the replication of damaged DNA by either arresting the cell cycle or by initiating apoptosis. Mutations occurring in *TP53* which have been linked to human cancers are thought to disrupt the DNA binding domain of the p53 protein preventing the activation of its target genes. This facilitates the replication of damaged DNA and subsequently could allow for the accumulation of the genetic aberrations necessary to result in a cancerous phenotype (Cho *et al.*, 1994). Further hypotheses suggest mutant *TP53* to have tumour promoter properties, founded by the maintenance of high levels of p53 within cancer tissues. This indicates that the cancer cells are selecting for mutant *TP53* expression. The exact

mechanisms for this selection are, however, unknown and further research need be carried out to elucidate the exact mechanisms (Hainaut, 1995).

The literature exploring the occurrence of *TP53* mutations in oesophageal cancer is extensive. Considering 240 cases of squamous-cell carcinoma, 45.8 % were found to contain mutations within *p53*, with the prevalence considering adenocarcinoma being even higher with 71.8 % of all cases including aberrant versions of the gene (Montesano *et al.*, 1996). The majority of mutations are found to be located within exons 5 – 8 of the 11 exons coding for the gene. Exons 5 – 8 are responsible for encoding the entire DNA-binding domain of the p53 protein including its flanking regions, with mutations occurring outside of this region being uncommon in oesophageal cancer associations (Wagata *et al.*, 1993).

Immunohistochemical staining reveals that the p53 protein is expressed in normal oesophageal cells and at heightened levels in the nuclei of proliferating ones (Bennett *et al.*, 1992). The protein detected, however, may be representative of mutant p53 protein or the functionally active wild-type protein possessing the ability to suppress the replication of cells containing damaged DNA (Hainaut, 1995).

1.10.1.1. The distribution of *TP53* mutations in OC

The distribution and occurrence of *TP53* mutations are different for the different types of OC, namely squamous-cell carcinoma and adenocarcinoma. In adenocarcinoma, mutations are predominantly located within exons 5 – 8 with mutational hot spots at codon 175 in exon 5 and codons 248 and 273 in exon 8 (Hollstein *et al.*, 1996). Codon 175 is also found to be frequently mutated in squamous-cell carcinoma; however, it is the relatively high levels of mutation attributed to codons 193, 194 and 195 that give squamous-cell carcinoma its characteristic *TP53* mutation profile. This specific mutation profile is not found to match any other form of cancer to date, in contrast the three exons discussed for adenocarcinoma being the most frequently mutated codons in all cancers (Hollstein *et al.*, 1996). The majority of the p53 mutations identified occur in a region encoding for the DNA-binding domain of the protein; mutations disrupting the core domain are rare. In squamous-cell carcinoma, however, the majority of mutations identified appear to disrupt the hydrophobic core of the

p53 protein and are believed to disrupt regions usually in the tertiary structure of the wild-type protein molecule. These kinds of mutations are most likely to be particularly disruptive (Montesano *et al.*, 1996).

Mutated p53 sequence may be as result of endogenous circumstances, or as result of exposure to exogenous or environmental carcinogens. The type of p53 mutations present may allow for insight into the mechanisms responsible for the DNA aberrations driving the tumourigenesis process, meaning that analyzing the spectrum of p53 mutations can be useful in identifying the carcinogens involved in the etiopathogenesis of OC (Hollstein *et al.*, 1996). Mutations of p53 associated with adenocarcinoma show highest frequency of transition at CpG dinucleotides (69 %). CpG transitions are less frequent in squamous-cell carcinoma with mutations at A: T bp being most frequent. This indicates that *TP53* mutations associated with squamous-cell carcinoma is most likely a result of exposure to exogenous carcinogens (Hollstein *et al.*, 1996; Montesano *et al.*, 1996).

It is therefore apparent that *TP53* gene mutations play a critical role in the development and/or progression of both the squamous-cell carcinoma and adenocarcinoma forms of OC, despite the two forms having distinct pathological entities at a molecular level. The two forms of cancer are also distinct in terms of the frequency, type and distribution of the *p53* mutations they possess indicating the diverseness of aberration that can lead to disease. Due to the highly conserved DNA-binding domain of the p53 protein, mutation of the regions interacting with this molecule could produce the same effects as mutations altering p53 protein function. Further studies exploring this and p53 protein aberration could prove particularly rewarding to our understanding of carcinogenesis, especially considering the fact that heightened p53 expression levels have been reported in the nuclei of proliferating cells.

1.11. Study objectives

Our research group has its focus on a number of genes involved in iron metabolism in the OC patient and control cohorts used in this study. The genes include *HFE* (see section 1.4.7.1.1), *SLC40A1* (see section 1.4.2), *CP* (see section 1.4.7.2), *HAMP* (see section 1.7) and *HEPH* (see section 1.9.1.3). The *HAMP* gene has recently been identified to be a crucial regulator in iron metabolism and has been associated with a variety of iron related disorders, cancer included. The promoter region of the *HAMP* gene has yet to be characterized, and therefore the focus of this study was the characterization of the 5' UTR of the *HAMP* gene in an OC cohort.

The objectives of this study were as follows:-

1. Identification of novel and previously identified promoter variants in the *HAMP* gene of an OC patient cohort.
2. Investigation of the effects of the identified variants in the promoter region of *HAMP* on gene expression.
3. Investigation of the iron parameters of an OC cohort relative to a healthy control population and the effect that the presence of a promoter variant in the *HAMP* gene may have on these parameters.
4. Assessment of the genotype-phenotype correlation between variant presence and absence, OC presentation and iron parameter indicators.

Chapter 2

Detailed Experimental Procedures

The study protocol was approved by the Ethics Review Committee of the University of Stellenbosch (N07/06/147)

2. Detailed Experimental Procedures

2.1. Subjects

The patient study population comprised 48 unrelated individuals of the Black Xhosa-speaking population of South Africa. Individuals were from the Transkei region of Southern Africa with cultural habits originating from Xhosa tribes.

Written and informed consent was obtained from each participating individual before obtaining blood samples. Blood samples were obtained from the 48 unrelated patients, all of which suffered from squamous cell carcinoma of the eosophagus. A barium swallow followed by theatre biopsies were performed on patients to confirm the presence of ADC or SCC on a histological basis (Hermanek and Sobin, 1987).

Following written and informed consent, blood samples were obtained from 51 healthy, unrelated population and sex matched individuals to serve as a control cohort. Brush biopsy, using a Nabeya capsule, was used for early screening of OC amongst the control individuals.

Iron overload could be classified under the following conditions: transferrin saturation greater than 45% and/ or serum ferritin exceeding 200µg/l in females and 300µg/l in males. The following iron parameters were obtained for all participating patient and control individuals: serum-iron, transferrin, transferrin saturations, ferritin, Alanine Transaminase (ALT) and C-reactive protein (CRP) levels. Transferrin saturation was determined on the basis of serum transferrin and serum iron concentrations. ALT and CRP levels were taken to differentiate between raised serum ferritin levels due to increased body iron stores or hepatocellular damage and inflammation respectively. Normal serum-iron ranges for males and females are 65 - 176µg/dL and 50 – 170µg/dL, respectively. Transferrin levels of between 0.21g/L to 0.36g/L are considered within the normal range for healthy adults. ALT reference ranges lie between 5 and 40 IU/L for healthy adults, with CRP reference ranges for

healthy adults of 10 – 40mg/L indicating mild inflammation and viral infection, 40 – 200 mg/L indicating active bacterial infection and >200mg/L indicating severe bacterial infection.

2.2. Polymerase chain reaction (PCR) amplification

2.2.1. Oligonucleotide primers

The primer pairs, melting temperatures and PCR conditions used for the amplification of the *HAMP* promoter region (1202bp) are shown in Table 2.1. Primers were designed using the Primer 3 program (Rozen and Skaletsky, 2000, available at http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi,2002). Similar parameters are shown for the amplification of the *TP53* gene (Table 2.2). These primer pairs were adapted, and modified from Audrezet *et al.* (1993).

Table 2.1 Oligonucleotide primers used for the amplification of the *HAMP* promoter region

Primer name	Primer Sequences 5' – 3'	T_m (°C)	Amplicon size (bp)	T_{A1}	T_{A2}	PCR cycle	$MgCl_2$ [mM]
PP2F	CATCGGACTGTAGATGTTAGC	60	256	60		A	1.5
PP2R	TCAAGACTAGCCTGGGCAAC	62					
PP3F	CACGCCTGGCTAAATTTGTT	58	326	55		A	2
PP3R	CACCACACGTGCATAGGTTC	62					
PP4F	TCAAGGGTCTGACACTGGG	62	312	60	55	B	1.5
PP4R	CCATCACGATGTCATTCTGC	60					
PP5F	AAGTGAGTGGAGGAGAGCG	62	285	55		A	2
PP5R	CTTTGCTCTGTCTCATTTCC	58					
PP6F	CTGAGGGTGACACAACCCT	60	291	56		A	1
PP6R	AGAGCCACTGGTCAGGCTG	62					

Abbreviations: 5', 5-prime; 3', 3-prime; °C, degrees Celcius; bp, base pair; F, Forward; mM, millimoles per liter; $MgCl_2$, magnesium chloride; PCR, polymerase chain reaction; PP, promoter primer; R, Reverse; T_{A1}, annealing temperature one; T_{A2}, annealing temperature two; T_m, melting temperature [$T_m = 2({}_nA + {}_nT) + 4({}_nG + {}_nC)$]. (For A and B cycles refer to section 2.2.2)

Table 2.2 Oligonucleotide primers used for the amplification of *TP53*

Primer name	Primer Sequences 5' – 3'	T_m (°C)	T_{A1}	T_{A2}	PCR cycle	$MgCl_2$ [mM]
Ex2F	GGTTGGAAGTGTCTCATGCTGGAT	59.1	Not optimized			
Ex2R	GACAAGAGCAGAAAGTCAGTCCCA	58.7				
Ex2-4F	CAGGTGACCCAGGGTTGGAAG	60.1	50	54	B	1.5
Ex2-4R	GGAAGGGACAGAAGATGACAG	54.8				
Ex4F	CCAGCAGCTCCTACACCGGC	63.1	55		A	2
Ex4R	GAATCCCAAAGTTCCAAACA	50.9				
Ex5aF	CTTTCAACTCTGTCTCCTTC	50.3	55		A	2
Ex5aR	CCTGGGCAACCAGCCCTGTC	63.6				
Ex5bF	TGGCCAAGACCTGCCCTGTG	62.7	55		A	2
Ex5bR	CCTGGGCAACCAGCCCTGTGGT	66.5				
Ex6F	TACAAGCAGTCACAGCACATGACG	59.4	Not optimized			
Ex6R	AGGGAGGTCAAATAAGCAGCAGGA	60.1				
Ex7F	TTGCCACAGGTCTCCCCAAG	59.8	54	50	B	1.5
Ex7R	GGGCACAGCAGGCCAGTGTG	64.1				
Ex8F	GGACAGGTAGGACCTGATTTC	54.6	55		A	2
Ex8R	AATCTGAGGCATAACTGCAC	52.8				

Abbreviations: 5', 5-prime; 3', 3-prime; °C, degrees Celcius; bp, base pair; Ex, exon; F, Forward; mM, millimoles per liter; $MgCl_2$, magnesium chloride; PCR, polymerase chain reaction; R, Reverse; T_{A1}, annealing temperature one; T_{A2}, annealing temperature two; T_m, melting temperature [$T_m = 2({}_nA + {}_nT) + 4({}_nG + {}_nC)$]. (For A and B cycles refer to section 2.2.2)

2.2.2. PCR amplification reactions and conditions

PCR reactions were performed in 25µl volumes, using the primer pairs listed in Table 2.1 and 2.2. The reactions comprised of 20ng DNA template, 0.2mM of each dNTP (dATP, dCTP, dGTP, dTTP) (Fermentas), 10pmol of each primer, 1 x Taq buffer (Bioline), $MgCl_2$ (Bioline) at concentrations indicated in Table 2.1 and 2.2, and 0.5U Taq polymerase enzyme (Bioline). Two different PCR cycling programs were used for amplification, labeled A and B:

Cycle A

An initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, 45 seconds at the annealing temperature specified in Table 2.1 or 2.2 (indicated as T_{A1}), and 30 seconds of extension at 72°C. This was followed by a final extension time of 10 minutes at 72°C.

Cycle B

An initial denaturation step at 95°C for 5 minutes, followed by 10 cycles of denaturation at 95°C for 30 seconds, 45 seconds at the annealing temperature specified in Table 2.1 or 2.2 (indicated as T_{A1}), and 30 seconds of extension at 72°C. This was followed by 30 cycles of denaturation at 95°C, 45 seconds at the annealing temperature specified in Table 2.1 or 2.2 (indicated as T_{A2}), and 30 seconds of extension at 72°C. A final extension time of 10 minutes at 72°C was then allowed.

2.3. Agarose gel electrophoresis

PCR amplification success was tested on a 1.5% (w/v) horizontal agarose gel [2.25g agarose in 150ml 1 x TBE (90mM Tris-HCl, 90mM H_3BO_3 and 0.1mM EDTA, pH 8.0) and 0.01% (v/v) ethidium bromide (EtBr)]. Prior to loading, 5µl of the PCR product was mixed with an equal volume of cresol red loading buffer (2mg/ml cresol red and 35% (w/v) sucrose) and

loaded onto the gel. A 100 bp ladder (Fermentas) was used as a molecular size marker in order to assess whether the size of the amplicon obtained was correct. The PCR products were resolved at 120V for one hour in 1 x TBE buffer solution and the DNA subsequently visualized by ultraviolet light transillumination assisted by a GeneSnap MultiGenius Bio Imaging System (Syngene).

2.4. Heteroduplex single stranded conformational polymorphism (HEX-SSCP) analysis

A 30cm vertical gel apparatus was used for HEX-SSCP analysis and samples were loaded on 30cm 12% polyacrylamide (PAA) gels [7.5% urea, 1.5 x TBE (135mM Tris-HCl, 90mM H_3BO_3 and 2mM EDTA, pH 8.0), 12% (w/v) PAA (1% C of a 40% stock (99 acrylamide (A): 1 bisacrylamide (BAA)), 0.1% (w/v) ammonium persulphate (APS) and 0.01% N, N, N', N' – tetramethylethylenediamine (TEMED)].

Prior to loading, PCR product was mixed with 15µl bromophenol blue loading dye (95% (v/v) formamide, 20mM EDTA, 0.05% (w/v) bromophenol blue and 0.05% (w/v) Xylene cyanol) and denatured at 95ºC for a duration of 10 minutes. Immediately after the denaturation step, tubes were snap cooled by incubation on ice for a period of 5 minutes.

Gels were electrophoresed at 250V for 18 hours at 4ºC in 1.5 x TBE buffer. Upon completion, gels were stained by submerging in a 0.01% (v/v) ethidium bromide solution by submergence therein for 10 minutes and subsequently de-stained in dH_2O for 5 minutes. Visualisation was performed using ultraviolet light trans-illumination [MultiGenius Bio Imaging System (Syngene)].

