REVIEW

Recent advances in understanding haemochromatosis: a transition state

K J H Robson, A T Merryweather-Clarke, E Cadet, V Viprakasit, M G Zaahl, J J Pointon, D J Weatherall, J Rochette

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Mutations in the hepcidin gene *HAMP* and the hemojuvelin gene *HJV* have recently been shown to result in juvenile haemochromatosis (JH). Hepcidin is an antimicrobial peptide that plays a key role in regulating intestinal iron absorption. Hepcidin levels are reduced in patients with haemochromatosis due to mutations in the *HFE* and *HJV* genes. Digenic inheritance of mutations in *HFE* and *HAMP* can result in either JH or hereditary haemochromatosis (HH) depending upon the severity of the mutation in *HAMP*. Here we review these findings and discuss how understanding the different types of haemochromatosis and our increasing knowledge of iron metabolism may help to elucidate the host's response to infection.

Mutations in the hepcidin gene *HAMP* and the hemojuvelin gene *HJV* have recently been shown to result in juvenile haemochromatosis (JH), whereas digenic inheritance of mutations in *HFE* and *HAMP* can result in either JH or hereditary haemochromatosis (HH). These findings are discussed below.

HLA LINKED HAEMOCHROMATOSIS AND HFE

In his monograph in 1935 Sheldon suggested that haemochromatosis might have a genetic basis.¹ It was later found to be an autosomal recessive disease.² After Marcel Simon demonstrated linkage of the haemochromatosis locus to the MHC class I molecule HLA-A3,³ it was presumed that hereditary haemochromatosis (HH) was a simple, autosomal recessive disorder restricted to people of north west European origin. In 1996, Feder and colleagues identified the *HFE* gene and showed that over 80% of patients were homozygous for the C282Y mutation in this gene.⁴ Other groups have subsequently confirmed that the majority of haemochromatosis patients are homozygous for the C282Y mutation in *HFE*.^{5–7} Among the remainder, over 75% of those who carry a single copy of the C282Y mutation are also compound heterozygotes for the more prevalent but mild H63D mutation in *HFE* (reviewed by Merryweather-Clarke *et al*⁸). Population studies have demonstrated that the C282Y mutation is restricted to people of north west European origin.^{8–9} Other, rarer *HFE* mutations and variants have been reported (fig 1) (reviewed by Pointon *et al*¹⁰), the majority of which have been

found in conjunction with the C282Y mutation. However, a Vietnamese patient has been described who is homozygous for a novel splice site mutation.¹¹ Another mutation appears to result in an autosomal dominant form of haemochromatosis (478delC) (Pointon *et al*, manuscript in preparation). It should be noted that *Hfe*^{-/-} mice have a much more severe phenotype than *Hfe*^{Cys/Cys} mice¹² and this may help explain the severity of iron loading in these patients. Haemochromatosis due to mutations in *HFE* is known as type 1 haemochromatosis (OMIM 235200). More males than females are affected. Onset is usually in the fourth decade for men and in the fifth for women.

The original paper describing the cloning of the *HFE* gene reported that the mRNA encoding HFE was most abundant in the liver, and that low levels of HFE mRNA were found in many other tissues.⁴ Subsequently, there has been a series of studies describing the use of antibodies raised against HFE peptides or bacterially expressed HFE for the localization of HFE in different tissues. Histochemical techniques have localized HFE to a number of different cell types, including Kupffer cells,¹³ the gastrointestinal tract,¹⁴ placenta,¹⁵ epithelial cells and macrophages and monocytes,¹⁶ and crypt cells of the small intestine¹⁷ although some reports conflict. A variety of monoclonal and polyclonal anti-peptide antibodies have been used in these studies. One problem with the work on human material has been the lack of quantitation of HFE in the tissues examined. This has been overcome in a very recent study that examined the expression of iron metabolism related mRNAs in the rat using quantitative PCR, in situ hybridization, and western blot analysis.¹⁸ The study showed that HFE is expressed primarily in hepatocytes, and at 10-fold lower levels in Kupffer cells.¹⁸

The cloning of the *HFE* gene paved the way for the identification of non-*HFE* related forms of

Abbreviations: ACD, anaemia of chronic disease; AD, Alzheimer's disease; β_2 M, β_2 -microglobulin; Cp^{-/-} mice, ceruloplasmin null mice; JH, juvenile haemochromatosis; HH, hereditary haemochromatosis; IEL, intra-epithelial lymphocytes; IL-6, interleukin 6; IRE, iron responsive element; MHC, major histocompatibility complex; NF- κ B, nuclear factor κ B; NRAMPI, natural resistance associated macrophage protein 1; Pcm, polycythaemic mice; PKAN, pantothenate kinase associated neurodegeneration; SLC11A1, solute carrier family 1 member 1; SLC11A2, solute carrier family 1 member 2; TfR2, transferrin receptor 2; TFR2, transferrin receptor 2 gene

See end of article for authors' affiliations

Correspondence to: Kathryn J H Robson, MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, Headley Way, Oxford, OX3 9DS, UK; kathryn.robson@imm.ox.ac.uk

Correspondence to: Alison T Merryweather-Clarke, MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, Headley Way, Oxford, OX3 9DS, UK; alison.hammer@imm.ox.ac.uk

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Figure 1 Representation of the HFE/β₂-microglobulin complex adapted from Feder *et al*⁴ illustrating the relative positions of the different amino acid mutations and sequence variants in HFE.^{4 10 42 156–170} The approximate positions of the two splice site mutations relative to the appropriate exons have also been included.^{11 171} The R6S mutation is in the signal sequence.¹⁷² The HFE protein is 343 amino acids long. The mature protein is 321 amino acids comprising three extracellular domains, α₁, α₂, and α₃, with a single membrane spanning domain and a short cytoplasmic tail. It interacts with β₂-microglobulin (β₂M) that is essential for the correct assembly of the HFE/β₂M complex. Unlike classical class I molecules there is no peptide binding groove between the α₁ and α₂ domains. The α₃ domain is key to the interaction with β₂M. The four invariant cysteine residues form two disulphide bridges, one in the α₂ domain and one in the α₃. It is the C282Y mutation found in the majority of HH patients that disrupts this second disulphide bridge. The correct assembly of HFE/β₂M results in cell surface presentation. The C282Y form of HFE becomes stuck in the endoplasmic reticulum and middle Golgi.^{173 174}

haemochromatosis, due to identification of mutations in a number of other genes.^{19–23} Analysis of these genes suggests that the pathway controlling the availability and uptake of iron is more complex than we had once thought. A list of genes with mutations leading to iron storage diseases together with their chromosomal locations, corresponding proteins, and previous names, is shown in table 1. Neonatal haemochromatosis may also have a hereditary component.²⁴

