IRON EXCESS AND RISK OF TYPE 2 DIABETES MELLITUS IN A PROSPECTIVE COHORT OF MEDITERRANEAN POPULATION

José Cándido Fernández Cao

JOSÉ CÁNDIDO FERNÁNDEZ CAO

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INTERNATIONAL DOCTORAL THESIS

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Department of Basic Medical Sciences

UNIVERSITAT ROVIRA I VIRGILI

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I STATE that the present study, entitled ”Iron excess and risk of type 2 diabetes mellitus in a prospective cohort of Mediterranean population” presented by José Cándido Fernández Cao for the award of the degree of Doctor, has been carried out under my supervision at the Medicine and Basic Sciences Department of this university.

Reus, 8th January 2016

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A mi madre

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ABSTRACT

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ABSTRACT

Background: In recent years iron has been related to diseases such as cancer, cardiovascular disease, metabolic syndrome (MetS), gestational diabetes and type 2 diabetes mellitus (T2DM), through an increase in oxidative stress.

Several studies have found an association between a high consumption of red and processed meat and the risk of T2DM. The intake of haem iron intake - an important component of meat – as well as body iron stores have also been associated with the risk of developing T2DM in U.S., Chinese, and northern European cohorts, however these relationships have not been studied in southern European populations. Although the potential mechanism that mediates these relationships is not well-established, iron plays a role in insulin resistance and β-cell dysfunction, which are two key events in the clinical development of T2DM. Osteocalcin (OC), a biomarker of bone metabolism, has recently been related to glucose homeostasis and iron metabolism. This relationship suggests a potential for mechanism for explaining iron-induced T2DM.

Main objectives: To assess, prospectively, the effect of elevated dietary iron intake and body iron status on the onset of T2DM in an adult Mediterranean population and to evaluate the relationship between iron status and OC levels as a potential mechanism for explaining iron-induced T2DM.

Material and methods: We conducted three studies on the PREDIMED (PREvención con DIeta MEDiterránea) cohort with data collected in men and women aged 55 to 80 from centres located in Reus-Tarragona, Pamplona and/or Barcelona.

The first was an observational cohort study of 1,073 individuals (131 incident diabetics and 942 non-incidents diabetics) to assess the association between iron intake and T2DM. Several proportional hazard regression models were fitted and adjusted for socio-demographic, anthropometric, lifestyle, and dietary variables.

Second was a cross-sectional study of 423 subjects, 250 normal glucose metabolism (NGM) subjects and 173 impaired fasting glucose (IFG) subjects, to assess the relationship between iron status and serum concentrations of total and uncarboxylated osteocalcin (OC). Several multiple linear regression were fitted.

The third was a prospective nested case-control study of 459 participants (153 incident diabetics and 306 non-incidents diabetics) to evaluate the association between body iron status (measured as serum ferritin (SF), soluble transferrin receptor (sTfR) and the sTfR/ferritin ratio) and the risk of T2DM. A conditional logistic regression model was fitted and adjusted for socio-demographic, anthropometric, lifestyle, dietary and inflammation variables, as well as for MetS components.

The methodology included the diagnosis of T2DM based on American Diabetes Association (ADA) criteria. Dietary intake was determined at baseline using a validated 137-item, semi-quantitative food frequency questionnaire (FFQ). Socio-demographic, anthropometric, lifestyle, iron status, lipid profile, glucose metabolism, and inflammation data were collected at baseline.

Results: In the first study we found that incident diabetics consumed more haem iron (3.9 ± 1.3 vs. 3.7 ± 1.3mg/day, P = 0.036), and less coffee (20.5 ± 30.9 vs. 31.5 ± 47.5cc/day, P = 0.001) than non-incidents diabetics. We also found that elevated haem iron intake (HR = 1.30, 95% CI, 1.02-1.66, P = 0.037), and alcoholic beverage consumption (HR = 1.02, 95% CI, 1.01-1.04, P = 0.005), increased the risk of T2DM but that this risk decreased with high coffee consumption (HR = 0.93, 95% CI, 0.89-0.98, P=0.005).