2.5. Semi-automated DNA sequencing analysis

PCR products showing aberrant conformers upon HEX-SSCP analysis were reamplified and sent to InQaba Biotec for bi-directional semi-automated sequencing analysis. The primers used for sequencing are the same as those used for PCR amplification in Tables 2.1 and 2.2. Sequencing results were then subsequently analyzed using ClustalW and BioEdit Sequence Alignment Editor software (Hall, 1999 and Thompson *et al*., 1997).

2.6. Construct preparation

Primer sets PP2, PP3, PP4 and PP5, outlined in Table 2.1, were modified to contain restriction enzyme recognition sites for the *NheI* (G*CTAG C) and *BglII* (A*GATC T) endonucleases (Table 2.3). Variants identified in the promoter region of the *HAMP* gene; namely -429 G/T, -582 A/G, and -188 C/T were amplified using the primers and reaction conditions specified in Table 2.3. These products were then purified according to procedures outlined in section 2.6.1 and the purified PCR product obtained was subsequently used for construct preparation.

The pGL4.10[*luc*2] (Promega) promoterless vector was used for preparation of constructs containing the entire *HAMP* promoter region. This was performed for the -429 G/T and -582 A/G variants only. The pGL4.23[*luc*2/minP] (Promega) minimal promoter-containing vector was used for the preparation of the constructs with fragments of the *HAMP* promoter containing the variant under investigation and flanking regions of +/- 200bp. This was performed for the -188 C/T, -429 G/T and -582 A/G variants. A whole promoter-containing construct could not be prepared for the -188 C/T fragment due to the occurrence of other variants within the vicinity of the variant of interest. Proliferation of a β-galactosidase (Promega) producing plasmid was performed for co-transfection with the promoter-less pGL4 constructs for normalization of the *firefly* luciferase readings obtained. Proliferation of the pGL4.73[*hRluc*/SV40] (Promega) vector producing *renilla* luciferase protein was used for co-transfection with the minimal promoter-containing constructs for normalization of the *firefly* luciferase readings obtained.

Table 2.3 Oligonucleotide primers used for the incorporation of endonuclease recognition sites

Primer name	Primer Sequences 5' – 3'	T_m (°C)	T_{A1}	T_{A2}	PCR cycle	$MgCl_2$ [mM]
PP2F	AGTGCTAGCCATCGGACTGTAGATGTTA	62	60		A	2
PP6R	AGAGCCACTGGTCAGGCTG	62				
PP3F	AGTGCTAGCCACGCCTGGCTAAATTTGTT	64	55		A	2
PP3R	TGAAGATCTCACCACACGTGCATAGGTT	61				
PP4F	AGTGCTAGCTCAAGGGTCTGACACTGGG	65	60	55	B	1.5
PP4R	TGAAGATCTCCATCACGATGTCATTCTGC	60				
PP5F	AGTGCTAGCAAGTGAGTGGAGGAGAGCC	65	55		A	2
PP5R	TGAAGATCTCTTTGCTCTGTCTCATTTCC	59				

Abbreviations: 5', 5-prime; 3', 3-prime; °C, degrees Celcius; bp, base pair; F, Forward; mM, millimoles per liter; $MgCl_2$, magnesium chloride; PCR, polymerase chain reaction; PP, promoter primer; R, Reverse; T_{A1}, annealing temperature one; T_{A2}, annealing temperature two; T_m, melting temperature [$T_m = 2({}_nA + {}_nT) + 4({}_nG + {}_nC)$].

2.6.1. Purification of DNA solutions

Purification of DNA solutions was performed using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences – GE Healthcare). A GFX column was placed within a collection tube for each purification to be performed and 500µl Capture Buffer (Amersham Biosciences: containing acetate and choatrope) added to each GFX column. The DNA solution to be purified was then added to the Capture Buffer and mixed thoroughly *via* pipetting action. Post a 30 second centrifugation step at 16000 x g, the elute was discarded. A mixture of 500µl Wash Buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, absolute ethanol to a final concentration of 80%) was subsequently added to the GFX column and subjected to centrifugation at 16000 x gravity for 30 seconds. The flow-through was discarded and the GFX column transferred to a clean 1.5ml Eppendorff tube. A volume of 50µl elution buffer

(10mM Tris-HCl, pH 8.0) was added and each column and subjected to a 1 minute centrifugation step at 16000 x gravity.

2.6.2. Digestion

The PCR product, pGL4.10 and pGL4.23 vectors were digested with restriction enzyme endonucleases *NheI* and *BglII* (NEB) allowing for the creation of sticky ends for ligation. The multiple cloning sites of pGL4 vectors include recognition sites for the aforementioned restriction endonucleases, allowing for insert incorporation in a specific orientation only.

The digestion of the PCR products and pGL4 vectors were performed in 20µl reactions, consisting of 10µl PCR product, 2U or the relevant enzymes (*NheI* and *BglII*) and 1X buffer, in a 37^{o}C waterbath, and digested overnight.

2.6.3. Ligation and dephosphorylation

Dephosphorylation of the digested pGL4 vectors used, and ligation of the *HAMP* promoter inserts and the dephosphorylated vector, was carried out using the Rapid DNA Dephos & Ligation Kit (Roche). Dephosphorylation and ligation were carried out according to the manufacturer's specifications where rAPid Alkaline Phosphatase and T4 DNA Ligase were used for dephosphorylation and ligation, respectively.

2.6.4. Test ligation

To test for successful ligation the ligated product and insert-less pGL4 vector was subsequently digested using only the *NheI* enzyme. The digested products were resolved on a 1% Agarose gel as specified in 2.3. Restriction enzyme digestion reactions were carried out using the same protocols outlined in section 2.6.2.

2.7. Transformation

The competent cell transformation procedure was performed using the EZ Competent cells (Qiagen) kit. A volume of 4µl of ligation mixture (as outlined in section 2.6.3) was added to pre-cooled tubes containing 25µl of Qiagen EZ Competent cells, and subsequently incubated on ice for 30 minutes. This was followed by a heat shock step at 42°C for 30 seconds, followed by incubation on ice for 2 minutes. Luria-Bertani (LB) (10g NaCl, 10g Tryptone, 5g Yeast Extract, brought to volume of 1L) medium (300 µl) was added to each tube and incubated on a shaker at 37°C, shaking at 180rpm for one hour. Following an overnight incubation at 37°C, 150µl culture was plated out on LB-Agar-Ampicillin (10g NaCl, 10g Tryptone, 5g Yeast Extract, 20g Agar brought to volume of 1L, with 0.06mg/ml ampicillin) plates and incubated overnight at 37°C.

2.8. Colony PCR

Colony PCR was performed on the colonies obtained in order to verify the presence and orientation of the insert. PCR reactions were performed using the primers and reaction conditions outlined in Table 2.4. The RV3 primer was specific to the pGL4 vector used. When used in conjunction with the respective *HAMP* promoter fragment primer; amplification would occur only in the presence of a *HAMP* promoter amplicon inserted in the correct orientation. Orientation of the insert was also confirmed using semi-automated bidirectional DNA sequencing analysis (see section 2.5).

Table 2.4 Primer and reaction specifications for colony PCR reaction

Primer name	Primer Sequences 5' – 3'	T_m (°C)	T_{A1}	T_{A2}	PCR cycle	$MgCl_2$ [mM]
RV3	CTAGCAAAATAGGCTGTCCC	54	55	N/A	A	2
PP3R	TGAAGATCTCACCACACGTGCATAGGTT	61				
PP4R	TGAAGATCTCCATCACGATGTCATTCTGC	60				
PP5R	CTTTGCTCTGTCTCATTTCC	58				

Abbreviations: 5', 5-prime; 3', 3-prime; °C, degrees Celsius; bp, base pair; F, Forward; mM, millimoles per liter; $MgCl_2$, magnesium chloride; PCR, polymerase chain reaction; PP, promoter primer; R, Reverse; T_{A1}, annealing temperature one; T_{A2}, annealing temperature two; T_m, melting temperature. $T_m = 2({}_nA + {}_nT) + 4({}_nG + {}_nC)$. Note: the RV3 primer was used as the forward primer in conjunction with each of the reverse primers listed in the table. (For cycle A refer to section 2.2.2)

2.9. Transformation and plasmid proliferation of pGL4 and β-Galactosidase (β-gal) plasmids

Proliferation of the pGL4 (Fig 2.1 – 2.3) and β-Gal plasmids (Fig. 2.4) were performed by the transformation of EZ Competent cells (Qiagen) as per section 2.7. A single colony was picked, and used to inoculate 150ml LB-Amp medium. Inoculated cultures were then incubated overnight at 37°C, shaking at 180rpm. Plasmid DNA extraction was then performed according to section 2.10.

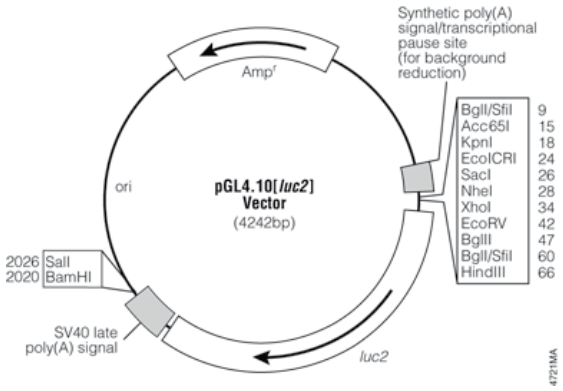

Fig 2.1 Schematic representation of the pGL4.10[*luc*2] promoterless vector (Promega)

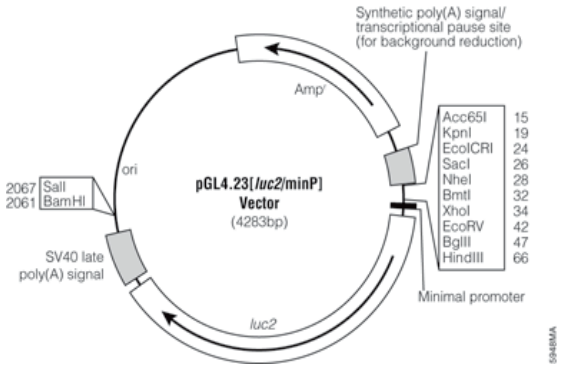

Fig 2.2 Schematic representation of the pGL4.23[*luc*2/minP] minimal promoter-containing vector. (Promega)

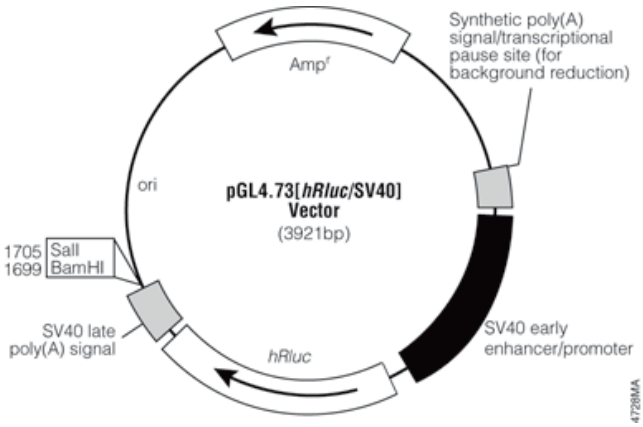

Fig 2.3 Schematic representation of the pGL4.73[*hRluc*2/SV40] vector (Promega)

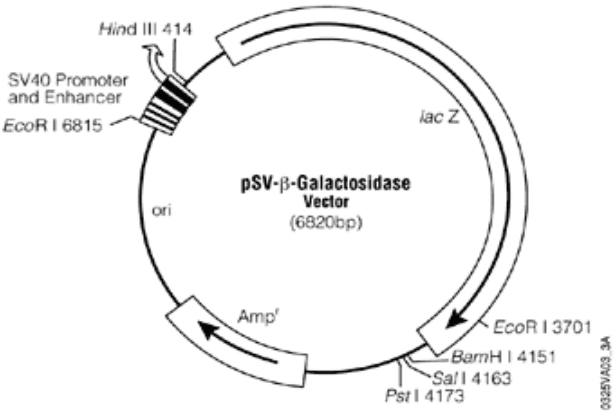

Fig 2.4 Schematic representation of the pSV-β-Galactosidase control vector. (Promega)

2.10. Plasmid DNA extraction

Plasmid DNA extraction was performed using the Qiagen Endofree Plasmid Maxi Kit. Cultures were allowed to proliferate to cell densities of approximately 3-4 x 10^9 cells per ml culture medium, then subsequently harvested by centrifugation at 6000 x gravity for 15 minutes at 4°C. Plasmid extraction was then executed according to the manufacturer's instructions (Qiagen Plasmid Maxi Protocol). The plasmid concentration obtained ranged between 1.8µg/µl and 3.0µg/µl.

2.11. Transfection

2.11.1. Cell preparation

A HepG2 (ATCC®) liver cell line was used to investigate the effect of the identified *HAMP* promoter variants had on gene expression. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% Foetal Bovine Serum (FBS) (v/v) (Sigma), 2mM L-Glutamine (Sigma) and 100U/ml Penicillin and Streptomycin (Sigma), incubated at 37ºC with an atmosphere of 5% CO_2; 95% air. HepG2 cells were washed using 5ml DMEM medium prior to use, scraped from the flask and subsequently centrifuged at 800rpm for 5 minutes at room temperature (Spectrafuge 24D, Labnet International). The supernatant was discarded, and cells resuspended in 5ml fully supplemented DMEM medium. This was then added to a flask containing a 10ml volume of fully supplemented DMEM medium and incubated under the atmospheric conditions as described above.

Prior to transfection the cells were transferred to a six-well plate. Fresh medium was added to the cells and subsequently evenly dispersed amongst the wells of a six-well plate. Plates were incubated for a period of 24 hours to allow for confluence.

2.11.2. Construct addition

GeneJuice (Novogen; Merck) was used as a transfection reagent to facilitate the uptake of the construct by the HepG2 cell line. GeneJuice was applied in a 1:1 µg DNA to µl GeneJuice ratio for effective transfection; and 50µl of fully supplemented DMEM medium was used for every 1µl of GeneJuice used during the transfection procedure. For each transfection 200ng construct DNA and 100ng co-transfectant were used (β-Gal in the case of the promoterless constructs and pGL4.73 in the case of minimal promoter-containing constructs). Each experiment comprised of triplicate runs (3 wells containing the same variant containing construct) and each experiment was performed three times.

2.11.3.Exogenous stimuli addition

Twenty-four hours after the transfection procedure, exogeonous stimuli were added to the construct containing cultures. Each construct was subjected to treatment with Ferric Ammonium Citrate (FAC) ($C_6H_{11}FeNO_7$) (Fluka) to a final volume of 200µg/ml per well. Constructs were also subjected to treatment with human recombinant Interferon-γ (INF-γ) (Sigma) to a final concentration of 10ng/ml per well and lipopolysaccharides (LPS) (Sigma) to a final concentration of 10ng/ml per well.