OTHER INHERITED FORMS OF HAEMOCHROMATOSIS AND IRON OVERLOAD INCLUDING JUVENILE HAEMOCHROMATOSIS AND FERROPORTIN DISEASE

Juvenile haemochromatosis (JH), or type 2 haemochromatosis, is an autosomal recessive disease due to mutations in either the hepcidin (*HAMP*)²² or hemojuvelin (*HJV*)²³ genes.

The clinical manifestations of JH and HH have been compared in detail.²⁵ Equal numbers of males and females are affected by JH, and cardiac involvement and hypogonadism are frequent in JH.

JH is a more severe form of iron loading disorder than HH, being characterised by an earlier age of onset, usually before the age of 30. A particularly severe form of JH is caused by autosomal inheritance of mutations in *HAMP* (OMIM 606464).²² The *HAMP* gene maps to 19q13 and comprises three exons; the processed hepcidin peptide is coded entirely by exon 3.²⁶ The first two JH mutations reported disrupt the reading frame for exon 3.²² The first, 166C→T, results in a premature termination codon R56X, and the second is a frameshift mutation (93delG) that alters the reading frame of exon 3.²² The hepcidin peptide has four disulphide bonds,²⁷ the third of which is disrupted by a missense *HAMP* mutation C70R resulting in JH.²⁸ High levels of hepcidin result in anaemia^{29 30} and hyperferraemia.^{31–35}

A second gene, *HJV*, has recently been identified that is involved in the same pathway as hepcidin. *HJV* mutations also result in JH, with an autosomal recessive mode of inheritance.²³ *HJV* maps to 1q21³⁶ and encodes hemojuvelin (OMIM 608374).²³ JH due to mutations in *HJV* appears to be less severe than that due to *HAMP* mutations.²² As is seen with mutations in the transferrin receptor 2 gene (*TFR2*),^{37–43} there is striking allele heterogeneity in JH due to mutations in *HJV*, with the majority of the mutations being private.^{23 44–46} Most mutations generate premature termination codons or are missense substitutions affecting conserved amino acid residues. The 24 mutations described to date are summarized in table 2. The protein sequence of hemojuvelin has no hallmarks that explain its role in iron metabolism. Its expression is restricted to liver, heart, and skeletal muscle. Hemojuvelin is predicted to be a transmembrane protein, having both an Arg-Gly-Asp (RGD) motif and a partial von Willebrand factor type D domain. Many RGD motifs occur in cell surface proteins that interact with integrins in protein–cell or cell–cell interactions.⁴⁷ Hemojuvelin undergoes alternative splicing,²³ the smallest of the spliced variants lacking the RGD motif. The longest isoform encodes a protein of 426 amino acids and shows sequence similarity with the repulsive guidance molecule.²³ Urinary hepcidin levels are depressed in individuals with *HJV* linked JH, suggesting that hemojuvelin

Table 1 Examples of some of the genes in which mutations give rise to inherited forms of iron overload including haemochromatosis						
Gene	Previous names	Protein	Chromosome locus	Disease	Mode of inheritance	Key reference(s)
<i>HFE</i>	<i>HLA-H</i>	HFE	6p21.3–6p22.1	Haemochromatosis type 1	Incomplete autosomal recessive	4
<i>HJV</i>	<i>HFE2</i>	Hemojuvelin	1q21	Juvenile haemochromatosis	Autosomal recessive	23
<i>HAMP</i>	<i>HEPC</i>	Hepcidin LEAP-1	19q13	Juvenile haemochromatosis	Autosomal recessive	22
<i>TFR2</i>		Transferrin receptor 2	7q22	Haemochromatosis type 3	Autosomal recessive	19
<i>SLC40A1</i>	<i>IREG1</i> , <i>MTP1</i> , <i>SLC11A3</i> , <i>FPN1</i>	Ferroportin	2q32	Haemochromatosis type 4 ferroportin disease	Autosomal dominant	20, 21
<i>H-ferritin</i>		H-ferritin	11q13	Autosomal dominant iron overload	Autosomal dominant	74
<i>L-ferritin</i>		L-ferritin	19q13.3–13.4	Hyperferritinaemia/cataract syndrome	Autosomal dominant	75, 76
<i>CP</i>		Caeruloplasmin	3q23–24	Acaeruloplasminaemia	Autosomal recessive	80, 81
<i>PANK2</i>		Pantothenate kinase 2	20p12.3–p13	PKAN	Autosomal recessive	109

Table 2 HJV mutations that have been identified in patients with JH

Nucleotide change	Exon	Residue	Alleles	Reference(s)
18G→C	2	Q6H	1	46
220delG	3	V74fsX113	1	44
*	3	C80R	1	45
253T→C	3	S85P	2	44
295G→A	3	G99R	1	44
296G→T	3	G99V	1	23
302T→C	3	L101P	2	44, 46
391–403del	3	R131fsX245	2	44
445delG	3	R149fsX245	6	44
503C→A	3	A168D	2	44
509T→C	3	F170S	4	44
516C→G	3	D172E	1	44
573G→T	3	W191C	2	44
615C→G	3	S205R	1	44
665T→C	4a	I222N	2	23, 45
739G→T	4a	G250V	1	44
806–807insA	4a	N269fsX311	1	44
842T→C	4a	I281T	2	23, 46
863C→T	4a	R288W	2	44
954–955insG	4a	G319fsX341	1	44
959G→T	4b	G320V	46	23, 44, 45
962G→A	4b	C321X	1	46
963C→A				
976C→T	4b	R326X	1	23
1079delC	4b	C361fsX366	14	23
1153C→T	4b	R385X	4	44