In the second study we observed that for every 50ng/mL increase in ferritin, total serum OC decreased significantly (by 2.5% [P = 0.004] in the whole sample and by 2.9% [P=0.010] in NGM subjects) but not in IFG subjects (2.2% [P = 0.080]). Otherwise, for every 1ng/mL increase in sTfR, total serum OC decreased by 13.0% (P = 0.038) and UOC decreased by 22.1% (P = 0.029) in the whole sample. In contrast, sTfR/ferritin ratio was not significantly associated with total OC or UOC.

In the third study, we found that incident diabetics had higher SF (127.6 ± 2.5 vs. 105.2 ± 2.5μg/L, P = 0.037), higher fasting insulin (36.5 ± 1.9 vs. 32.0 ± 1.8pmol/L, P = 0.022) and higher glucose levels (mean: 6.1, interquartile range: 5.6-6.8 vs. 5.2, 4.8-5.5mmol/L, P <0.001), and higher HOMA-IR (homeostasis model assessment of insulin resistance) (1.4 ± 1.9 vs. 1.0 ± 1.8, P <0.001) than non-incident diabetics. We also observed that SF values >257 μg/L in males and >139μg/L in females increased the risk of T2DM (OR=3.6, 95% CI, 1.3-9.9, P-trend = 0.014). We also found an association between low sTfR/ferritin ratio levels and the risk of T2DM (OR = 3.0, 95%CI, 1.1-8.4, P-trend = 0.042) but found no association with sTfR (OR = 1.3, 95% CI, 0.5-3.2, P-trend = 0.722).

Conclusions: In an adult Mediterranean population at high cardiovascular risk, high dietary haem iron and body iron stores increase the risk of T2DM after adjustment for potential confounding variables. The relationship between sTfR and the risk of developing T2DM is not evident, while the relationship between iron status and serum OC levels is controversial.

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ABBREVIATIONS

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ABBREVIATIONS

ACLS	Aerobics Center Longitudinal Study
ADA	American Diabetes Association
AI	Adequate Intake
ARIC	Atherosclerosis Risk in Communities
BMP	Bone Morphogenetic Protein
CO	Carbon monoxide
DCYTB	Duodenal CYTochrome B
DPP	Diabetes Prevention Program
DMT1	Divalent Metal Transporter 1
DRIs	Dietary Reference Intakes
EAR	Estimated Average Requirement
EPIC	European Prospective Investigation of Cancer
FFQ	Food Frequency Questionnaire
FLVCR	Feline Leukaemia Virus subgroup C Receptor
FPG	Fasting Plasma Glucose
FPN	Ferroportin
GDM	Gestational Diabetes Mellitus
GLUT	GLUcose Transporter
GWAS	Genome-Wide Association Studies
HAMP	Hepcidin AntiMicrobial Peptide
HCP1	Haem Carrier Protein 1
HH	Hereditary Haemochromatosis

HJV	Hemojuvelin
HO-1	Haem oxygenase-1
HOMA-β	Homeostasis Model Assessment of β-cell
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HPFS	Health Professionals' Follow-up Study
ID	Iron Deficiency
IDA	Iron Deficiency Anaemia
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
IOM	Institute of Medicine
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
IREs	Iron-Responsive Elements
IRPs	Iron-Regulatory Proteins
IWHS	Iowa Women's Health Study
JIN	Jiangsu Nutrition Study
KIHD	Kuopio Ischemic Heart Disease Risk Factor
LIP	Labile Iron Pool
LPI	Labile Plasma Iron
LRP/CD91	Low density lipoprotein Receptor-related Protein/ CD91
MAPK	Mitogen-activated protein kinases
MCV	Mean Corpuscular Volume
MESA	Multi-Ethnic Study of Atherosclerosis
MetS	Metabolic Syndrome
NEFA	Non-Esterified Fatty Acids