2.11.4.Luciferase assay

The luciferase assay was used for the promoterless construct containing cultures. Following induction, cells were harvested, lysed and assayed using a luciferase assay kit (Sigma). Medium was removed from the wells and each well washed three times with 1 x PBS solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4). A volume of 350ml cell culture lysis reagent (Sigma) (Sigma reagent constituents not available) was added to each well and plates incubated at room temperature for a period of 15 minutes. The cells were subsequently scraped off the bottom of each well using a glass scraper and the contents of each well transferred to an appropriate 1.5ml Eppendorff tube for centrifugation at 16 000 x g 1 minute at 4ºC. The supernatants were removed and transferred to a new Eppendorff tube for storage at -80ºC. The luciferase substrate and cell lysate containing luciferase were allowed to equilibrate to room temperature before use. Lysate (20µl per well) was pippetted into a 96-well microtiter plate and the plate transferred to a luminometer (Veritas™ Microplate Luminometer) for automated luciferase readings. An amount of 50µl luciferase substrate was automatically added to the appropriate wells and the relative light units (RLU) measured using the Veritas v2.0.4401 coupled software program (2sec delay, 8sec integration time).

2.11.5.Beta-Glo assay

Beta-Glo assays were used for normalisation of the luciferase values obtained from the promoterless construct containing cultures, using a Beta-Glo Assay kit (Promega). Briefly, the contents of the Beta-Glo Assay substrate and Beta-Glo Assay buffer bottles were combined and gently mixed. A volume of 100µl of the cell lysate solution described in section 2.11.4 was added to the appropriate wells of a 96-well microtiter plate and subsequently 100µl of the combined Beta-Glo solutions added to each filled well. A 30 minute incubation period at room temperature was allowed giving the signal time to stabilise after which the signal was measured using a luminometer.

The relative light units of the luciferase assay were normalised to the β-galactosidase readings obtained for each construct within an experiment. This reading was subsequently normalised to the relevant wild-type sequencing containing construct used. This allowed for a luciferase reading normalised to a co-construct, relative to the a WT.

2.11.6.Dual-luciferase reporter assay

Dual luciferase assays were performed using the Dual-Luciferase Reporter Assay Kit (Promega). Medium was removed from each well of the six-well plate, and each well subsequently washed three times with 1 x PBS solution. Active lysis of the cells was achieved by applying 300µl of 1 x Passive Lysis Buffer (PLB) (Promega) to each well and then immediately scraped using a sterile glass scraper. The lysate was subsequently transferred to 1.5ml Eppendorff tube and frozen at -20ºC. Following 1 – 2 freeze-thaw cycles, active cell lysis was completed.

Cell lysates (20µl per well) were transferred to a 96-well microplate. The plate was then transferred to a luminometer (Veritas™ Microplate Luminometer) for automated luciferase readings (Veritas v2.0.4401 software program). The luminometer was programmed to add 50µl of Luciferase Assay Reagent II (LARII) (kit supplied lyophilized Luciferase Assay Substrate in 10ml kit supplied Luciferase Assay Buffer II) (Promega) to each cell lysate with

a 2 second delay and 8 second reading time. Luminometer, injector 2, was then utilised to add 50µl Stop & Glo® Substrate (1 x dilution of kit supplied Stop & Glo® Substrate using kit supplied Stop & Glo® Buffer) (Promega) to obtain *renilla* luciferase readings, present due to co-transfection with the pGL4.73 construct.

2.12. Statistical analysis

Allele and genotype frequencies were estimated and statistical differences between patient and control groups were tested for significance by the Fisher exact test and/or chi-squared (χ^2) analysis. The Hardy-Weinberg equilibrium (HWE) was performed to test equilibrium for the genetic traits investigated. Box plots were used to identify outliers in the iron parameter data of patients and controls. The F-test for variance was used to assess the type of *t*-test to be used and subsequently the student's *t*-test for equal or unequal variances was carried out to determine statistically significant associations between patients and controls, males and females and within male and female cohorts, accordingly. All tissue culture experiments were repeated three times with triplicate runs of each construct within each experiment. The statistical analysis software R was used to investigate potential differences between constructs, separately for untreated as well as in the presence of exogenous stimuli. The dependent variable throughout was the log-ratio of luciferase to Beta-Glo/*Renilla*. For each stimulus, a hierarchy of linear mixed-effects models was fitted (Pinheiro & Bates, 2000), with the most appropriate model used to test for significant differences between constructs. This allowed various forms of heterogeneity to be modeled; runs were entered as random effects. Graphs of the multiplicative effects of the various constructs, in the original units, were produced in each case, together with 95% confidence intervals. Each run was investigated separately if there were significant inconsistencies between runs. Probability (*P*) values smaller than 0.05 were regarded as statistically significant.

Chapter 3

Results

Presented in the form of a full-length manuscript in preparation of future publication in a scientific journal

CHARACTERIZATION OF THE PROMOTER REGION OF THE *HAMP* GENE IN OESOPHAGEAL CANCER

ABSTRACT

The hepcidin antimicrobial peptide gene (*HAMP*) encodes for a 25 amino acid protein, which has been found to be primarily expressed in liver tissue and is crucial to the regulation of bodily iron status. Defects occurring in the promoter region of the *HAMP* gene may contribute to the dysregulation of the gene, resulting in an iron overload status. Iron overload is a previously described risk factor in the development of various cancers, including oesophageal cancer (OC).

The aim of this study was to investigate the promoter region of the *HAMP* gene in the Black South African population. The *HAMP* promoter region of Black South African OC patients and a population-matched control group were subjected to mutation screening using polymerase chain reaction (PCR) amplification methods and heteroduplex single-stranded conformational polymorphism (HEX-SSCP) analysis. One previously described (-582A/G) and two novel (-188C/T and -429G/T) variants were identified in the 5'UTR region of this gene. All variants were confirmed by bi-diectional semi-automated sequencing analysis.

The novel variant located at position -429, relative to the transcriptional start site, was found to occur at a high frequency within the patient cohort and was absent from the control group. The variant was found to disrupt a recently identified p53 response element; linking it directly to the cancer progression phenotype. The use of this variant in the development of risk assessment and/or diagnostic test development, as well as cancer drug therapies, warrants further consideration. Bioinformatic analysis of the -188C/T variant did not reveal it to

create or abolish any known transcription factor binding sites, where as the presence of the -582A/G variant resulted in the disruption of a USF binding site.

INTRODUCTION

Oesophageal cancer (OC) is the sixth leading cause of cancer related deaths in the world (Pisani *et al.*, 1999), with the majority of OC classified as squamous-cell carcinoma (SCC) and adenocarcinoma (ADC). The precise mechanisms of disease pathogenesis remain unclear. However, several factors have previously been associated with OC development, including irritation of the oesophageal tissues by oxidative damage, which may result from a variety of factors such as smoking and gastroesophageal reflux. This, in turn, can lead to inflammation of the oesophageal tissues and contribute to the carcinogenic process (Terry *et al.*, 2000).

The amount of dietary iron taken up by the body is tightly regulated as the body does not possess an active mechanism for iron excretion. Free radicals produced as a result of iron partaking in the Fenton Reaction have been shown to be damaging, therefore illustrating the vital role iron plays in facilitating cellular proliferation. Iron containing enzymes are responsible for the catalization of numerous metabolic reactions crucial to the efficient and effective progression of DNA synthesis and energy provision. It is thus apparent that iron is vital for cellular proliferation and its availability therefore directly correlates to cancer cell development (Larrick and Cresswell, 1979; Chitambar *et al.*, 1983). Cancerous tissues have been shown to absorb increased amounts of iron to sustain the rapid levels of their proliferation (Chan *et al.*, 1987). The use of iron chelators, such as desferrioxamine, have also been used to inhibit the growth of aggressive tumours, highlighting the dependence of the tumour tissues on this trace element (Kwok and Richardson, 2002).

The hepcidin protein, encoded by the hepcidin antimicrobial peptide gene (*HAMP*), has been shown to be crucial for the regulation of iron metabolism. The protein is involved in the inhibition of the intestinal absorption of iron, induction of iron sequestration by macrophages and the blockage of iron transport across the placental barrier (Atanasiu *et al.*, 2006). The *HAMP* gene is up-regulated in response to high levels of bodily iron and inflammation, resulting in increased production of the hepcidin protein, therefore preventing any further assimilation of iron at the intestinal lumen. The hepcidin protein functions by binding to the membrane-bound protein, ferroportin (FPN), resulting in its internalisation and subsequent degradation. With FPN being the only known mammalian iron exporter, iron is unable to be transported from the intestinal enterocytes and subsequently to the plasma.

Recently, a p53 response element (p53RE) has been identified in the promoter region of the *HAMP* gene (Weizer-Stern *et al.*, 2007). By means of deletion constructs, this response element has been identified to span positions -435 to -413 relative to the transcription start site. It has been illustrated that the binding of the p53 tumour suppressor protein to the p53RE site results in the increased transcriptional activity of the *HAMP* gene. This results in reduced iron export amongst FPN expressing tissues and at the intestinal lumen. The resulting iron deprivation experienced by cancer cell lines could result in cell cycle arrest or apoptosis, highlighting an alternative pathway for the tumour suppressor protein, p53, involved in cancer prevention and/or treatment (Weizer-Stern *et al.*, 2007).

In this study, we investigated the possible involvement of *HAMP* promoter variants in OC development by mutation analysis of the promoter region of the gene in a Black South African cohort.

MATERIALS AND METHODS

Study cohort

The study cohort consisted of 48 unrelated OC patients from the Black Xhosa-speaking population of South Africa, presenting with SCC of the oesophagus and displaying symptoms such as severe weight loss and dysphasia. A barium swallow followed by a tissue biopsy was implemented to confirm the presence of a SCC phenotype on a histological basis. Blood samples were obtained from the study patient cohort and from 51 healthy, unrelated population-matched individuals, the control cohort. Control individuals were subjected to early OC screening by brush border biopsy. In this study "Black" refers to South Africans of central African descent.

Methods

See chapter 2 for detailed experimental procedures. For simplicity, the luciferase constructs are numbered as shown in Table 3.1 for the rest of this section.

RESULTS AND DISCUSSION

Mutation analysis, by means of HEX-SSCP, followed by bi-directional sequencing analysis, demonstrated the presence of three variants in the -1202 bp *HAMP* gene promoter fragment investigated. These included one known (-582A/G: rs10421768) and two novel (-188C/T and -429G/T) variants. The allele and genotype frequencies of these variants in the Black South African population are shown in Table 3.1. A statistically significant association was observed for the -429G/T variant when comparing the patient (10 of 41) and population-matched control (0 of 48) groups. This variant was only observed in the heterozygous state.

Table 3.1: Allele and genotype frequencies of *HAMP* variants identified in the Black South African population

	Allele Frequencies*		**Gentoype Frequencies**			
Variant	**Patients**	**Controls**	**Genotype**	**Patients**	**Controls**	***P***
-188 C/T	0.1	0.08	CC	0.82 (37)	0.84 (51)	0.870
(this study)	(n = 45)		CT	0.16 (7)	0.16 (10)	
			TT	0.02 (1)	0.00 (0)	
-429 G/T	0.12	0.00	GG	0.75 (31)	1.00 (48)	*0.0002*
(this study)	(n = 41)		GT	0.25 (10)	0.00 (0)	
			TT	0.00 (0)	0.00 (0)	
-582 A/G	0.09	0.08	AA	0.83 (34)	0.84 (72)	0.811
(rs10421768)	(n = 41)		AG	0.15 (6)	0.16 (14)	
			GG	0.02 (1)	0.00 (0)	

*Allele frequency representative of the polymorphic allele only; actual numbers are shown in parenthesis

Iron parameters were obtained for all the patients and controls in this study. To further assess the effect of iron on disease expression, we performed statistical analyses on the iron parameters obtained. The analyses were performed for patients and controls, in combination and separately for males and females. This was performed because of the difference in "normal" parameters between males and females. The data was further analyzed by grouping individuals with and without the presence of the variants and then analyzing iron parameters based on these groupings. These groups included individuals with and without the identified variants within the patient group; as well as the variant presence and absence considering both patients and control individuals.

Males and Females

When comparing the female patient and female control groups, the serum-iron, transferrin, ferritin and C-reactive protein levels (CRP) were found to be statistically significant (Table 3.3). Mean serum-iron and mean transferrin levels were found to be higher in the control females relative to the female patient group, whereas the mean ferritin and CRP levels were higher in the female patient groups relative to the female control group (Addendum A: Table 6.2). This corresponds to a higher level of iron being stored as ferritin in OC patients, as well as a higher level of inflammation as indicated by the increased levels of CRP protein (Kalantar-Zadeh *et al.*, 2004). The amount of transferrin present is dependent on the amount of serum-iron and therefore low serum-iron levels are paired to low transferrin levels. The high level of iron stored as ferritin occurring amongst OC patients correlates with the reduced serum levels of iron available. Male patients exhibit the same pattern of statistical significance as the females despite variating normality in iron parameters between sexes.

Table 3.2: Iron parameter statistics for female and male groups individually

Description	t-Test *P*-value	Measured levels relative to controls
Females		
serum iron	2.83712E-08	Lower in patients
transferrin	1.75763E-27	Lower in patients
%transferrin saturation	0.085466439	Not significant
ferritin	0.000282725	Higher in patients
CRP	0.000354578	Higher in patients
Males		
serum iron	2.83712E-08	Lower in patients
transferrin	5.04404E-07	Lower in patients
%transferrin saturation	0.376404575	Not significant
ferritin	2.08292E-05	Higher in patients
CRP	1.53923E-06	Higher in patients

Variant Influence on iron parameters

Analyses were performed by grouping patients and controls with the variant (irrespective of homozygous or heterozygous state) vs. patients and controls without the variant.

When ignoring OC presence, and grouping the patient and control groups into one, we considering focused on the presence and absence of variants for iron parameters (Table 3.3). No statistically significant associations were found considering the presence or absence of the -188C/T and -582A/G variants, however statistical significance was identified for serum-iron, transferrin, ferritin and CRP levels in the presence of the -429G/T variant. Mean serum-iron and transferrin levels were elevated in the absence of the variant whereas mean ferritin and CRP levels were elevated in individuals heterozygous for -429G/T (Table 6.2). Elevated ferritin levels are coupled to increased iron-bodily iron status (Kalantar-Zadeh *et al.*, 2004) suggesting that the presence of the -429G/T variant results in under expression of the *HAMP* gene, since this would lead to an increase dietary iron assimilation.

Considering only the patient group, variant presence appeared to have a statistically significant association with transferrin levels and only in the case of the -188C/T and -429G/T variants (Table 3.4) Borderline significance was visualized for transferrin levels for the -582A/G variant (indicated in blue) (Table 3.4). Mean values for transferrin were higher in patients without a heterozygous or homozygous version of the variant (including both the heterozygous and homozygous variants), which can be explained by a lower level of free transferrin available in the presence of the variant should the presence of the variant result in under-expression of the *HAMP* gene. Reduced levels of the transferrin protein in the patient presenting with a variant, relative to patients without a variant suggests an interaction of the *HAMP* gene product with the *Transferrin* (*TF*) gene. Further investigation is required to ascertain whether this is occurring, and this is warranted by statistics indicating that the presence of one of the identified variants in the *HAMP* promoter region results in a significant/ marginally significant reduction in transferrin levels in patients without the variant.

Table 3.3: Iron parameter statistics representing variant presence or absence in the patients and controls.