*Information available at the time of final publication.⁴⁵

modulates hepcidin expression.²³ Similar observations have been made for haemochromatosis expressing C282Y homozygotes.^{33–35} *HAMP* mRNA levels in the liver of HH patients are inappropriately low for body iron stores and urinary levels are correspondingly low.^{33–34}

In southern Italy, where a minority of haemochromatosis patients are C282Y homozygotes, genome-wide screening of affected families led to the identification of another rare form of haemochromatosis caused by autosomal inheritance of mutations in the gene for *TFR2*.¹⁹ This is known as haemochromatosis type 3 (OMIM 604250) and is due to a number of private mutations.^{37–43} The first mutation, Y250X, was identified in two Sicilian families who were homozygous for a C→G transversion at nucleotide 750 in the coding sequence of the mRNA.¹⁹ A second nonsense mutation, missense and frameshift mutations have now been reported: E60X,³⁷ M172K,³⁷ R455Q,³⁹ Q690P,³⁸ V221I,⁴² and the deletion of AVAQ (residues 594–597).^{39–40, 43} The clinical phenotype is similar to that of type 1 haemochromatosis, with iron deposition in the liver having a periportal distribution. However, the clinical features vary depending upon the reported mutation.^{37–43}

Interestingly, transferrin receptor 2 (TfR2) is expressed in hepatocytes⁴⁸ and in duodenal crypt cells¹⁷ as well as in platelets.⁴⁹ Griffiths and Cox have demonstrated that HFE and TfR2 localise in duodenal crypt cells, and that within the crypt areas the distribution of TfR2 differed from that of transferrin receptor 1.¹⁷ Little or no HFE was present in the duodenal crypt cells of an HH patient and unlike the observed intracellular localisation of wildtype HFE, TfR2 localised to the basolateral surface of the duodenal crypt cell. These authors suggest that the interaction of HFE with TfR2 within this vesicular compartment was stimulated by the presence of holotransferrin (iron loaded transferrin).¹⁷ TfR2 plays a role in cellular iron transport although it has a 25-fold lower affinity for transferrin relative to that of TfR1.⁵⁰

Type 4 haemochromatosis (OMIM 606069) is an autosomal dominant form of iron overload associated with

mutations in the gene encoding ferroportin/IREG1/MTP1 (*SLC40A1*, previously *SLC11A3*).^{20–21, 51} The first description of this form of haemochromatosis was in a large kindred from the Solomon Islands.⁵² Mutations in *SLC40A1* have a wide geographical distribution.^{53–58} One in particular, 162delVal, has been found in six unrelated families.^{59–62} Ferroportin is a membrane spanning transporter that exports iron from cells and is predicted to have nine or more transmembrane domains. Ferroportin is predominantly found in Kupffer cells and the basolateral membrane of the duodenal enterocyte.^{63–65} It is also expressed in reticulo-endothelial macrophages, placental syncytiotrophoblasts, and hepatocytes.

The majority of ferroportin mutations localize to the external face of the protein in the model of Devalia *et al*,⁵⁹ many of them mapping to the extracellular loop between transmembrane domains 3 and 4 (fig 2). It has been suggested that ferroportin expressed in the membrane of macrophages may interact with caeruloplasmin, a ferroxidase, to provide Fe³⁺ for transferrin.^{66–67} The accumulation of iron by macrophages in patients with type 4 haemochromatosis suggests some loss of ferroportin function, possibly through a failure to interact with another protein such as caeruloplasmin. This is consistent with the phenotype of the ceruloplasmin null (*Cp*^{−/−}) mouse in which iron accumulates in the Kupffer cells⁶⁸ in a manner similar to that seen in patients with ferroportin mutations. Hephaestin plays an important role in intestinal iron absorption and is predicted to be a ferroxidase based on significant sequence identity to the serum multi-copper ferroxidase, ceruloplasmin.⁶⁹ A recent report has shown that, in the rat, hephaestin is expressed both in the enterocyte and the macrophage, suggesting that caeruloplasmin may not be required as the ferroxidase in macrophages.¹⁸ Other mutations elsewhere in ferroportin may have the reverse effect, resulting in loss of regulation and enhanced export of iron from the macrophage (Viprakasit *et al*, unpublished observations). Disruption of ferroportin gene regulation causes dynamic alterations in iron homeostasis and erythropoiesis in polycythaemic mice.⁷⁰ A 58 bp microdeletion in the *Slc40a1* promoter, identified in radiation induced polycythaemic mice (*Pcm*), altered the transcription start sites and eliminated the iron responsive element (IRE) in the 5′ untranslated region.⁷⁰ This resulted in increased duodenal and hepatic ferroportin protein levels during early postnatal development. *Pcm* mutant mice were iron deficient at birth and went on to develop reticulo-endothelial iron overload as young adults.⁷⁰ They also showed an erythropoietin dependent polycythaemia in heterozygotes and a hypochromic, microcytic anaemia in homozygotes.⁷⁰ It was also observed that the defects in erythropoiesis were transient and that the delayed upregulation of hepcidin during postnatal development correlated with increased ferroportin levels and polycythaemia in *Pcm* heterozygotes.⁷⁰

These findings will no doubt modify current models of how HFE mutations result in iron loading.^{67–71–73} In the model proposed by Townsend and Drakesmith HFE has two mutually exclusive activities in cells: inhibition of both uptake and release of iron from cells.⁷¹ The balance between transferrin saturation and transferrin receptor concentration would determine which of these two functions predominates. The inhibition of iron release may involve ferroportin. HFE present in the duodenal crypt cells and the reticuloendothelial system interprets the body's need for iron and thus controls iron absorption by the gut enterocyte and its subsequent distribution. Hence mutations in HFE would be predicted to result in over-absorption of dietary iron or its distribution.