NGM	Normal Glucose Metabolism
NHS	Nurse's Health Study
NHSurv	National Health Survey
NTBI	Non-Transferrin Bound Iron
OC	OsteoCalcin
PCFT	Proton-Coupled Folate Transporter
PCFT/HCP1	Proton-Coupled Folate Transporter/Haem Carrier Protein 1
PI3K	PhosphatidylInositol 3-Kinase
PREDIMED	PREvención con DIeta MEDiterránea
RIs	Reference Intakes
ROS	Reactive Oxygen Species
SF	Serum ferritin
SSB	Sugar-Sweetened Beverages
STEAP3	Six Transmembrane Epithelial Antigen of the Prostate
sTfR	Soluble Transferrin Receptor
T2DM	Type 2 Diabetes Mellitus
TIBC	Total Iron-Binding Capacity
TfR	Transferrin Receptor
UL	Tolerable Upper Intake Level
UOC	Uncarboxylated OsteoCalcin
U.S.	United States
UTR	UnTranslated Region
WHO	World Health Organization
WHS	Women's Health Study
ZIP	ZRT/IRT-like Protein

	Biliverdin	
	Bilirubin	
	CD163	
	DCYTB	Duodenal CYTochrome B
	DMT1	Divalent Metal-ion Transporter 1
	Ferric iron	
	Ferrous iron	
	Ferritin	
	FLVCR	Feline Leukaemia Virus subgroup C Receptor
	FPN	Ferroportin
	Haem iron	
	Haptoglobin	
	HO-1	Haem oxygenase-1
	Hephaestin	
	Hepcidin	
	Hb	Haemoglobin
	Hpx	Haemopexin
	Insulin	
	Insulin receptor	
	LRP/CD91	Low density lipoprotein Receptor-related Protein/CD91
	Mb	Myoglobin
	OC	Osteocalcin
	PCFT/HCP1	Proton-Coupled Folate Transporter/Haem Carrier Protein 1
	Tf	Transferrin
	TfR1	Transferrin Receptor 1
	STEAP3	Six Transmembrane Epithelial Antigen of the Prostate

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INTRODUCTION

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1. IRON

Functions

The essential role of iron in living organisms is demonstrated by the fact that this is the fourth most abundant element in the earth's crust, and the most plentiful transition element in organisms. Iron is an essential trace element required by all organisms except for a few species of bacteria (Chua et al., 2007). It is present in many metabolic and cellular processes, including molecular oxygen transport, electron transfer, key metabolic reactions and DNA synthesis (Hentze et al., 2004), making it a subject of much interest, especially because of its role in health and disease.

These physiological and vital iron functions revolve around its ability to exist in two stable oxidation states, ferric (Fe^{+3}) and ferrous (Fe^{+2}) iron. This chemical property of iron underlies in its ability to participate in reduction-oxidation (electron transfer) reactions, and also leads to its potential toxicity. Thereby, iron can participate in the production of oxygen free-radicals, and these in turn can damage numerous biological molecules (e.g., membrane lipids, proteins, DNA). Mammals have thus developed complex regulatory mechanisms that control iron absorption, transport, storage and recycling (Gulec et al., 2014).

Distribution and body iron content

The amount of iron in the body depends on age, gender, nutrition, and general state of health. There are limited iron stores in the new-born and these gradually increase with age until adulthood. Body iron content is approximately 4.0g in men and 3.5g in women; 55mg and44 mg per kilogram of body weight, respectively. In adults, most body iron is present in haemoglobin (60–70%) in circulating erythrocytes where it is essential for oxygen transport, and in muscle myoglobin (10%). The remaining body iron, around 20–30%, is found mainly stored in reticuloendothelial system and the liver as ferritin and haemosiderin (figure 1). Approximately 1% is incorporated in the iron-containing enzymes and less than 0.2% of body iron is in the plasma transport pool. The major

content of ferritin is in the liver, 25% of body iron, two-thirds of this as ferritin and up to one-third as haemosiderin (Geissler and Singh, 2011).