Description	t-Test *P*-value	In Patients
-188C/T Presence vs. -188C/T Absence		
serum iron	0.33174998	
transferrin	0.351071062	
%transferrin saturation	0.237498835	
ferritin	0.427797616	
CRP	0.218144757	
-429G/T Presence vs. -429G/T Absence		
serum iron	0.001002562	lower
transferrin	4.33521E-09	lower
%transferrin saturation	0.244405236	
ferritin	0.043101444	higher
CRP	0.02123364	higher
-582A/G Presence vs. -582A/G Absence		
serum iron	0.239810997	
transferrin	0.382999266	
%transferrin saturation	0.11662121	
ferritin	0.45994801	
CRP	0.38771611	

Table 3.4: Iron parameter statistics representing variant presence or absence in patients.

Description	t-Test *P*-value	Variant Presence
-188C/T Presence vs. -188C/T Absence in Patients		
serum iron	0.386134878	
transferrin	0.031668925	lower
%transferrin saturation	0.426561774	
ferritin	0.289366507	
CRP	0.179977693	
-429G/T Presence vs. -429G/T Absence in Patients		
serum iron	0.077178176	
transferrin	0.03485538	lower
%transferrin saturation	0.459804835	
ferritin	0.198577288	
CRP	0.469491084	
-582A/G Presence vs. -582A/G Absence in Patients		
serum iron	0.213079178	
transferrin	0.057160041	
%transferrin saturation	0.357047402	
ferritin	0.4048286	
CRP	0.217360868	

Functional analyses were performed on the three variants identified: These variants were tested in the absence and presence of exogenous stimuli, including FAC, INF-γ and LPS, respectively.

The introduction of FAC mimics the occurrence of high intracellular iron levels. INF-γ, a cytokine involved in innate and adaptive immunity against viral and intracellular bacterial infections and tumour control, should increase hepcidin production to facilitate the sequestering of iron by activated macrophage cells (Hayward *et al.*, 2000). LPS are large molecules found in the outer membranes of gram-negative bacteria. They act as endotoxins eliciting a strong immune response upon detection by the body. An increase in hepcidin

production would be advantageous in this situation reducing the amount of iron available to invading pathogens (Ulevitch and Tobias, 1999).

The -188C/T variant

The basal expression profile obtained for the -188T variant indicated a significant (P = 0.0019) reduction in the amount of expression occurring relative to the -188C construct (Fig 3.1). When analysing the expression profiles for the various treatments, statistically significant under-expression was observed for both, INF-γ (P = 0.0293) and LPS (P = 0.0493) addition relative to the WT, which has been subjected to the same relevant treatment. No statistically significant under-expression occurred relative to the WT construct upon FAC induction, but expression was observed to occur to a lesser degree compared to the WT sequence also FAC induced. This suggests the presence of the -188C/T variant to significantly alter the regular expression profile of the *HAMP* gene under normal, and immune response conditions (Figures 3.1 – 3.4). Data indicates the presence of the variant does not hamper the *HAMP* expression under high-iron conditions, even though under-expression is observed relative to the WT in untreated experiments. It is important to consider that the presence of the minimal promoter, designed to bring about construct expression, may be overshadowing the presence of a single variant within the promoter region being investigated. The section of the *HAMP* gene included for minimal promoter investigation may not be able to function independently of its larger surrounding sequence region, and therefore the results obtained may be minimal-promoter driven. Whole-promoter constructs containing the -188C/T variant need be investigated to confirm the results obtained using minimal-promoter containing constructs.

No whole-promoter constructs were prepared for the -188C/T variants as no individual presented with only the -188C/T variant for the -1202bp region under investigation. Sit-directed mutagenesis may be employed to allow for whole-promoter construct preparation, but due to time and funding constraints this was not performed for this study. Future investigation considering whole-promoter analysis, however, need be carried out.

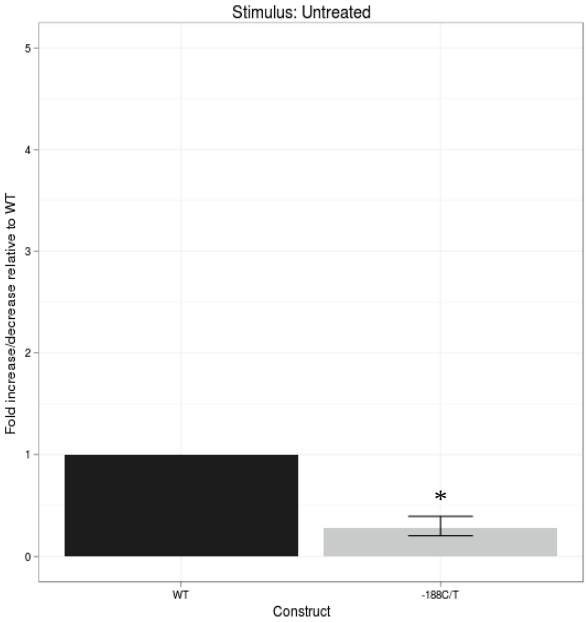

Fig 3.1 Luciferase assay results for minimal-promoter pGL4 vectors containing WT and -188C/T promoter fragment. Data represent means of at least three independent experiments (+/- SEM) and statistically significant *P* values are represented by *. *P* values less than 0.002 are considered to be statistically significant.

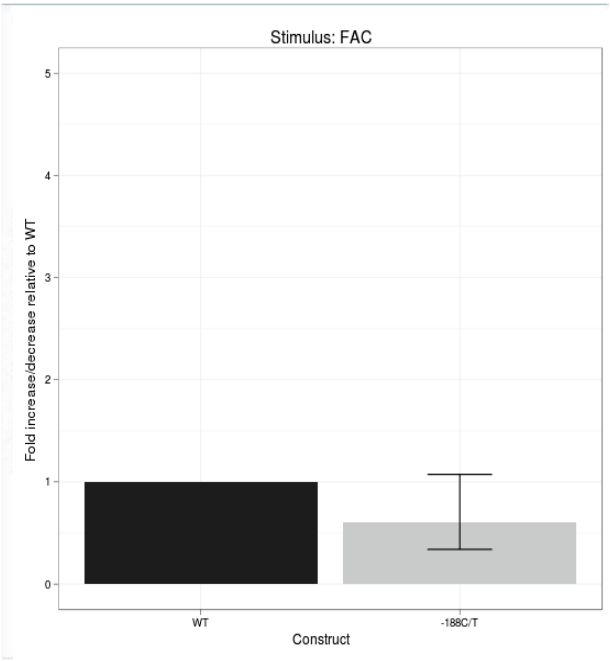

Fig 3.2 Luciferase assay results for minimal-promoter pGL4 vectors containing WT and -188C/T promoter fragment, FAC treatment. Data represent means of at least three independent experiments (+/- SEM).

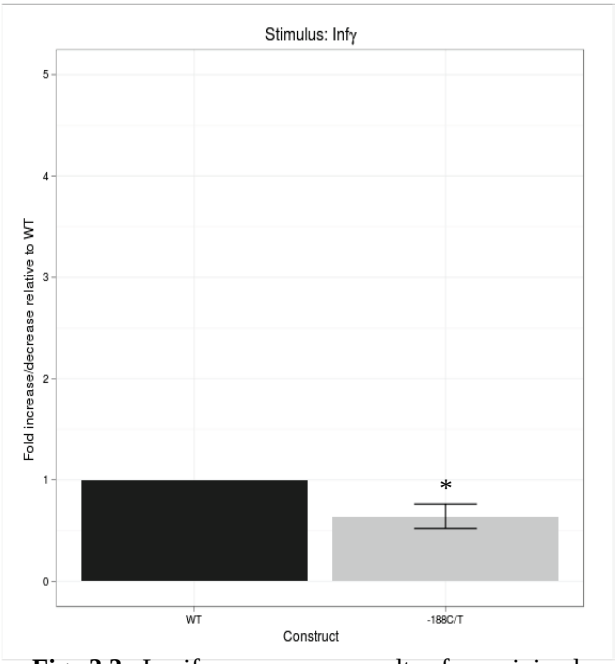

Fig 3.3 Luciferase assay results for minimal-promoter pGL4 vectors containing WT and -188C/T promoter fragments, INF-γ treatment. Data represent means of at least three independent experiments (+/- SEM) and statistically significant *P* values are represented by *. *P* values less than 0.05 are considered to be statistically significant.

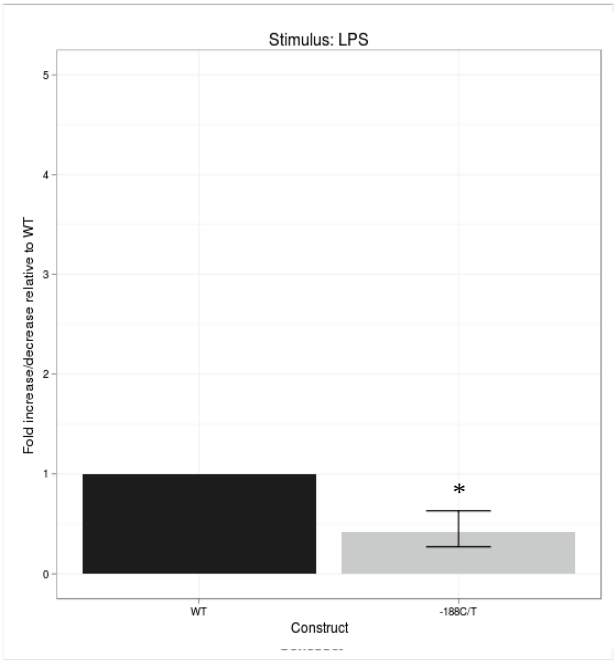

Fig 3.4 Luciferase assay results for minimal-promoter pGL4 vectors containing WT and -188C/T promoter fragments, LPS treatment. Data represent means of at least three independent experiments (+/- SEM) and statistically significant *P* values are represented by *. *P* values less or equal to than 0.05 are considered to be statistically significant.

The introduction of exogenous stimuli, showed under expression in the presence of the -188T variant relative to the WT, for untreated and all treatments. Statistically significant associations were demonstrated for run1 and run3 of untreated, INF-γ and LPS addition. Bioinformatic analysis did not reveal the creation or abolishment of a known transcription factor binding sites for the variant containing region; however the presence of the variant notably hampers transcriptional regulation by resulting in reduced expression of the *HAMP* gene. It can therefore be hypothesized that variant presence permits lower levels of hepcidin expression possibly allowing for a higher level of iron influx.

The -429G/T variant

A novel variant, a G to T transition at nucleotide position -429, demonstrated statistically significant differences between the patient (10 of 41) and control (0 of 51) cohort and was only identified in a heterozygous state. Although this variant has not been detected in our control population, it has been identified at a frequency of 1% (1 of 100) in a previous study performed in our laboratory in individuals of a South African Mixed Ancestry population (Panton *et al.*, 2008). The electropherogram indicating the -429G/T variant can be visualized in Figure 3.5.

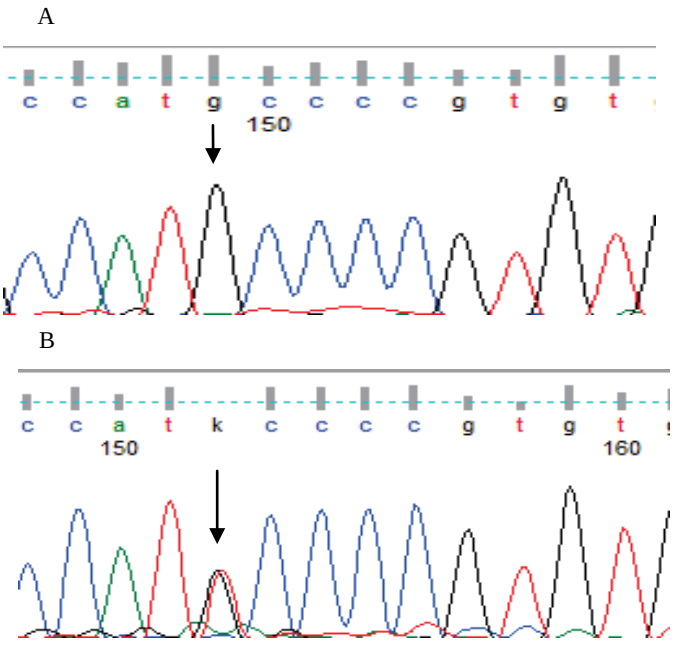

Fig 3.5 Electropherograms indicating (A) the wild-type sequence (B) the -429G/T variant in heterozygous state. The arrows indicate the point of variation.

The -429G/T variant is localised within an area previously identified as a response element for the p53 tumour response protein (p53RE) (Weizer-Stern *et al.*, 2007). The response element was found to span the region -435 to -413, relative to the transcription start site. Applications using deletion construct preparations and electromobility shift assays confirmed

binding of the p53 protein to this region. Weizer-Stern *et al.* (2007) also showed that the induction of single nucleotide polymorphisms within this region resulted in reduced binding affinity of the p53 protein. The presence of the -429G/T variant could therefore result in reduced p53 binding, or may inhibit binding completely.

The identification of a p53RE in the promoter region of the *HAMP* gene suggests the potential involvement of *HAMP* in cancer phenotype presentation. It was shown that binding of the p53 tumour suppressor to the p53RE region results in up-regulation of *HAMP* gene expression and increased production of the hepcidin protein. The presence of increased hepcidin protein subsequently allows for decreased absorption of iron, cutting off a vital resource required for the proliferation of cancer cells. Iron parameter statistics confirm this hypothesis indicating a large increase in the amount of ferritin in patients presenting with the -429G/T mutation; and increased CRP levels, a measure of inflammation in individuals heterozygous for the variant (Table 3.3).

The *TP53* gene was investigated for this study. A subset of 20 patients (WT and heterozygous for the -429G/T variant) and 20 controls were selected for, and subjected to mutation analysis for *TP53*. Screening of the *TP53* gene revealed no detectable variation in patients or controls. Tumour tissue samples (subset of 20 patients) were sent to an appropriate laboratory authority for p53 immunohistochemical and iron staining. No aberrant results were obtained but this may be due to the age of the tumour samples used and the fact that they were frozen beforehand. No conclusive results could therefore be deduced from this data.

Luciferase activity from the -429T whole-promoter construct showed a significant (P = 0.0329) reduction when compared to that of the -429G whole-promoter construct

demonstrating that the presence of the variant within the recently identified p53RE decreased transcriptional activity relative to the WT (Figure 3.6). Induction with FAC should result in an upregulation of the *HAMP* gene in response to a higher level of iron present, and subsequently a reduction in the amount of iron absorption required. The introduction of FAC still allowed for statistically significantly reduced levels of expression in the presence of the -429G/T variant relative to Luc9 ($P < 0.0001$) (Figure 3.7).

In the case of INF-γ addition, the same trend in expression was observed as in the cases of untreated and FAC addition; however there was statistically significant variation between the three experimental runs carried out. This inconsistency could account for the lack of significance observed between -429G and -429T constructs, and therefore individual runs were considered (Figures 3.8 – 3.9). Run 1 exuded aberrant results to what is theoretically expected (we expect an under-expression in the presence of the variant); however runs two and three showed the same trend of under-expression in the presence of the variant, although only run 2 showing statistical significance ($P = 0.0006$). Repeat experiments for this stimulus are warranted to confirm the results obtained. INF-γ, a cytokine involved in innate and adaptive immunity against viral and intracellular bacterial infections and tumour control, increases hepcidin production to facilitate the sequestering of iron by activated macrophage cells. Under-regulation relative to the -429G construct suggests more iron available to invading pathogens during infection in the presence of the -429T variant.