Other rare forms of inherited disorders of iron metabolism have been reported. These include mutations in the IRE of H-ferritin,⁷⁴ and mutations in the IRE of L-ferritin which result

Figure 2 Schematic representation of the structure of ferroportin, based on the model proposed by Devalia *et al.*⁵⁹ The filled circles correspond to the cysteine residues. The relative positions of the reported mutations are indicated by open circles.^{20 21 53-62 95} A third mutation at amino acid position 144 has just been reported associated with parenchymal loading and cirrhosis.¹⁷⁵ We include two novel unpublished mutations, C326Y (Viprakasit *et al*, manuscript in preparation) and D270V (Zaahl *et al*, manuscript in preparation). The length in amino acids of the predicted loops and the position of three potential glycosylation sites (Y) are indicated.

in the hyperferritinaemia-cataract syndrome.^{42 54 75-78} This syndrome has been reviewed recently.⁷⁹ It is associated with low serum iron and elevated serum ferritin levels. Acaeruloplasminaemia results from mutations in caeruloplasmin.^{80 81} This disease is characterised by iron accumulation in the brain and visceral organs; the involvement of the central nervous system distinguishes it from other inherited iron storage disorders. Acaeruloplasminaemia is associated with anaemia and low serum iron levels (for reviews see Gitlin⁸² and Miyajima *et al*⁸³). A total of 21 mutations in the caeruloplasmin gene have been identified in 24 families worldwide.⁸² Neurological problems such as ataxia and dementia are common, suggesting that iron efflux from storage sites within the CNS requires caeruloplasmin. The latter has a direct role in mobilising iron from parenchymal tissues by oxidising Fe²⁺ to Fe³⁺, enabling it to bind to circulating apo-transferrin.⁸³

POPULATION GENETICS AND SCREENING

The genetics of haemochromatosis is complicated by incomplete penetrance and digenic inheritance. Studies of various mouse models suggest that the inheritance of iron loading is more complex than was initially believed. A number of human population studies have demonstrated that there is a direct relationship between the HFE C282Y genotype and transferrin saturation.⁸⁴⁻⁸⁶ Transferrin saturation reflects the amount of transport iron that is available to cells via uptake through the transferrin receptor cycle.⁸⁷ An unexplained raised transferrin saturation is usually an early biochemical indicator of haemochromatosis. The normal range has been the subject of debate. For men it is usually between 15-55% and for women 15-50%. The normal serum iron concentration is 10-30 µmol/l. Patients who have haemochromatosis due to mutations in the *HFE* gene have transferrin saturations above 55% and also have a raised serum ferritin concentration (men >300 µg/l, women >200 µg/l).⁸⁸ However, not all C282Y homozygotes will show signs of iron loading. The age of onset is variable and is on average a decade later in women compared to men due to blood loss during menstruation and pregnancy. The presenting features are variable and lead to difficulties in diagnosis. Disease penetrance in haemochromatosis has been the subject of much recent discussion in the literature.⁸⁹⁻⁹³

It appears that disease is associated with a raised serum ferritin concentration, suggesting that there are genetic modifiers that contribute to increased serum ferritin concentrations in the presence of a raised transferrin saturation. This is supported by two observations. Firstly, mutations in ferroportin result in a phenotype that differs from that of HFE haemochromatosis. Patients with ferroportin mutations have a raised serum ferritin in the absence of a raised transferrin saturation. They rapidly become anaemic when treated by venesection unless they are also heterozygous for the H63D variant of HFE, and tend to have microcytic red cells. Iron loading in the liver is initially restricted to Kupffer cells, unlike that of HFE haemochromatosis in which iron is found predominantly in hepatocytes, with a periportal distribution; Kupffer cells are spared until the late phase of the disease progression. As a consequence, type 4 haemochromatosis has been referred to as "ferroportin disease".^{94 95} Patients with HFE haemochromatosis tolerate venesection to mobilise their iron stores and their red cells tend to be macrocytic.^{96 97}

The second observation suggesting that genetic modifiers influence serum ferritin concentration in the presence of increased transferrin saturation comes from work with mouse models of haemochromatosis. The degree of iron loading varies depending on the genetic background of the mouse.^{98 99} Results presented at Bioiron 2003 suggested that there are at least four independent loci contributing to iron loading in the mouse model.¹⁰⁰

Unfortunately, the phenotypic markers for haemochromatosis lack specificity and sensitivity. Transferrin saturation and serum ferritin levels are best evaluated together. A raised serum ferritin is not unique to a patient with haemochromatosis. Serum ferritin concentrations can be increased and transferrin saturation decreased in both acute and chronic disease.¹⁰¹ Due to variability in the age of onset of disease in HFE haemochromatosis, biochemical tests need to be repeated on a regular basis to identify those in the presymptomatic phase. Genetic screening for haemochromatosis is not currently deemed an acceptable alternative to phenotypic screening because of the incomplete penetrance of the C282Y homozygous state. The acceptability of screening must also be considered, although it may be more acceptable to screen for haemochromatosis than is currently thought (Cadet *et al*, submitted). Genetic screening for

haemochromatosis may become more valuable when disease penetrance is better understood and it may be more appropriate in populations with a higher degree of penetrance. Reports from South Wales and California suggest low penetrance whereas much higher penetrance is seen in the Picardy region of northern France.^{89 91 93 102 103} Environmental as well as genetic factors may help explain some of these differences.

In the future, screening for the C282Y mutation may have other advantages. Although the C282Y allele is not a high risk factor for diseases such as Alzheimer's or cardiovascular disease in isolation, in women who smoke or who have hypertension, it increases the risk of developing cardiovascular disease.¹⁰⁴ The risk of myocardial infarction and atherosclerosis is related to iron stores.¹⁰⁵ The HFE C282Y mutation is also a risk factor for Alzheimer's disease (AD) when present with the apolipoprotein E4 allele, a risk that is increased in the presence of the C2 allele of transferrin.¹⁰⁶ Over 30 polymorphisms have been reported in the human transferrin gene.¹⁰⁷ Of these, the C2 allele has a slightly reduced affinity for iron. If genetic screening for susceptibility to AD becomes a reality it may be worth including both HFE C282Y and TF C2 or routine monitoring of serum iron levels as part of a disease prevention programme. Increased circulating iron may induce the oxidative damage to lipids implicated in the genesis of common diseases such as cardiovascular disease and AD, both of which involve plaque formation. Iron misregulation is known to play a role in pantothenate kinase associated neurodegeneration (PKAN; previously known as Hallervorden-Spatz disease),¹⁰⁸ and has been implicated in other neurodegenerative disorders.^{109–112}