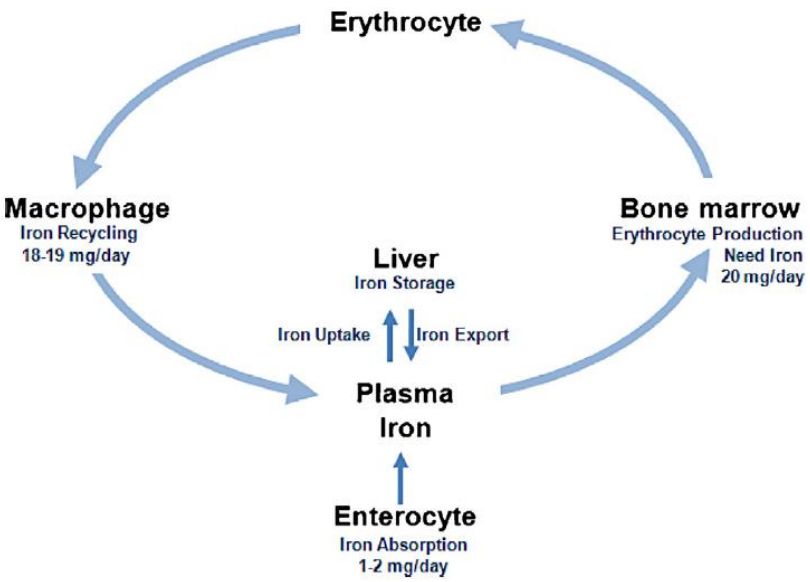
1.1 IRON METABOLISM

1.1.1 Iron digestion and absorption

1.1.1.1 Digestion process of food containing iron

Approximately 20-30mg of iron is required daily for the de novo production of haemoglobin, 90%–95% of which is derived from iron that is recycled from senescent and effete erythrocytes by reticuloendothelial macrophages in the spleen (see figure 1). The normal diet contains 15-20mg of iron, and approximately 1–2mg of iron is absorbed in the proximal duodenum to replace iron losses from bleeding, desquamation of epithelial cells, sweating and urinary excretion (Steinbicker and Muckenthaler, 2013).

Figure 1. Distribution of body iron.



Source: (Yun and Vincelette, 2015)

Iron is found in a large variety of foods (see table 1). It can be found in the diet in two forms: non-haem iron (inorganic iron), which is present in both plant foods and animal tissues, and haem iron (organic iron), which comes only from animal source foods. Meats, fish, and seafood are rich sources of haem iron, the remainder is non-haem. Moreover, iron in dairy foods, eggs, and all plant-based foods are entirely non-haem. It is estimated that the bioavailability of iron from a vegetarian diet is approximately 10 percent, rather than the 18 percent from a mixed Western diet. Hence, the requirement for iron is 1.8 times higher for vegetarians (Otten et al., 2006).

In Western countries, iron derived from haem sources make up two-thirds of the total iron absorbed, despite only constituting one-third of the iron ingested (West and Oates, 2008). In this regard, different research studies have estimated that the bioavailability of haem iron may be 2- to 7-fold higher than non-haem iron (Bjorn Rasmussen et al., 1974) (Hurrell, 1997)(Ekman and Reizenstein, 1993) (Reizenstein, 1979). It is believed that 25% of dietary haem iron intake is absorbed, and only 5–15% of non-haem iron (Rajpathak et al., 2009a).

Non-haem iron digestion

Non-haem iron is found in both animal and plant foods mainly as ferric iron. This iron form is highly insoluble, and produces insoluble non-absorbable complexes in alkaline environment. Thereby, the process of its absorption has to be preceded by the reduction of ferric ion (Fe^{+3}) to ferrous ion (Fe^{+2}) (Przybyszewska and Żekanowska, 2014). Gastric acid, ascorbic acid and other organic acids (Gulec et al., 2014) and sugars such as fructose (Christides and Sharp, 2013) contained in foods promote reduction and solubilisation of dietary ferric iron and thus improve its subsequent absorption. Interestingly, research has shown that the presence of an elevated gastric content increases the bioavailability of dietary iron more than in the sole presence of isolated hydrochloric acid (Sobala et al., 1989).