LPS are large molecules found in the outer membranes of gram-negative bacteria. They act as endotoxins eliciting a strong immune response upon detection by the body. An increase in hepcidin production would be advantageous in this situation reducing the amount of iron available to invading pathogens. The addition of LPS resulted in a significant increase ($P = 0.0001$) in the amount of expression occurring relative to the -429G construct, surpassing that

of -429G levels (Figure 3.10). This suggest the presence of the -429G/T variant, although detrimental to *HAMP* regulation, will allow for appropriate action under threat of bacterial infection. Increased hepcidin expression in the presence of invading pathogens will result in a restriction in the amount of iron available to them for survival.

When using minimal promoter constructs no significant differences were found comparing variant containing and wild-type sequence containing constructs (Figures 3.11 – 3.14). It can be hypothesised that the presence of a strong minimal promoter overshadows the effect the presence a single variant may have on gene expression. The theory of the particular region being incorporated into the vector not being able to function adequately without its larger respective adjacent 5'UTR regions to facilitate its function, must also be considered. The region incorporated may be lacking the adjacent sequences necessary to adequately regulate expression of the proceeding gene.

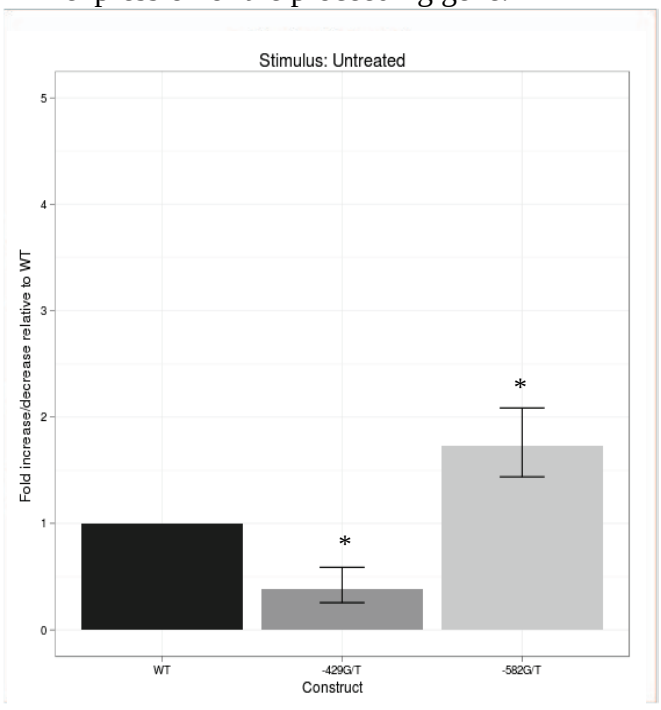

Fig 3.6 Luciferase assay results for promoterless pGL4 vectors containing WT, -429G/T and -582A/G whole promoter inserts. Data represent means of at least three independent experiments (+/- SEM) and statistically significant *P* values are represented by *. *P* values less than 0.05 are considered to be statistically significant.

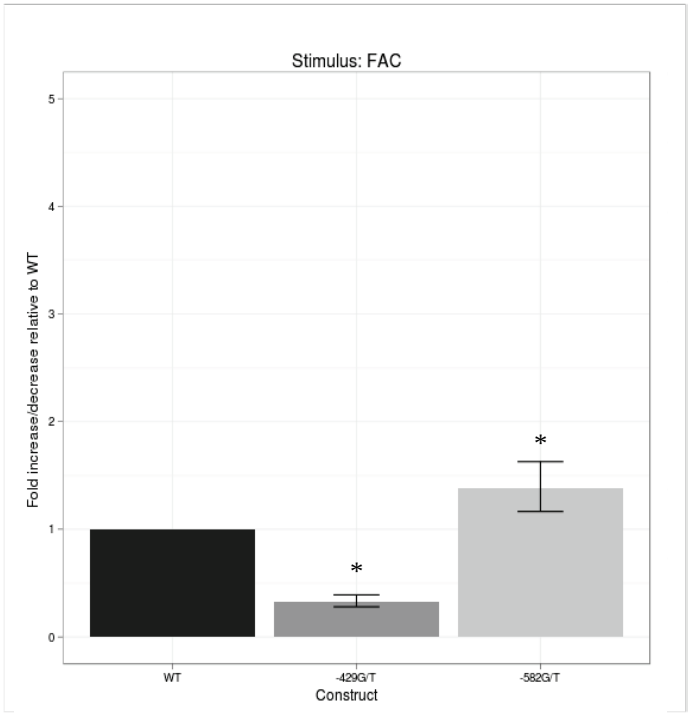

Fig 3.7 Luciferase assay results for promoterless pGL4 vectors containing WT, -429G/T and -582A/G whole promoter inserts, FAC treatment. Data represent means of at least three independent experiments (+/- SEM) and statistically significant *P* values are represented by *. *P* values less than 0.05 are considered to be statistically significant.

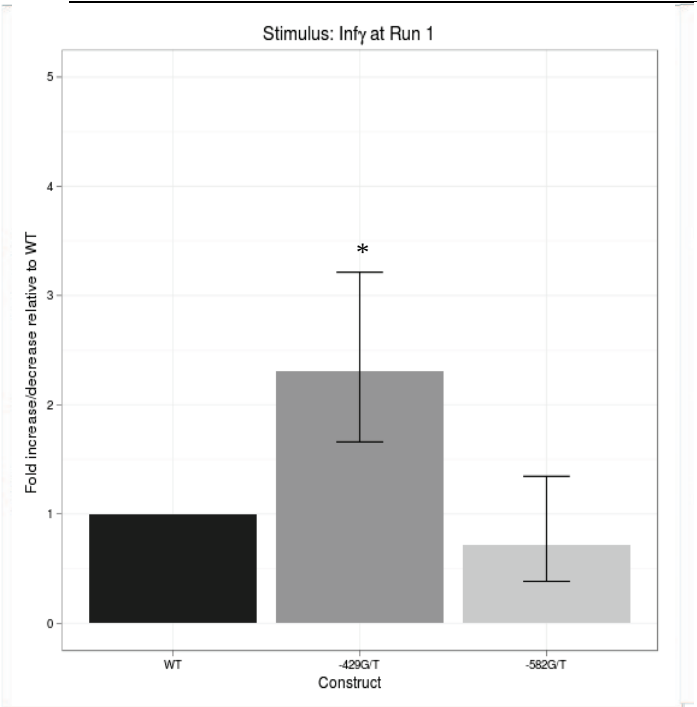

Fig 3.8 Luciferase assay results for promoterless pGL4 vectors containing WT, -429G/T and -582A/G whole promoter inserts, INF-γ treatment (Run1). Data represent means of at least three independent experiments (+/- SEM) and statistically significant *P* values are represented by *. *P* values less than 0.05 are considered to be statistically significant. (Fitted using ML instead of REML)

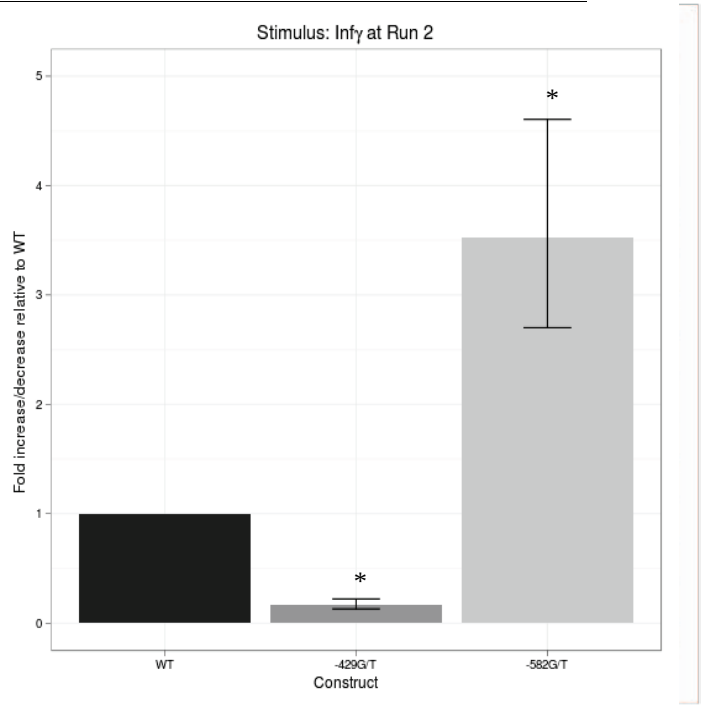

Fig 3.9 Luciferase assay results for promoterless pGL4 vectors containing WT, -429G/T and -582A/G whole promoter inserts, INF-γ treatment (Run2). Data represent means of at least three independent experiments (+/- SEM) and statistically significant *P* values are represented by *. *P* values less than 0.005 are considered to be statistically significant. (Fitted using ML instead of REML)

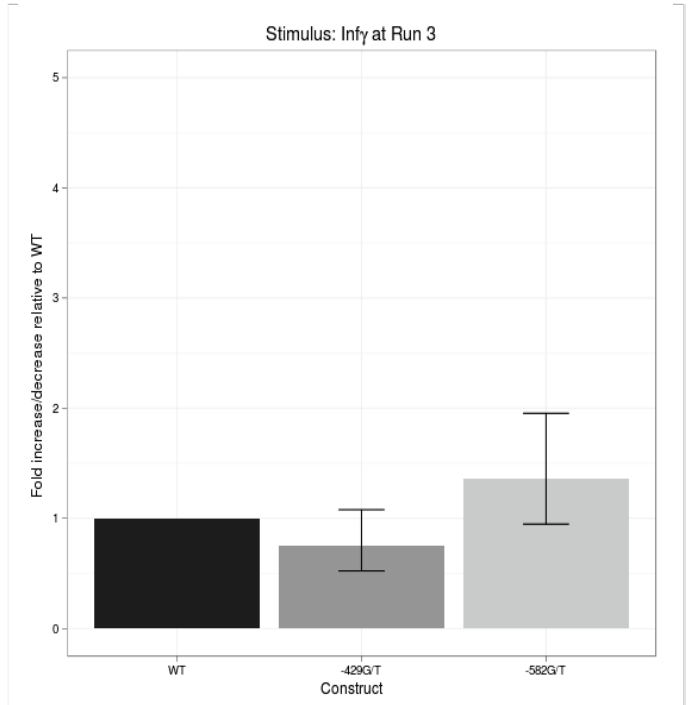

Fig 3.10 Luciferase assay results for promoterless pGL4 vectors containing WT, -429G/T and -582A/G whole promoter inserts, INF-γ treatment (Run3). Data represent means of at least three independent experiments (+/- SEM) (Fitted using ML instead of REML)

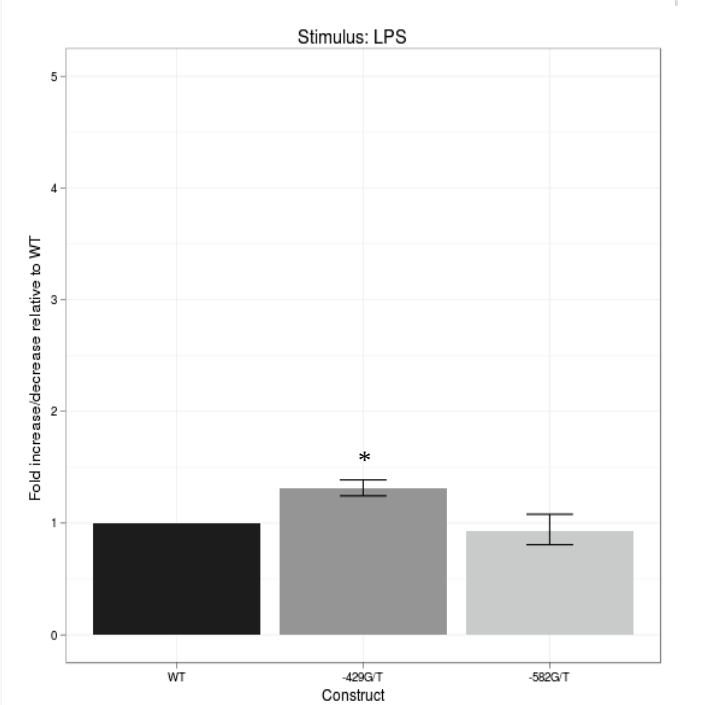

Fig 3.11 Luciferase assay results for promoterless pGL4 vectors containing WT, -429G/T and -582A/G whole promoter inserts, LPS treatment. Data represent means of at least three independent experiments (+/- SEM) and statistically significant *P* values are represented by *. *P* values less than 0.005 are considered to be statistically significant.

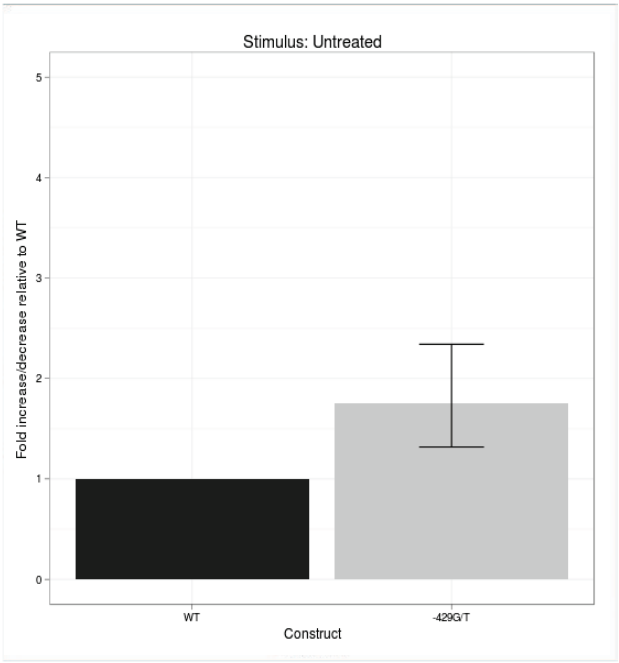

Fig 3.12 Luciferase assay results for minimal-promoter pGL4 vectors containing WT and -429G/T promoter fragments. Data represent means of at least three independent experiments (+/- SEM).

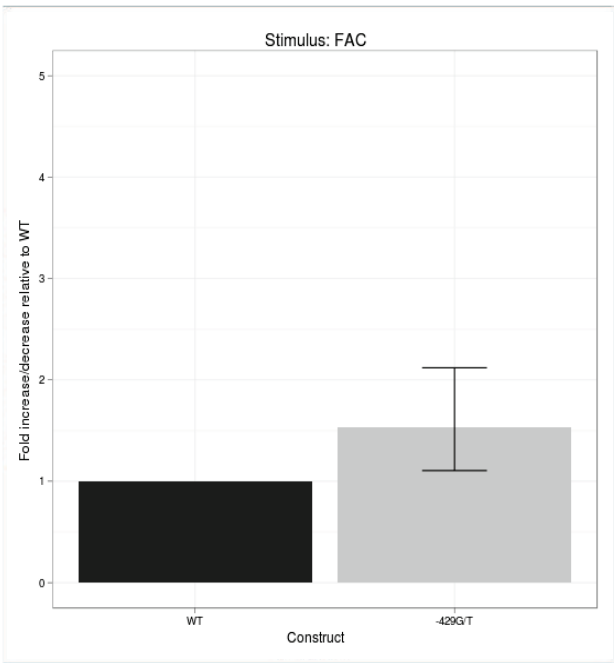

Fig 3.13 Luciferase assay results for minimal-promoter pGL4 vectors containing WT and -429G/T promoter fragments, FAC treatment. Data represent means of at least three independent experiments (+/- SEM).