GENETIC MODIFIERS AND DIGENIC INHERITANCE

Clues as to the complexity of factors resulting in iron loading disorders have come from recent work investigating haemochromatosis patients who carry only one copy of the HFE C282Y mutation. We and others have found that some of them are heterozygous for mutations in *HAMP*.^{42 113 114} This suggests that haemochromatosis can result from digenic inheritance of mutations in *HFE* and *HAMP*. The severity of disease correlates with the type of *HAMP* mutation.¹¹³ We identified two different mutations in *HAMP*, the more severe being a four nucleotide deletion, ATGG, at the 3' end of exon 2 (Met50del IVS2+1(-G)) which removes the splice site consensus GT but creates a second splice site consensus leading to a different open reading frame in exon 3.¹¹³ *HAMP* encodes hepcidin, which is synthesized in the liver as a pre-peptide and then processed to produce hepcidin. As the mature peptide is entirely encoded by exon 3,³² no hepcidin is expressed by the Met50del IVS2+1(-G) mutant allele, and heterozygosity for this and the C282Y mutation in HFE

results in JH.¹¹³ Such patients presumably have half the functional levels of HFE and hepcidin, which act synergistically to produce disease. Double heterozygosity for the reported missense *HAMP* mutations (fig 3) and the HFE C282Y mutation results in a milder disease that resembles the classic adult onset HFE haemochromatosis.^{42 113 114} Consistent with these observations, hepcidin has been shown to act as a genetic modifier in the mouse model for haemochromatosis.¹¹⁵

Several groups have suggested that the ancestral haplotype carrying the C282Y mutation is associated with more severe disease.^{116–118} Others have not been able to confirm this observation.¹¹⁹ The ancestral haplotype extends into the major histocompatibility complex (MHC) class I region. The extended MHC region includes genes involved in modulating the immune response, one of which is tumour necrosis factor α . There are number of tumour necrosis factor α polymorphisms two which have been studied in relation to expression of HH.¹²⁰ These findings suggest that tumour necrosis factor α plays a role in HH by modulating the severity of the liver damage.¹²⁰ One of the problems encountered in identifying the HFE gene was that it occurred on an extended haplotype.⁴ It is not clear whether other genes on this extended haplotype have provided a selective advantage. It has been proposed that the MHC class I genes may themselves be genetic modifiers and so explain the extended linkage disequilibrium.¹²¹

We have reported that the 16189 variant of mitochondrial DNA may also act as a modifier.¹²² Its frequency in the normal UK population is 8.8%, increasing to 14.4% in disease expressing C282Y homozygotes and decreasing to 3.2% in non-expressing homozygotes.¹²²

Studies such as these need independent confirmation in a second population. Sampling error can sometimes lead to false disease associations.

IRON, HOST DEFENCE, AND INFECTION

The complex and controversial literature on the roles of iron deficiency or overload in susceptibility to infection is reviewed elsewhere.^{121 123 124} It is not clear whether, in evolutionary terms, genetic diversity which favours increased iron absorption would have been advantageous. However, it is generally accepted that the hypoferraemia that occurs in acute infections, reflecting redistribution of iron rather than iron deficiency, may be an important mechanism for limiting iron availability for micro-organisms or parasites.

It is uncertain at present how mutations in some of the proteins described in this review result in iron overload. It is clear, however, that they are likely to be involved in a novel pathway of iron metabolism. That hepcidin is an acute phase protein³³ and HFE an MHC class I-like molecule⁴ suggests that other players in the pathway may well be involved in a host defence pathway that limits iron availability and restricts growth of invading pathogens.

Two groups identified hepcidin as an antimicrobial peptide.^{26 125} Two separate approaches identified hepcidin as having a key role in iron homeostasis.^{31 32} Loss of hepcidin expression in the mouse leads to severe iron overload³¹ and in humans a severe form JH is due to autosomal recessive inheritance of mutations in *HAMP*.²² The human gene encodes a pre-peptide of 84 amino acids,²⁶ which after cleavage of the signal sequence yields a propeptide of 60 amino acids that is cleaved further to give the 25 and 22 amino acid forms of hepcidin which is found in serum¹²⁵ and urine.²⁶ It is these 22 and 25 amino acid forms that have antibacterial²⁶ and antifungal activities.¹²⁵ Hepcidin is a member of a family of proteins which includes the defensins, some of which have been shown to be involved in inflammation. Hepcidin is a cysteine-rich, cationic antimicrobial peptide and is a type II acute phase protein.³³

Figure 3 Schematic representation of the structure of hepcidin.²⁷ The eight cysteine residues are indicated by black circles. The positions of the missense mutations G71D (black background) and C70R (dark grey) are indicated. The only amino acid with an acidic side chain in the mature peptide, D60 (white background) is also indicated. The remaining amino acids are represented by light grey filled circles.

Hepcidin regulates intestinal iron absorption.¹²⁶ Elegant work by Nemeth and colleagues has shown that interleukin 6 (IL-6) mediates hypoferraemia of inflammation by inducing synthesis of hepcidin.¹²⁷ This is a key finding as it helps explain the anaemia of chronic disease (ACD, also called the anaemia of inflammation). ACD is a hypoproliferative anaemia commonly found in patients with chronic infections, cancer, injury, and inflammatory disorders.^{128–129} Clinically, patients with ACD have a low serum iron (hyposideraemia) and reduced erythropoiesis due to low levels of erythropoietin. Inflammation increases retention of iron by the macrophage and restricts duodenal iron uptake. Hepcidin is key to this response. Mice lacking Hfe mount a general inflammatory response after injection of lipopolysaccharide but lack the appropriate hepcidin response and so do not develop hyposideraemia.¹³⁰ Understanding the basis of ACD may help in developing new treatments.