In addition, the bioavailability of non-haem iron can be impaired by pathological conditions of the alimentary tract, such as Helicobacter pylori infection and

inflammatory diseases. Also, the chronic use of proton pump inhibitors for gastric acid reflux also decrease non-haem iron absorption (Gulec et al., 2014).

Table 1. Iron content in food (mg/100g).

Meats	1-3
Liver	13
Lean meat	1.3
Spanish cured ham	2.3
Fish and seafood	0,4-2,7
Lean fish	1
Oily fish	1.1
Seafood	2.5
Eggs	2.2
Dairy products	0,004-0,9
Natural yoghurt	0.1
Cheese	0.6
Whole milk	0.04
Legumes	5,5-8,2
Lentil	8.2
Chickpea	6.8
Nuts	5-7,9
Pistachio	7.2
Cereals	0,8-2,1
Bread	1.6
Spaghetti	2.1
Vegetables	0,3-3
Tomato	0.7
Potato	0.8
Chard and Spinach	3
Fruits	0,1-0,7
Banana	0.6
Orange	0.5
strawberry	0.7

Source: Mataix et al., 2002

Haem iron digestion

Haem consists of protoporphyrin IX with an iron atom bound in the centre, coordinated to the pyrrole rings. It acts as prosthetic group of haem-proteins such as haemoglobin, myoglobin, cytochromes, catalase, peroxidase, and nitric oxide synthase, and also participates in metabolic processes such as transcription, translation, and cellular differentiation (Furuyama et al., 2007).

Haem iron from diet comes primarily from haemoglobin and myoglobin, thus it is only present in animal sources. The low pH in the stomach and the action of proteolytic enzymes in the stomach and small intestine release haem from these proteins (West and Oates, 2008). The haem released in the stomach is poorly absorbed, because pure haem is poorly soluble at the low gastric pH, but the availability of haem is unaffected by gastric secretion (West and Oates, 2008) (Hooda et al., 2014). The presence of protein increases the solubility of haem iron. Thus, it interaction with peptides produced from proteolytic digestion prevents the formation of insoluble haem polymers, which could reduce its bioavailability. Hence, the amino acids and peptides released from digestion of meat and by-products can enhance subsequent absorption of haem iron (West and Oates, 2008) (Hooda et al., 2014).

1.1.1.2 Intestinal iron absorption

Iron is absorbed actively in epithelial cells of the duodenum and first portion of the jejunum, decreasing in to the distal intestine (Przybyszewska and Żekanowska, 2014). The epithelium of the small intestine is organised into large numbers of self-renewing crypt-villus units. Villi are finger-like protrusions of the gut wall that project into the gut lumen to maximise available absorptive surface area. Crypts are home to a population of proliferating epithelial cells, which fuel the active self-renewal of the epithelium (Clevers, 2013). Thus, the epithelium is composed of stem cells and transit amplifying cells, which differentiate into several types of mature intestinal epithelial cells and move upward along the side of the villi to be ejected when they reach the villi tips (Clevers, 2013). Six differentiated epithelial cell types are distinguished. The most populous cell on the villus is the absorptive enterocyte, a highly polarised columnar cell (Clevers, 2013). This cells

reach absorptive maturity during their migration from duodenal crypts towards the apex of the intestinal villus (Przybyszewska and Żekanowska, 2014). It is precisely in these mature cells where iron is absorbed, although the mechanism of absorption is different for haem and non-haem iron.