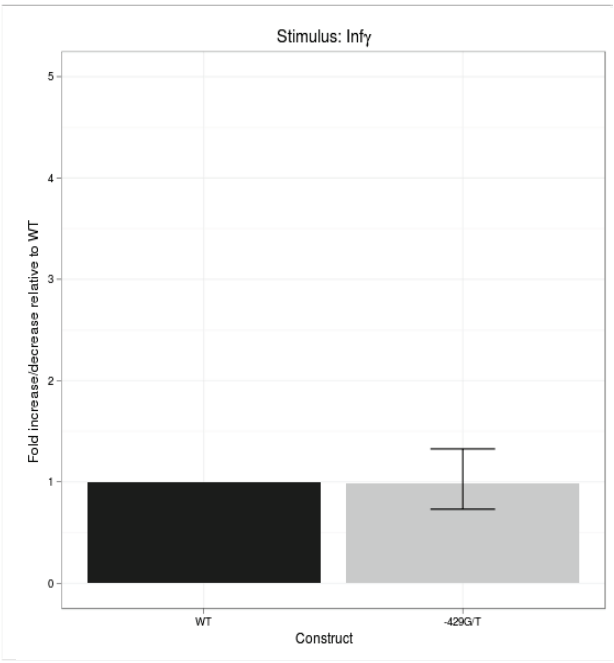

Fig 3.14 Luciferase assay results for minimal-promoter pGL4 vectors containing WT and -429G/T promoter fragments, INF-γ treatment. Data represent means of at least three independent experiments (+/- SEM).

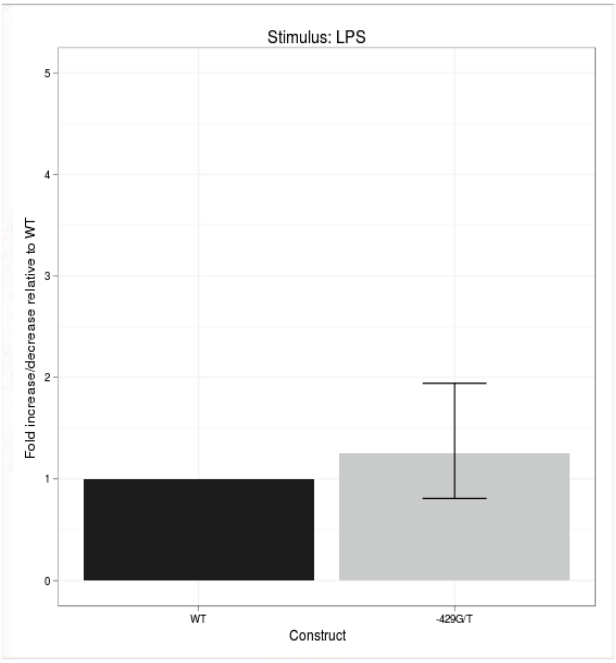

Fig 3.15 Luciferase assay results for minimal-promoter pGL4 vectors containing WT and -429G/T promoter fragments, LPS treatment. Data represent means of at least three independent experiments (+/- SEM).

The -582A/G variant

Bioinformatic analysis of the -582A/G variant predicted that the presence of this variant may disrupt a vertebrate transcriptional binding site for the upstream stimulatory factor (USF) transcription factor (Heinemeyer *et al.*, 1998). USF proteins are members of the basic-helix-loop-helix family of transcription factors directly involved in the up-regulation of transcriptional activity. They exhibit divergence in their N-terminal sequences; however, C-terminal sequences show high levels of conservation attesting to a high degree of necessity (Jiang *et al*., 2000). Luciferase activity for the -582G plasmid construct showed a statistically significant ($P = 0.0073$) increase in the amount of expression occurring in the presence of the variant relative to the WT plasmid construct (Figure 3.6). The addition of FAC resulted in decreased transcriptional activity; however, these values were still above that of the wild-type expression levels obtained (Figure 3.7).

wwwIn the case of INF-γ addition there was the same general trend of over-expression relative to the WT sequence of -582A. No statistically significant values were obtained, but this could be due to statistically significant deviation found between the three experimental runs performed for INF-γ. Considering the runs individually (Figures 3.8 – 3.10), run 1 exhibited an aberrant expression profile to what is expected, however runs 2 and 3 showed over – expression relative to the WT construct with run 2 at statistically significant levels ($P = 0.0032$). Replicate runs for INF-γ addition are required to verify these findings. LPS addition yielded no significant differences from the WT construct suggesting, as in the case -429T, the presence of the -582A/G variant will allow for an appropriate *HAMP* expression profile under threat of bacterial infection (Fig 3.11). The presence of the -582A/G variant therefore appears to result in an upregulation in the basal expression of the *HAMP* gene, to

levels equal to those observed with WT sequence, suggesting that the presence of the variant will not alter the *HAMP* expression profile in response to bacterial infection.

Minimal promoter results for the -582A/G variant relative to its wild-type sequence construct did not indicate any statistically significant deviations. This suggests, as in the case with the -429G/T minimal-promoter containing construct, that the presence of a strong minimal promoter overshadows the effect of a single variant on gene expression (Figures 3.16 – 3.19). The fragment containing the -582A/G variant may also not be able to function independently of a larger region of its adjacent sequence necessary for it to effectively regulate the expression of a proceeding gene. Further studies investigating the importance of regions of the 5'UTR of the *HAMP* gene are required to access the necessity of particular 'blocks' for transcriptional regulation of the *HAMP* gene it precedes.

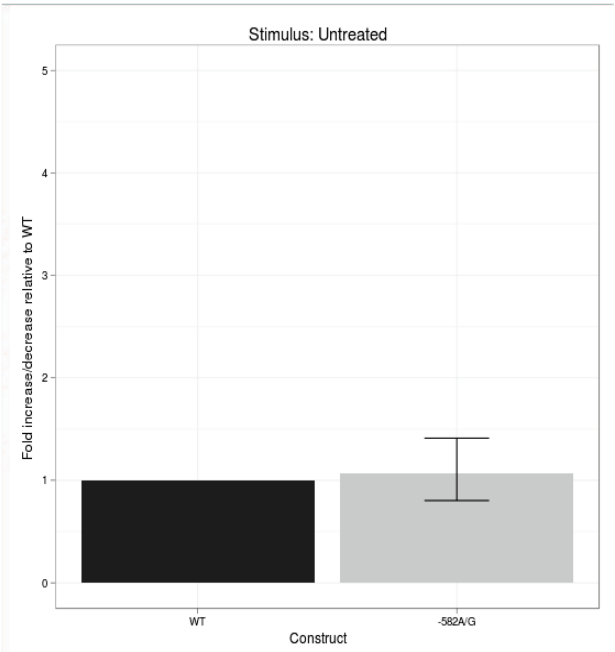

Fig 3.16 Luciferase assay results for minimal-promoter pGL4 vectors containing WT and -582A/G promoter fragments. Data represent means of at least three independent experiments (+/- SEM).

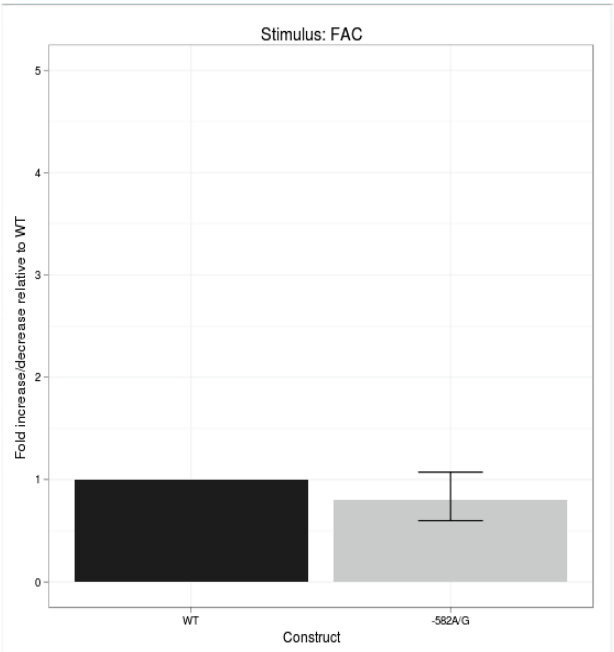

Fig 3.17 Luciferase assay results for minimal-promoter pGL4 vectors containing WT and -582A/G promoter fragments, FAC treatment. Data represent means of at least three independent experiments (+/- SEM).

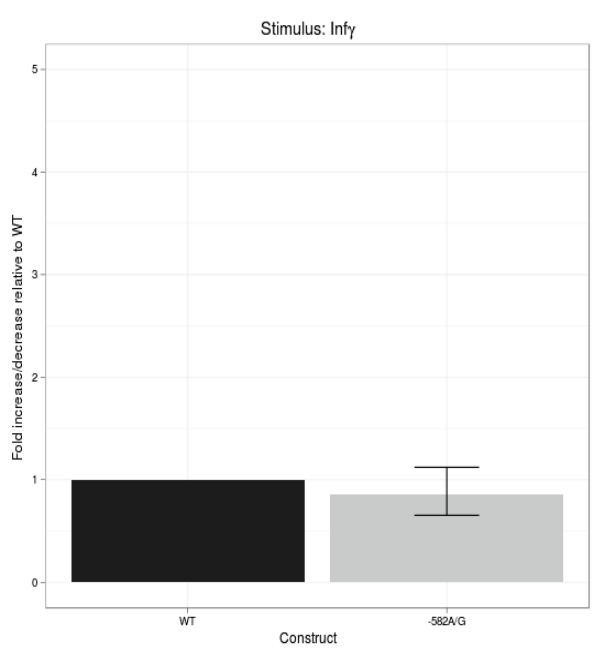

Fig 3.18 Luciferase assay results for minimal-promoter pGL4 vectors containing WT and -582A/G promoter fragments, INF-γ treatment. Data represent means of at least three independent experiments (+/- SEM).

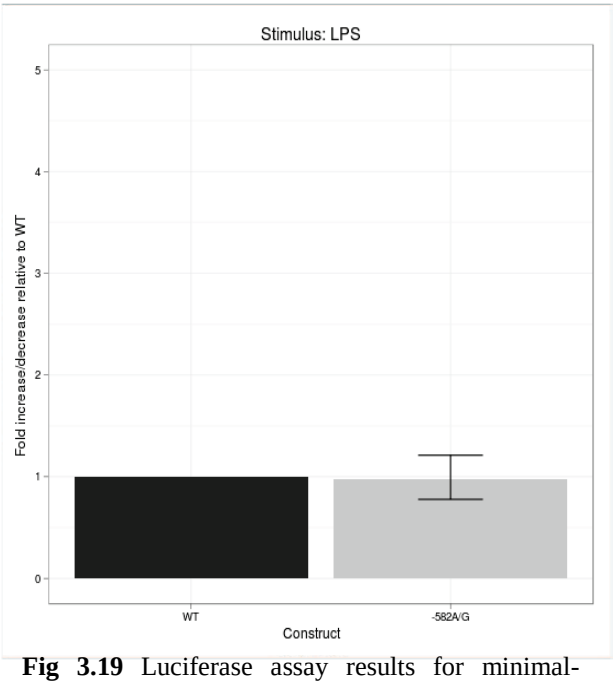

Fig 3.19 Luciferase assay results for minimal-promoter pGL4 vectors containing WT and -582A/G promoter fragments, LPS treatment. Data represent means of at least three independent experiments (+/- SEM).

Concluding remarks

Iron parameter statistics indicate an overall higher level of ferritin storage in male individuals compared to female individuals. This can be explained by females losing iron with each menstruation cycle resulting in a slower progression to the ferritin levels seen in males. This coincides with statistics putting male individuals at an increased likelihood to develop OC.

Iron parameter statistics also indicate elevated ferritin and CRP levels in individuals heterozygous for the -429G/T variant. This correlates with the decreased transcriptional activity observed with luciferase constructs possessing the variant relative to wild-type constructs. Under expression of the *HAMP* gene would ultimately lead to elevated ferritin stores, due to the increased level of iron absorption that would result. The disruption of the

p53RE site preventing upregulation of the gene and the involvement of this variant in cancer phenotype progression is noteworthy.

The -582A/G and -429G/T variants were identified exclusively in the heterozygous state in the Black South African OC population screened. Luciferase analyses of these variants indicate that the variants have opposing expression profiles. Statistical analyses of these two variants, in relation to the WT construct, yielded statistically significant results ($P = 0.001$) (Figure 3.6) and therefore the investigation of the simultaneous presence of these variants within a single patient is warranted.

Overall results suggest that mutations in the *HAMP* promoter may alter iron homeostasis, and in the case of the -188C/T and -429G/T variants could result in an iron burden which may prove to be advantageous to cancer phenotype presentation and/or progression. The presence of the -429G/T mutation within a p53RE could also represent an alternative pathway whereby the p53 tumour suppressor protein is able to control/prevent tumour development.

DISCLOSURES

Recognition is to be given to the National Research Foundation (NRF) for providing funding for project completion. INNOVUS completed the processing of a provisional patent (ZA2008/09634).

Chapter 4

Conclusion

4. Conclusion

The study objectives consisted of the identification of novel and/or previously described variants in an OC patient cohort and subsequent functional analyses of these variants to identify the effect variants may have on gene expression. It also involved the assessment of iron parameter statistics regarding patient and control cohorts in order to better assess the genotype-phenotype correlations observed between healthy and OC afflicted individuals, taking into account the presence and absence of the identified variants.

In this study, in accordance with the first objective, three variants were identified in the promoter region of the *HAMP* gene, of which one was known (-582A/G) and two which were novel (-188C/T and -429G/T). This was accomplished using PCR amplification of 1202bp of the 5' UTR of the *HAMP* gene and subsequent HEX-SSCP analysis and semi-automated bi-directional sequencing analyses. The HEX-SSCP technique has a 70% sensitivity rating (Kotze *et al.*, 1995) and it is therefore possible that other variants within the promoter region may not have been detected using this type of screening method.