HFE has a structure similar to MHC class I molecules.¹³¹ Importantly, these molecules form a heterodimer with β_2 -microglobulin (β_2 -M). When mutations in HFE were first proposed to be responsible for HH, supporting evidence came from the β_2 -M null mouse.¹³² The iron loading observed in these mice recapitulated the iron loading observed in HH. Mice lacking both Hfe and β_2 -M have significantly greater iron overload than mice lacking either gene alone.¹³³

Other proteins involved in iron metabolism include DMT1, the divalent metal ion transporter,¹³⁴ which was originally identified as a gene with homology to natural resistance associated macrophage protein 1 (NRAMP1) and so given the name NRAMP2.¹³⁵ NRAMP1 is now classified as solute carrier family 1 member 1 (SLC11A1) and NRAMP2 as solute carrier family 1 member 2 (SLC11A2). SLC11A1 is expressed in spleen, liver, and lungs, but is most abundant in monocytes/macrophages and polymorphonuclear leukocytes.^{136–137} The G169D mutation in the murine gene, *Slc11a1*, is associated with susceptibility to infection with intracellular parasites such as *Leishmania*, *Mycobacteria*, and *Salmonella*.¹³⁸ SLC11A1 has a role in the transport of divalent metals and so restricts the availability of divalent metal ions to phagocytosed pathogens in the phagosome.¹³⁹ Within the phagolysosome SLC11A1 is predicted to function in a pH dependent fashion, transporting divalent ions from the lumen of the phagolysosome thus restricting the growth of the pathogen.¹⁴⁰

The iron transporter SLC11A2 has a major role in the transport of iron across the brush border into the enterocyte of the duodenum. It is also involved in transporting the iron released from transferrin in the lumen of recycling endosomes into the cytoplasm.¹⁴¹ Mutations in SLC11A2 result in anaemia.¹³⁴

Further evidence suggesting that there is a pathway involving host defence and iron comes from the work of de Sousa and colleagues.¹⁴² They noted that the relative proportions of CD4+ and CD8+ T lymphocytes were abnormal in patients with HH. Those with abnormally high iron stores have CD4:CD8 >3 (normal: 0.61–1.77). The abnormal ratio, due to abnormally low numbers of CD8 cells, remains unchanged after treatment by phlebotomy.¹⁴³ In patients whose abnormal ratios persist after intensive treatment, the transferrin saturation starts to increase earlier than in those whose CD4:CD8 ratios are closer to the normal range.¹⁴⁴ Anomalies of the T cell receptor repertoire have also been described in C282Y carriers.¹⁴⁵

Nuclear factor κ B (NF- κ B) is a transcription factor that plays a pivotal role in transactivation of genes involved in the hepatic acute phase response and innate and adaptive immunity.¹⁴⁶ One of the genes that it regulates, IL-6, is known to activate hepcidin.³³ As discussed above, hepcidin plays a significant role in iron metabolism.^{30–32} Endotoxin induced NF- κ B activation in macrophages is inhibited by iron

chelators, suggesting that intracellular iron plays a signalling role in NF- κ B activation.¹⁴⁶ Tumour necrosis factor α expression is also induced by NF- κ B. A model proposed by Chorney *et al*⁷³ suggests that HFE may either signal directly to the intra-epithelial lymphocytes (IEL) or induce the expression of cell-surface or secreted molecules that carry out the signalling process. They suggest that iron loaded enterocytes would be viewed by a neighbouring $\gamma\delta$ IEL as being oxidatively stressed, in keeping with the role of $\gamma\delta$ T cells in responding to tissue damage.¹⁴⁷ The tumour necrosis factor α released could lead to an upregulation of ferritin expression and hence increased iron stores.¹⁴⁸ This may provide yet another link between genes involved in iron metabolism and host immunity.

The haemoglobin scavenger receptor, CD163, is expressed on monocytes at low levels and at high levels on macrophages.¹⁴⁹ IL-6, glucocorticoids, and IL-10 are acute phase mediators that strongly induce CD163 mRNA and its expression at the cell surface.^{150–151} LPS, IL-4, tumour necrosis factor α , and interferon- γ downregulate its expression.^{150–151} It has been suggested that haptoglobin-haemoglobin complexes may crosslink CD163 on the macrophage surface and so trigger a cascade that increases the production and secretion of anti-inflammatory cytokines.¹⁵² This may provide further clues as to IL-6 production resulting in hepcidin expression and ACD.¹²⁷ CD163 may also play a role in atherosclerosis,¹⁵³ again implicating iron in chronic disease.

HFE and hemojuvelin clearly operate upstream of hepcidin. Mice administered lipopolysaccharide have reduced levels of ferroportin mRNA and protein in the liver, spleen, and duodenum.¹⁵⁴ Recent data suggest that hepcidin regulates ferroportin expression in the liver and intestine of the rat, suggesting that ferroportin is downstream of hepcidin in this pathway.¹⁵⁵ Hepcidin could function by blocking the ferroportin linked iron export from enterocytes and macrophages. Some mutations in ferroportin would be predicted to alter this interaction, resulting in either enhanced or reduced export of iron from macrophages, as discussed above. The hepcidin receptor and the proteins involved in transcriptional control of ferroportin remain to be identified. From our experience there are still patients who have unexplained iron overload and may have mutations in other genes in the hepcidin pathway. Identification of these genes and their disease causing mutations will help fill the gaps in this pathway.

Clearly, the regulation of iron homeostasis and the inflammatory and immune responses are linked in a highly complex interactive system, many facets of which must have come under intense evolutionary pressure and which may show broad homology over many species. Hence they are likely to exhibit wide genetic heterogeneity, the further study of which may have important implications for a better understanding of both disorders of iron metabolism and their effects and the variability of response to infection among different ethnic groups.

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ELECTRONIC-DATABASE INFORMATION

Accession numbers and URLs for data in this article are as follows: GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for the haemochromatosis gene HFE); Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/omim/> (for HH [OMIM 235200]; for juvenile haemochromatosis, HJV, HFE2A [OMIM 606374], HAMP, HFE2B [OMIM 606464]; for haemochromatosis due to mutations in TFR2, HFE3 [OMIM 604250]; for mutations in SLC40A1, HFE4 haemochromatosis [OMIM 606069]).

Authors' affiliations

K J H Robson, A T Merryweather-Clarke, V Viprakasit, D J Weatherall, MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, Headley Way, Oxford, OX3 9DS, UK

E Cadet, J Rochette, Génétique Médicale-CHU, Faculté de Médecine, Université de Picardie Jules Verne, UMR-INERIS, Amiens, France

V Viprakasit, Department of Paediatrics, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand

M G Zaahl, Department of Genetics, University of Stellenbosch, Stellenbosch, South Africa

J J Pointon, Institute of Musculoskeletal Sciences, University of Oxford, The Botnar Research Centre, Nuffield Orthopaedic Centre, Windmill Road, Headington, Oxford, OX3 7LD, UK

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PostScript

LETTERS

Correction: no evidence of an association between the T16189C mtDNA variant and late onset dementia (Gibson *et al*)

We believe that the title of Chinnery *et al*'s paper should be corrected because the data the authors present do not include an analysis of the 16189 variant of mtDNA (Table 1).