Cells of gastrointestinal mucosa synthesise mucins, a glycoprotein that constitutes the main component of mucus. These molecules can bind ferric and ferrous iron and form soluble complexes, which can be directly absorbed from the duodenum (Przybyszewska and Żekanowska, 2014). The presence of alkaline pH in the duodenal content is a prerequisite for the absorption of iron ions by the mucin-integrin system. Acidification of the duodenal content changes the physicochemical properties of mucins, stimulating the formation of aggregates thereby causing the resultant loss of iron ion-binding capacity (Przybyszewska and Żekanowska, 2014).

Iron in duodenum can be found as organic iron (haem), inorganic iron (non-haem), but also as ferroproteins, e.g. lactoferrin or phytoferritin. It has been seen in humans that ferritin from both, animal and plant sources (Davila-Hicks et al., 2004) (Lönnerdal et al., 2006) (Lv et al., 2015), is absorbed, but the exact mechanism is not known yet. It has observed that ferritin, at least partially, survives digestion (Theil et al., 2012), and could be absorbed through a different mechanism from inorganic or organic iron (Theil et al., 2012), possibly by endocytosis (San Martin et al., 2008).

Non-haem iron absorption

The non-haem iron absorption starts in apical membrane of differentiated enterocytes of the mid and upper zones of the villus of duodenal epithelium (Lane et al., 2015). The first step in this process is the reduction of non-haem iron, from ferric iron to ferrous iron, since non-haem iron is rapidly oxidised into its ferric form in the presence of dissolved oxygen. Importantly, there is also evidence that the reduction of non-haem iron in the extracellular environment may involve non-enzymatic ferrireduction driven by endogenous and secreted reductants, such as ascorbate (secreted or supplied in the diet) and/or superoxide (Lane et al., 2015). Congruently, duodenal cytochrome B (DCYTB)

appears to be regulated by iron, hypoxia, erythroid activity and by increased systemic iron requirements (McKie, 2008).

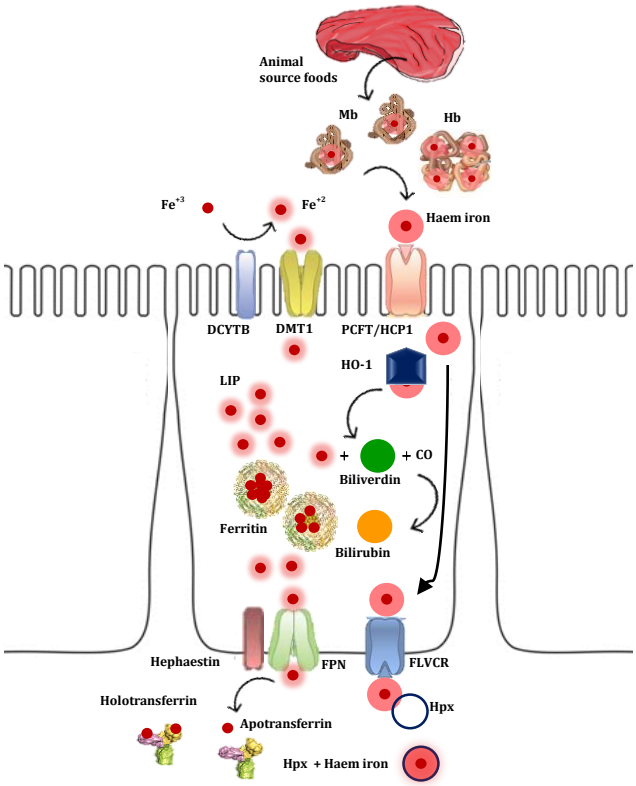
Ferrous iron is then transported across the apical membrane of the enterocyte via divalent metal transporter 1 (DMT1), and possibly other divalent metal transporters, such as ZRT/IRT-like Protein (ZIP) 14 (Liuzzi et al., 2006). DMT1 is a 12-transmembrane domain protein, transports several divalent metals but operates mostly as a cotransporter of protons and ferrous iron (Brasselagnel et al., 2011).