The three variants identified within the *HAMP* promoter region were functionally investigated by transfection into a HepG2 cell line of variant containing pGL4 minimal and promoterless vector constructs. This was performed in both the absence and presence of exogenous stimuli, namely FAC, INF-γ and LPS. Statistically significant effects were found with all three variants investigated, relative to their WT sequencing constructs included in functional investigations. Considering the basal levels of expression observed in untreated expression runs, statistically significant effects in the presence of a variant, relative to the respective WT construct used. A variant within the promoter region of the *HAMP* gene is therefore able to alter its expression, depending on its location. Hepcidin plays an absolutely critical role in iron regulation, and therefore the disruption of the wild-type gene expression profile within tissues could result in an iron burden or iron starvation of cells. Each of these could have severe consequences. The presence of the -582 G/T variant, based on the luciferase results obtained, appears to result in an increase in the basal (i.e. un-induced) amount of hepcidin produced. This would subsequently result in reduced iron influx,

contributing to an anemia phenotype, and iron sequestration by macrophages. The presence of the -429 G/T and -188 C/T variants, based on the luciferase results, appear to result in reduced basal levels of *HAMP* expression. The resulting reduced levels of hepcidin could result in a higher influx of iron in the intestinal lumen leading to an iron overload phenotype. Iron overload is associated with a variety of disorders including haemochromatosis. Mutation of the *HAMP* gene has previously been linked to a severe form of haemochromatosis, namely juvenile haemochromatosis. The presence of high iron also facilitates the production of free radicals, increasing susceptibility to cancer. It is also important to note that cancerous cell lines require large amounts of iron for propagation. The hypothesis that a higher iron status may promote the creation of cancerous cells or facilitate increased propagation of already existing cancerous cells merits further investigation.

The -429G/T variant was identified exclusively in the heterozygous state in the Black Xhosa-speaking South African OC population screened, and the -582A/G variant as exclusively heterozygous with the exception of a single patient that was homozygous. Luciferase analyses of promoter reporter constructs containing these variants indicate that the variants have opposing expression profiles. Statistical analyses of these two variants yielded statistically significant results (Untreated: $P = 0.001$; FAC: $P < 0.0001$; INF-γ; run2, $P < 0.0001$; LPS: $P = 0.0002$) considering the simultaneous effect of both variants in relation to WT constructs (Figures 3.6 – 3.11) and therefore the investigation of the simultaneous presence of these variants within a single patient is warranted. The opposing expression profiles observed indicates the possibility of compensation occurring, considering the simultaneous presence of the -429G/T and -582A/G variant in a single patient. The same theory can be assigned to the simultaneous presence of the -188C/T and -582A/G variants based on the expression profiles obtained. Considering the simultaneous presence of the -188C/T and -429G/T variants, an additive effect may be observed resulting in sever iron overload in patients possessing these two variants. The simultaneous presence of any of the three variants identified in this study occurring within a single individual was not identified within this study. The merits of the additive or compensatory effect combinations of these variants may have on expression could prove useful to the further understanding of the transcriptional regulation of the *HAMP* gene and possibly be beneficial for future genetic application.

This study investigated the role of promoter dysregulation of the *HAMP* gene in OC development and/or progression. Iron parameters were statistically correlated to provide further insight into OC presentation between patients and controls, males and females, and individuals presenting with and without variation in their *HAMP* promoter region. The significant association identified with the -429G/T variant suggests its possible association with OC susceptibility. This is further supported by its statistically significant frequency in the patient cohort, relative to the controls as well as statistically significant under-expression relative to WT sequence in culture. Genotype-phenotype correlations were therefore investigated and significant correlations were found between certain variants and iron parameter values observed.

Limitations of this study and future prospects

The study cohorts used were representative of a pilot study. Expansion of the patient and control groups to ascertain whether the obtained frequencies remain constant in the Black Xhosa-speaking South African population may contribute to strengthening of the results obtained in this study. Identification of raised frequencies upon cohort expansion would further strengthen the correlation of the identified variants to OC susceptibility. Expanding the study population to include South African Caucasian and Mixed Ancestry individuals could provide valuable statistical information on the identified variants and contribute to the improvement of diagnostic and counseling services for the unique populations of this country.

Considering the presence or absence of significant variance between runs, different statistical models were used to assess the effect that a particular variant has in relation to the respective WT. This hampered our ability to combine data sets to ascertain the fold-expression increases or decreases of variants relative to each other, and whether the same level of increase or decrease in expression was occurring upon treatment addition as was visualized in untreated cases for each variant considered. Replicate experiments aimed at reducing the level of variance observed are required. These will allow for the investigation of the aforementioned comparisons, and will provide valuable insight into the degree to which transcriptional up-regulation or down-regulation occurs in the presence a variant.

Further research including screening for novel and previously described variants in other genes involved in iron metabolism, including *TF*, *TRF1* and *TFR2* need be carried out to investigate the potential role these genes may have in OC development. Genes which have already been subjected to mutation screening within our research group (*HFE*, *CP* and *SCL40A1*) may be correlated to assess the interaction of gene mutation in contributing to the cancer phenotype and progression, and iron status. Investigation of the regulation of the *HAMP* gene will contribute to this investigation. Iron metabolism is an intricate combination of pathways involving numerous genes and protein interactions. The correlation of multiple genes involved in iron metabolism could provide necessary insight into the understanding of OC susceptibility and iron.

In this study there were noteworthy differences between the mean ages of patient and control groups, with control individuals having mean age higher than that of the patient cohort. This is not optimal considering iron loading in patients is age dependent in most cases. Investigation of the ages of individuals in the patient and control cohorts, however, indicated individuals of both groups, to be over the age of 50 years. Mutations within the *HAMP* gene are associated with early onset iron disorders, lessening complications caused by the discrepancy between ages. Individuals above the age of 50 should be showing iron loading symptoms to a certain degree. This discrepancy, however, remains something to be considered in future studies.

This study successfully identified novel and previously described variants in the promoter region of the *HAMP* gene in the Black Xhosa-speaking South African population. Significant associations were observed considering variant presence in relation to WT sequence, implicating the variants identified in OC susceptibly. In conjunction with further research this study may prove an important contribution to understanding the current association of iron dysregulation in the development and/or progression of OC.

Chapter 5

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5. References

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Chapter 6

Addenda

Addendum A

6.1. Mutation analysis, construct preparation and iron parameter statistics

i. *HAMP* promoter

Sequence chromatograms and 12% PAA HEX-SSCP gel depictions of the variants identified.

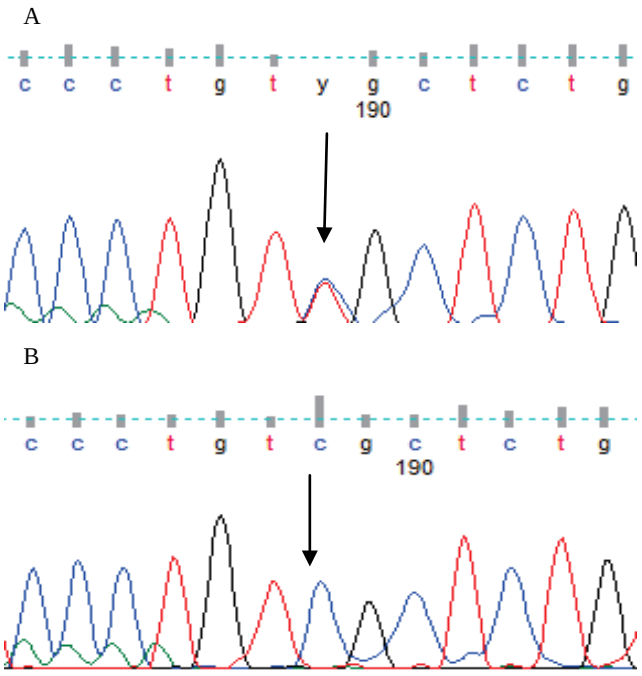


Fig 6.1 Electropherograms indicate (A) the -188 C/T variant in heterozygous state and (B) the wild-type sequencing pattern. Arrows indicate the point of change.

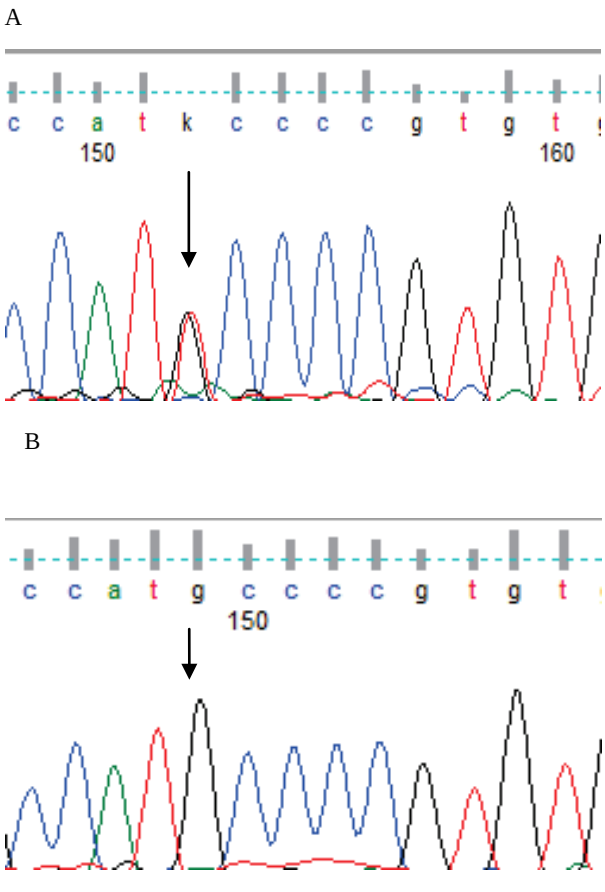


Fig 6.2 Electropherograms indicate (A) the -429 G/T variant in heterozygous state and (B) the wild-type sequencing pattern. Arrows indicate the point of change.

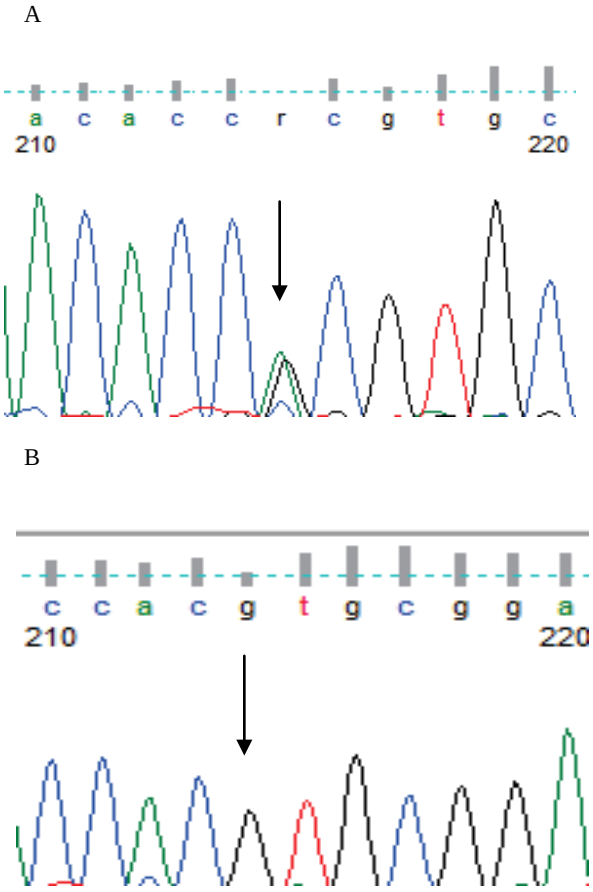


Fig 6.3 Electropherograms indicate (A) the -582 A/G variant in heterozygous state and (B) the wild-type sequencing pattern. Arrows indicate the point of change.

ii. TP53

Screening of the *TP53* gene revealed no detectable variation in the screened regions (Table 2.2). Semi-automated bi-directional sequencing performed on select samples depicting HEX-SSCP variation confirmed individuals to be wild-type for the particular sequences under investigation.

iii. Construct Preparation

PCR amplification of the *HAMP* promoter region in question was carried out using the protocol outlined in section 2.6. Successfully amplified PCR products were purified and subjected to digestion according to section 2.6.2. Post digestion products were once again purified and subsequently subjected to ligation according to section 2.6.3. Ligation success was tested enzymatically by digesting with a single enzyme, namely *NheI.* The results of these digestions are shown in Figs 6.4 and 6.5. Standard plasmid (without insert) is shown as the smaller band with successful digestions yielding bands higher up in the gel. The increase in band size correlates directly with the size of the incorporated insert.

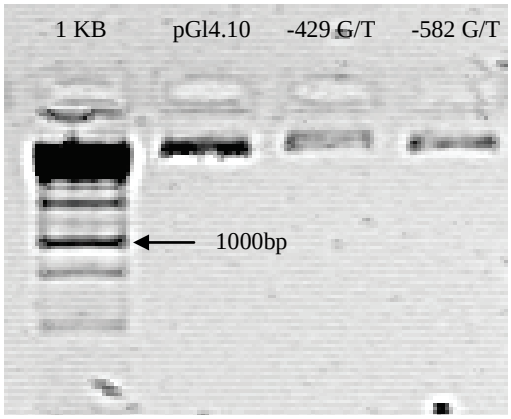


Fig 6.4 Agarose gel depiction (1.5% w/v) of the test ligation digest using the *NheI* enzyme for promoterless containing constructs. Annotations -429G/T and -582A/G refer to constructs containing the relevant variant containing inserts. The pGL4.10 depiction represents digested plasmid with the absence of insert. The pGL4.10 lane represents a fragment of 4242bp with the other lanes indicating band sizes approximately the relevant insert size larger. (-429G/T: 4554bp and -582A/G: 4568bp)

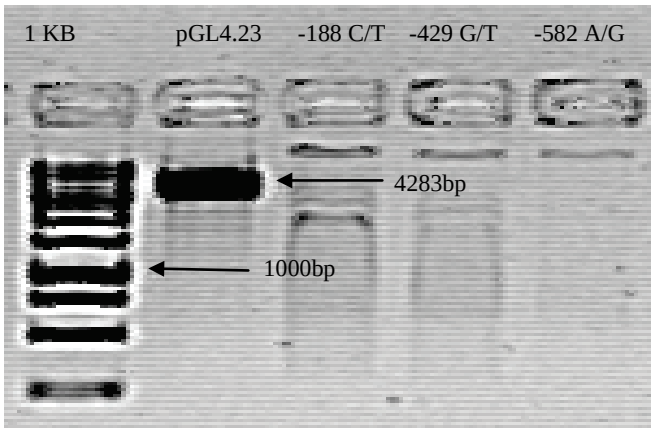
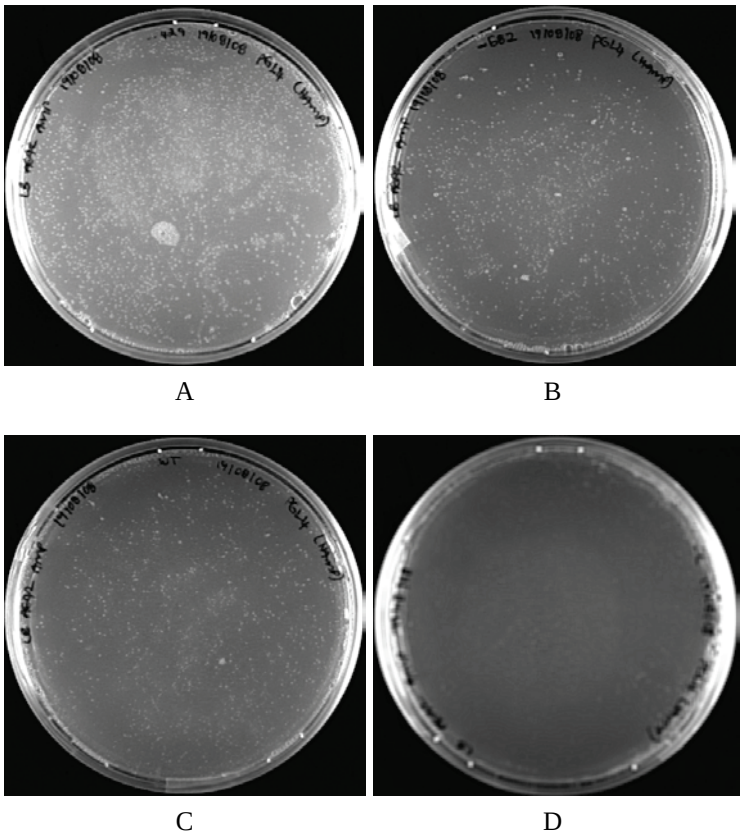


Fig 6.5 Agarose gel depiction (1.5% w/v) of the test ligation digest using the *NheI* enzyme for minimal promoter containing constructs. Annotations -188C/T, -429G/T and -582A/G refer to constructs containing the relevant variant containing inserts. The pGL4.23 depiction represents digested plasmid with the absence of insert. The pGL4.23 lane represents a fragment of 4283bp with the other lanes indicating band sizes the relevant insert size larger. (-188C/T: 4568bp, -429G/T: 4595bp and -582A/G: 4609bp)

iv. Transformation and Insert Confirmation

With ligation confirmed, transformation was performed according to the methodology outlined in section 2.9. Colonies were visualized on all plates with no colonies observed on the negative control plates prepared for each transformation procedure carried out (Fig 6.6). The colonies visualized must, at the very least, contain the pGL4 constructs as they contain the gene conferring ampicillin resistance allowing only ampicillin resistant bacterial strains to grow. This was confirmed by the absence of any colonies on the negative control plates and multitudes of colonies observed on the positive plates prepared.