We defined the 16189 variant as the DNA sequence associated with a polydC tract,² resulting from a T16189C transition that may generate heteroplasmic length variation, table 1. Heteroplasmic length variation does not occur when the polymeric tract is interrupted by a C→T transition, which occurs at several different sites but commonly at nucleotide 16186 or 16192. Individuals with these additional polymorphisms are excluded from our definition of the 16189 variant because they no longer have a long homopolymeric C tract. The variant does not alter any coding sequences yet lies near to mtDNA control sequences, which can explain its effects on mitochondrial function. In studies of disease associations with variants in this region we chose to investigate the 16189 variant rather than any other sequence change, because of the likely functional effects of the homopolymeric C tract and heteroplasmic length variation.

Gibson *et al*¹ have shown that the overall prevalence of the T16189C allele in their population is 12.6%, which is substantially higher than the 6.4–8.8 % prevalence of the 16189 variant reported in other studies.^{2,3} This is because they have quantified the prevalence of the T16189C transition *per se* rather than the variant. Including these additional polymorphisms may dilute out a real association with the 16189 variant. The authors have shown that the T16189C transition *per se* is not a risk factor for late onset dementia,¹ but to our knowledge this has not, in any case, been implicated with any disease phenotypes. However, they found that the heteroplasmic length variation, which implies the presence of the 16189 variant, was associated with a 2.2 fold increased risk. They did not, however, quantify the relative risk for the 16189 variant *per se*, which could well be significant, in direct contradiction of their title. From their data, it is possible that the variant might in fact predispose to late onset dementia.

The 16189 variant is a risk factor for type 2 diabetes,⁴ thinness at birth,⁵ and aged 20 years⁶ iron loading in haemochromato-

sis,⁷ dilated cardiomyopathy,⁸ endometrial cancer,⁹ and other multifactorial disorders.¹⁰ This variant may be mildly detrimental.¹¹ Unlike many other mtDNA polymorphisms implicated in type 2 diabetes, this variant probably has bona fide functional consequences because it has arisen many times independently in the various populations studied,^{2,4,12} excluding a founder effect. Because the authors did not perform mitochondrial haplotyping¹ to exclude a founder effect, their results may reflect the consequences of other co-segregating genes.

J Poulton, S Das

Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Women's Centre, John Radcliffe Hospital, Oxford, UK

Correspondence to: Professor J Poulton, Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Women's Centre, John Radcliffe Hospital, Oxford, OX3 9DU, UK; joanna.poulton@obs-gyn.ox.ac.uk

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No evidence of an association between the mtDNA 16184-93 polyC tract and late onset dementia

We are grateful to Professor Poulton and Dr Das for clarifying their definition of the 16189 variant. In our study we determined the allele status at position 16189 of mitochondrial DNA (mtDNA), and found no evidence of an association between the 16189C polymorphic sequence variant and late onset dementia.¹ The title of our manuscript therefore reflects our observations and does not need to be corrected. Part of the confusion seems to have arisen because of different definitions of the "16189 variant" in the literature.

The standard "Cambridge" reference mtDNA sequence^{2,3} has a run of cytosine residues from nucleotide position (np) 16184 to 16193 interrupted by a thymidine residue at nucleotide position 16189. In approximately 12% of the UK population, there is a T→C substitution at np 16189.¹ In most individuals this results a tract of 10 C residues (polyC tract). This sequence is unstable and is associated with length variation of the polyC tract, probably because of slippage during genome replication,⁴ generating larger and smaller polyC tracts within the same individual during life (heteroplasmy).⁴ However, occasional individuals have other polymorphisms between np 16184 and 16193, which appear to stabilise the tract and do not lead to the generation of length variants.⁴ We suggest a more accurate definition should be used when referring to the homopolymeric C tract that is present in the majority of individuals with the T16189C substitution. The term "mtDNA 16184-93 polyC tract" will hopefully prevent confusion in the future.

Poulton and Das comment on the results of our logistic regression analysis (table 1 in our paper¹), and suggest that individuals with homopolymeric tract length heteroplasmy have a 2.2 fold increased risk of developing Alzheimer's disease (AD) compared with controls. This would imply an association between their definition of the 16189 variant and late onset dementia. However, the confidence intervals for the relative risk of 2.2 are 0.85 to 5.81, comfortably including 1. The relative risk is therefore not statistically significant and does not support a link between AD and homopolymeric length tract heteroplasmy.

In our original study,¹ we measured homopolymeric tract length heteroplasmy using a trimmed PCR approach with a fluorescent forward primer. However, not all individuals with a 16184-93 polyC tract also have length heteroplasmy. Therefore, to address the concerns of Poulton and Das experimentally, we directly sequenced the relevant region of

Table 1 Sequences of identified variants

Variant	Nucleotides
Wild type sequence	ccccctccc
Sequences included in Chinnery's analysis	ccccccctcc ctcccccccc cccccccccc etc cccccccccc
16189 variant ²	cccccccccc
Heteroplasmic length variants included within 16189 variant	cccccccccc cccccccccc etc

Table 1 Frequency of the mtDNA 16184-93 polyC tract in control individuals, patients with dementia with Lewy bodies (DLB) and Alzheimer's disease (AD)

Group	Frequency of the mtDNA 16184-93 polydC tract		Comparison with controls	
	n	% (95% CI*)	Odds ratio (95% CI)	p†
Controls (n = 129)	9	6.98 (3.24 to 12.83)	—	—
DLB (n = 97)	10	10.31 (5.06 to 18.14)	1.53 (0.60 to 3.93)	0.46
AD (n = 182)	11	6.04 (3.06 to 10.56)	0.85 (0.34 to 2.13)	0.81

*Exact 95% confidence intervals for the percentage were calculated using the method of Clopper and Pearson. †Fisher's two tailed exact test. CI, confidence interval. Each group corresponds to the cohort of neuropathologically confirmed cases and controls reported in our paper.[1] Note that table 2 of our original report[1] only includes subjects where the complete APOE genotype was also known. This data was not available for two controls, one DLB case, and eight AD cases. The data from these cases are included in this table.

mtDNA in all of the cases and controls that harboured the 16189C variant in our original study,¹ using an established protocol.⁵ The frequency of the mtDNA 16184-93 polyC tract in our original control population corresponded to values reported in other studies^{6,7} (table 1); 59% of control individuals with the T16189C polymorphic variant had a 16184-93 polyC tract, corresponding to publicly available control sequence data (<http://www.genpat.uu.se/mtDB/>). We found no evidence of an association between AD or dementia with Lewy bodies and 16184-93 polydC tract in a cohort or neuropathologically defined cases and controls, either by logistic regression analysis, or by directly comparing cases and controls with Fisher's exact test (table 1).

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**S M Keers, A M Gibson, D M Turnbull,
P F Chinnery**

Correspondence to: Dr P F Chinnery, Department of Neurology, The Medical School, Framlington Place, Newcastle Upon Tyne, NE2 4HH, UK; p.f.chinnery@ncl.ac.uk

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BOOK REVIEWS

Statistical Methods in Genetic Epidemiology

By D C Thomas. Oxford University Press, 2004, \$42.50, pp 435. ISBN 0-19-515939-X

The contemporary research principles of genetic epidemiology are outlined in this book. The author clearly explains the research methodology and statistical analyses required to investigate important genetic epidemiological research questions. These include the following questions: Does a disease cluster in families? (familial aggregation); How does a disease cluster in families? (segregation analysis); Can familial aggregation be explained by genetic or environmental factors? (gene-environment interaction); Can we localise the genetic defect? (linkage and association studies). The theory is mainly illustrated with examples on the genetic epidemiology of cancer. As genetic epidemiology is a hybrid discipline, basic chapters on molecular genetics, epidemiology, statistics, and population genetics are included for those readers who need an introduction to any of these topics.

This book fascinates me because of its high didactic quality. The text is well organised and is easy to read. The content is interesting both to novices and to more advanced readers. The strength of the book is that it gives a complete overview of the different methods used in genetic epidemiology. Owing to its completeness, I would not be surprised if it were used in many semesters or courses on genetic epidemiology around the world. I would expect it also to be very useful for the more advanced genetic epidemiologist as an up to date reference text. Readers interested in closely related disciplines such as population genetics, molecular genetics, behaviour genetics, statistical genetics, genomics, and bioinformatics will not find enough detail here and should look elsewhere.

I consider this text to be a standard work in genetic epidemiology and would advise both teachers and researchers in the field to read and use it.

M P Zeegers

Human Evolutionary Genetics: Origins, Peoples & Disease

Edited by M A Jobling, M E Hurles, C Tyler-Smith. Garland Science, 2003, £35.00, pp 458. ISBN 9-780815-341857

With the near completion of the human genome sequence, and the exponential increase in associated information on inter-individual variability, there are enormous opportunities for using these data. Evolutionary geneticists are attempting to understand our origins and revisit the questions of the relative role of selection and drift. For medical geneticists, the search is now on for the genetic causes of complex disease, while forensic science is increasingly exploiting our interindividual differences to solve crimes. All these topics are inextricably linked. This super textbook covers almost everything an undergraduate student in human genetics would need as a basis for any of these areas, but will in fact have a much wider readership. It starts as far back as the structure of DNA, chromosome structure, meiosis, mitosis, and so on, while covering in some depth anthropological and archaeological evidence for the origins of modern humans, the extent and nature of genomic variation, and the principles of human population genetics. It explains clearly how the genome can be considered in blocks, owing to the pattern of historic and prehistoric recombinations and that these pieces of DNA, as well as the Y chromosome and mitochondrial DNA, track back to many ancestors who may have lived in different parts of the world.

This publication is timely, up to date, and has enormous and comprehensive coverage, without being too heavy to carry or costing too much. It is more than just a textbook, because, by using opinion boxes, it discusses contentious and problem issues. Thus as well as providing factual information, the book will stimulate the undergraduate to appraise observations and their interpretation critically. An important example is the discussion of error rates, and the potential impact of errors on interpretation of data. It answers questions that many of us get asked by our non-genetics friends, such as what exactly do we mean when we say that there is 1-2% sequence difference between humans and chimpanzees? How much do the different population groups of the world differ and is the term race meaningful? Clearly, as far as humans are concerned, it is not.

The book will also serve as a very useful introduction to molecular and population genetics for epidemiologists, anthropologists, and others who are new (or indeed not so new) to the field. It contains a wealth of information relevant for medical geneticists who are embarking on association studies. This, for example, includes the effects of selection and the effects of population admixture.

The book is very well laid out, with chapters grouped in six main sections, each of which aims to answer a question (Why study evolutionary genetics? How do we study genome diversity? How do we interpret genetic variation? Where and when did humans originate? How did humans colonise the world? How is an evolutionary perspective helpful?) There is a

good index and glossary, so that it is easy to look things up, and there are extensive references at the end of each chapter and recommendations for further reading. Indeed there are but few short-comings. The figures and tables, which are on the one hand very useful and informative, do also have some weaknesses. Whether it is my failing eyesight or heterozygous manifestation of a colour vision anomaly, I often found the blue grey and black shadings and characters extremely difficult to distinguish. The figure legends could also sometimes have been more informative. However Mark Jobling and colleagues are to be congratulated—this book is a good buy, an excellent read, and is to be strongly recommended.

D M Swallow

CORRECTIONS

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In the paper Recent advances in understanding haemochromatosis: a transition state (*JMG* 2004;**41**:721–30) figures 1 and 2 were incorrect. Below are the corrected figures.

A176CV has been changed to A176V and R224G has been changed to R224Q in figure 1.

N114D has been changed to N144D in figure 2. The journal apologises for these errors.

Figure 1

Figure 2

doi: 10.1136/jmg.2004.022111corr1

In the paper Recent advances (*JMG* 2004; 41:814–25) figure 2 was incorrect. Below is the corrected figures.

A small black bar has been inserted on the line representing the deletion present in the patient number 3273. The author apologies for this error.

Figure 2