A B

C D

Fig 6.6 Successful transformation plating grown overnight at 37ºC. The (A) -429 G/T, (B) -582 A/G, (C) WT plating indicates growth on LB-agar plates containing ampicillin as selective marker. (D), the negative control shows no growth indicative on the absence of pGL4.10 vector bestowing antibiotic resistance. Similar results were obtained for minimal promoter transformations using pGL4.23 (not shown).

Colony PCR was performed on randomly selected colonies on individual plates and the products subjected to semi-automated DNA sequencing analysis to confirm the presence and orientation of the desired insert (Fig 6.7 and 6.8). Colonies exhibiting positive results for the desired variants were then cultured and subjected to plasmid extraction according to section 2.10.

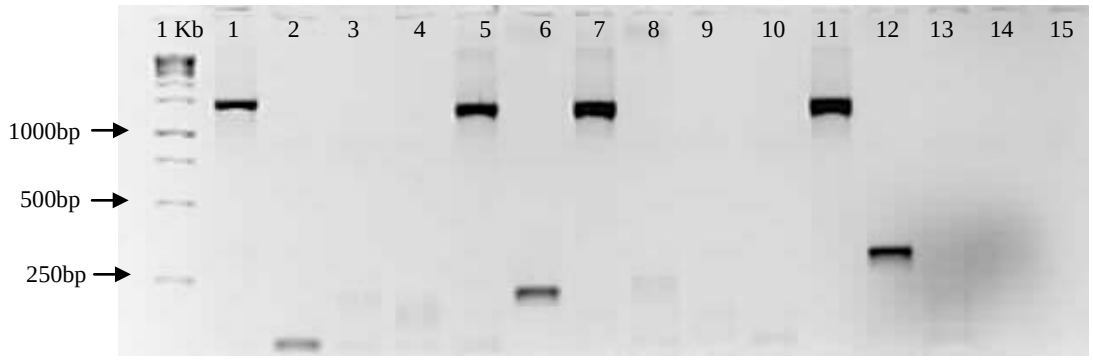


Fig 6.7 Agarose gel depiction (1.5% w/v) of colony PCR amplification for minimal promoter constructs containing -188 C/T. A WT for the PP5 fragment was also prepared and subjected to colony PCR although not depicted here. Amplifications were performed using RV3 and PP5R primers.

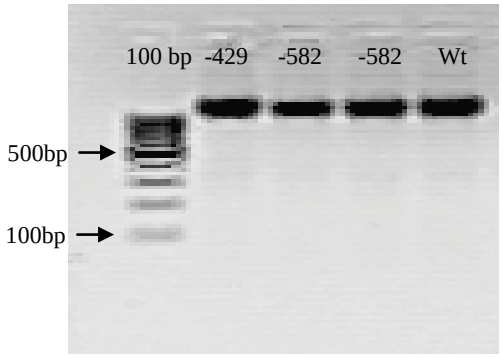


Fig 6.8 Agarose gel depiction of colony PCR amplifications for promoterless constructs containing -429 G/T, -582 A/G and WT. Amplifications were performed using RV3 and PP5R primers. Successful results were achieved for all colonies picked.

v. Iron parameter descriptive statistics for male and female patients

Table 6.1 Two Sample *t*-Test for iron parameters considering male and female groups individually.

t-Test: Two-Sample Assuming Unequal Variances

Serum-Iron

	Female Patients	*Female Controls*
Mean	6.581818182	11.355
Variance	7.798701299	15.50934177
Observations	22	80
Hypothesized Mean Difference	0	
df	47	
t Stat	-6.445814285	
P(T<=t) one-tail	2.83712E-08	
t Critical one-tail	1.677926722	
P(T<=t) two-tail	5.67424E-08	
t Critical two-tail	2.01174048	

t-Test: Two-Sample Assuming Equal Variances

Serum-Iron

	Male Patients	*Male Controls*
Mean	7.376	12.9625
Variance	6.6144	10.9545
Observations	25	16
Pooled Variance	8.283669231	
Hypothesized Mean Difference	0	
df	39	
t Stat	-6.062705825	
P(T<=t) one-tail	2.83712E-08	
t Critical one-tail	1.684875122	
P(T<=t) two-tail	4.23769E-07	
t Critical two-tail	2.022690901	

t-Test: Two-Sample Assuming Unequal Variances

Transferrin

	Female Patients	*Female Controls*
Mean	1.017142857	2.001168831
Variance	0.028740629	0.186921984
Observations	21	77
Hypothesized Mean Difference	0	
df	84	
t Stat	-15.97108168	
P(T<=t) one-tail	1.75763E-27	
t Critical one-tail	1.66319668	
P(T<=t) two-tail	3.51526E-27	
t Critical two-tail	1.988609629	

t-Test: Two-Sample Assuming Unequal Variances

Transferrin

	Male Patients	*Male Controls*
Mean	1.227041667	2.2195625
Variance	0.071725259	0.281061196
Observations	24	16
Hypothesized Mean Difference	0	
df	20	
t Stat	-6.922806428	
P(T<=t) one-tail	5.04404E-07	
t Critical one-tail	1.724718218	
P(T<=t) two-tail	1.00881E-06	
t Critical two-tail	2.085963441	

t-Test: Two-Sample Assuming Unequal Variances

Ferritin

	Female Patients	*Female Controls*
Mean	243.3605	76.655625
Variance	32212.08475	1302.89985
Observations	20	80
Hypothesized Mean Difference	0	
df	19	
t Stat	4.133035547	
P(T<=t) one-tail	0.000282725	
t Critical one-tail	1.729132792	
P(T<=t) two-tail	0.000565449	
t Critical two-tail	2.09302405	

t-Test: Two-Sample Assuming Unequal Variances

Ferritin

	Male Patients	*Male Controls*
Mean	283.8484	95.357
Variance	33983.76081	1690.036712
Observations	25	10
Hypothesized Mean Difference	0	
df	29	
t Stat	4.821470861	
P(T<=t) one-tail	2.08292E-05	
t Critical one-tail	1.699126996	
P(T<=t) two-tail	4.16584E-05	
t Critical two-tail	2.045229611	

t-Test: Two-Sample Assuming Unequal Variances

CRP

	Female Patients	*Female Controls*
Mean	24.82105263	4.032133333
Variance	490.0561988	16.60087377
Observations	19	75
Hypothesized Mean Differ	0	
df	18	
t Stat	4.075962794	
P(T<=t) one-tail	0.000354578	
t Critical one-tail	1.734063592	
P(T<=t) two-tail	0.000709155	
t Critical two-tail	2.100922037	

t-Test: Two-Sample Assuming Unequal Variances

CRP

	Male Patients	*Male Controls*
Mean	18.06666667	1.941666667
Variance	133.6123333	4.873560606
Observations	21	12
Hypothesized Mean Difference	0	
df	22	
t Stat	6.197971702	
P(T<=t) one-tail	1.53923E-06	
t Critical one-tail	1.717144335	
P(T<=t) two-tail	3.07847E-06	
t Critical two-tail	2.073873058	

Table 6.2: Descriptive statistics considering the presence and absence of the -429G/T variant among the combined patient and control groups

	t-Test: Two-Sample Assuming Equal Variances			
	Serum-Iron		CRP	
	429G/T	*WT*	*429G/T*	*WT*
Mean	5.89	10.04868421	21.0875	9.96880597
Variance	9.601	15.67693158	319.1955357	195.294044
Observations	10	76	8	67
Pooled Variance	15.02593891		207.175009	
Hypothesized Mean Difference	0		0	
df	84		73	
t Stat	-3.189280405		2.065079898	
P(T<=t) one-tail	0.001002562		0.02123364	
t Critical one-tail	1.66319668		1.665996224	
P(T<=t) two-tail	0.002005123		0.04246728	
t Critical two-tail	1.988609629		1.992997097	
	t-Test: Two-Sample Assuming Unequal Variances			
	Ferritin		Transferrin	
	429G/T	*WT*	*429G/T*	*WT*
Mean	309.5066667	139.1478873	0.988777778	1.777554054
Variance	66604.65378	13198.69463	0.044436694	0.377040881
Observations	9	71	9	74
Hypothesized Mean Difference	0		0	
df	8		30	
t Stat	1.955898189		-7.874953728	
P(T<=t) one-tail	0.043101444		4.33521E-09	
t Critical one-tail	1.859548033		1.697260851	
P(T<=t) two-tail	0.086202887		8.67042E-09	
t Critical two-tail	2.306004133		2.042272449	

Addendum B

6.2. Conference outputs

Paper presented as an oral presentation:

SASHG Conference, Spier Stellenbosch (5 – 8 April 2009) Abstract Submission

Characterization of the promoter region of the *HAMP* gene implicated in iron metabolism and its possible association with Oesophageal cancer in the Black South African population

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*Department of Genetics, University of Stellenbosch, JC Smuts Building, De Beer Street, Stellenbosch, 7600

ABSTRACT

OBJECTIVES: The *HAMP* gene codes for the 25 amino acid, hepcidin protein, crucial to the regulation of the bodily iron status. Defects occurring within the promoter region of this gene may contribute to its dysregulation, subsequently resulting in an iron overload status. Iron overload status is a previously described risk factor in the development of numerous cancers, oesophageal cancer (OC) included, and therefore the dysregulation of the *HAMP* gene may be involved in cancer phenotype exhibition.

METHODS: Approximately 1500 bp of the 5'UTR of the *HAMP* gene was subjected to mutational analysis in order to determine to effect of promoter variants on gene expression. Fifty Black, Xhosa-speaking South African individuals were screened against a cohort of 50 population matched controls.

RESULTS: Four variants were identified within the screened promoter region: -153 C/T, -188 C/T, -429 G/T and -582 A/G; with the -429 G/T variant being of particular interest. The -429 G/T variant is found to disrupt a previously identified p53 response element required for the up-regulation of *HAMP* gene expression.

CONCLUSIONS: Variants occurring within the promoter region of the *HAMP* gene are indeed able to alter gene expression, provided they occur at points crucial to the transcriptional regulation of the gene. Dysregulation of the *HAMP* gene results in an increased uptake of dietary iron, which in turn could feed the proliferation of carcinogenic cells lines.

Paper presented as an oral presentation:

Bio-Iron: Porto, Portugal (7 – 11th June, 2009) Abstract Submission

CHARACTERIZATION OF THE PROMOTER REGION OF THE *HAMP* GENE IN THE BLACK SOUTH AFRICAN POPULATION AND ITS POSSIBLE ASSOCIATION WITH OESOPHAGEAL CANCER

[1]N.W McGregor, [2]A Louw, [1]L Warnich, [1]M. G Zaahl

Departments of [1]Genetics and [2]Biochemistry, University of Stellenbosch, JC Smuts Building, De Beer Street, Stellenbosch, 7600

ABSTRACT

OBJECTIVES: The *HAMP* gene; encoding for the 25 amino acid hepcidin protein, is paramount in the regulation of the bodily iron status. Mutations disrupting the regular expression of this gene may therefore result in an irregular bodily iron standing, i.e. iron overload. An iron overload status is a previously described risk factor in the development of numerous cancers, oesophageal cancer (OC) included, and therefore the dysregulation of the *HAMP* gene may be involved in cancer phenotype exhibition.

METHODS: Mutational analysis of approximately 1500 bp of the 5'UTR of the *HAMP* gene was performed to determine the effect of promoter variants identified on gene expression. Initially, fifty Black, Xhosa-speaking South African individuals were screened against a cohort of 50 population matched controls. Immunohistochemical staining was performed on a subset of 20 patients to assess the presence of the p53 protein within cells. These results were correlated to the mutation analysis of the *TP53* gene for the above mentioned subset patients.

RESULTS: Variants identified within the promoter region of the *HAMP* gene comprise: -153 C/T, -188 C/T, -429 G/T and -582 A/G; with the -429 G/T variant being of particular interest. The -429 G/T variant is found to disrupt a previously identified p53 response element required for the up-regulation of *HAMP* gene expression. The -429 G/T variant was found to occur at a low frequency within the general Mixed Ancestry, Caucasian and Black South African populations contradictory to the high frequency identified in the Black South African patient cohort. Immunohistochemical results did not correlate high levels of p53 protein to the occurrence of the -429 G/T variant.

CONCLUSIONS: Variants found within regions crucial to the transcriptional regulation of the gene are able to alter gene expression, possibly to a level of disease phenotype exhibition. Dysregulation of the *HAMP* gene results in an increased uptake of dietary iron, which in turn could feed the proliferation of carcinogenic cells lines. The presence of the -429 G/T and -582 A/G variants indicate a trend in the alteration of *HAMP* gene expression. Whether this alteration is causative or merely accessory to the cancer phenotype exhibition is a hypothesis that warrants further investigation. Failure of the Immunohistochemical staining procedures to correlate the -429 G/T variant presence to altered p53 protein levels does not completely exclude the existence of a link concerning the two. A longer time period may be required for p53 protein build-up to occur.

Addendum C

6.2. Patents

Title:	***Biomarker for Diagnosing Cancer***
Arthors:	N. W. McGregor, M. G. Zaahl, T. Matsha
Field of Invention:	This invention relates to the fields of cancer biology and nucleic acid biochemistry. Specifically, this invention provides a diagnostic kit for use in detecting a susceptibility to Oesophageal Cancer in a subject, use of a biomarker for Oesophageal Cancer, a method of detecting a susceptibility to Oesophageal Cancer in a subject, and nucleic acid segments and compositions for use therefor.
Patent Number:	ZA2008/09634
Legal:	VON SEIDELS, Intellectual Property Attorneys