Ferrous iron absorbed can be guided to ferritin protein complex, to different intracellular compartments or to the basolateral membrane by means of an unknown mechanism. In basolateral membrane iron can be exported to the blood circulation by ferroportin (FPN), the only known mammalian iron exporter (see figure 2). This transporter is expressed not only in cells that absorb (enterocytes), but also in cells that store iron (hepatocytes and reticuloendothelial macrophages), consistent with its established roles in iron absorption and recycling (Gulec et al., 2014).

FPN transcription has been shown to be regulated by iron deficiency (ID), hypoxia, transition metals, haem and inflammatory cytokines (Ward and Kaplan, 2012). It is noteworthy that not only non-haem, but also haem iron can induce FPN transcription (Marro et al., 2010). At the posttranslational level FPN expression is regulated via the small hepatic peptide hormone hepcidin that binds FPN to cause its internalization and proteolysis (De Domenico et al., 2007). However, it has seen that hepcidin also induces the proteasomal-mediated degradation of DMT1 by a signalling pathway that remains to be identified. This pathway seems more sensitive to changes in hepcidin concentration than the FPN (Brasselagnel et al., 2011).

Beside FPN, a trans-membrane ferroxidase called hephaestin is located in the basolateral membrane and, in combination with plasma ceruloplasmin, helps to oxidise the exported ferrous iron back to ferric iron (Chen et al., 2004). This iron is then complexed to the major plasma iron transport protein, transferrin, for transport through the circulation and uptake by distal tissues (Lane et al., 2015).

Figure 2. Iron absorption mechanisms in the intestinal enterocyte.



Adapted from (Hooda et al., 2014)

Haem iron absorption

Haem iron from diet comes primarily from haemoglobin and myoglobin, thus it is only present in animal sources. It is well established that haem is an efficient source of iron in the mammalian diet, markedly more effective than non-haem iron. Its major site of absorption in mammals is duodenal epithelium, through a different route from the absorption of non-haem iron. However, the identity of the putative haem transporter expressed by the proximal duodenal was unknown until recently when Shayeghi et al. proposed haem carrier protein 1 (HCP1) as a candidate protein (Shayeghi et al., 2005). HCP1 transports haem, and its analogues such as zinc protoporphyrin, from the gut lumen into intestinal epithelial cells. This protein was initially reported to be a pH-

independent, low-affinity (K_m ~ 125µM), intestinal haem transporter (Shayeghi et al., 2005). Subsequent studies suggested that it was most likely a high-affinity (Km= 1.67µM) proton-coupled folate transporter (PCFT) and not a physiologically relevant haem transporter (Qiu et al., 2006) (Umapathy et al., 2007) (Nakai et al., 2007), and Qiu et al. proposed to amend the name to PCFT/HCP1 (Qiu et al., 2006). Recent evidence have argued that PCFT/HCP1 receptor was involved in haem iron uptake and not just in the transport of folate (Laftah et al., 2009) (Le Blanc et al., 2012). Other different mechanisms have been proposed for haem uptake, such as via pinocytosis (Wyllie and Kaufman, 1982), and via receptor-mediated endocytosis (Gräsbeck et al., 1979), but the exact mechanism still remains elusive. Irrespective of the exact mechanism, it has been seen that haem iron absorption from haemoglobin is saturable, with a daily maximum amount absorbed of 2 mg of iron (Pizarro et al., 2003). It has been suggested that the saturability of haem iron absorption might be a protective factor to avoid iron overload when iron intake is provided primarily by consumption of animal food.

It has been observed that haem iron appears to be transported intact from the gut lumen into enterocytes (Shayeghi et al., 2005). Inside enterocytes, haem iron is mostly catabolised by the microsomal enzyme haem oxygenase-1 (HO-1) in biliverdin, carbon monoxide (CO) and free iron. It is likely that, after its disassembled by HO-1, the liberated iron enters the same storage and transport pathways taken by inorganic iron. Hence, free iron would form part of the labile iron pool (LIP), which is delivered either to ferritin and eliminated from the organism with exfoliated enterocytes, or to the basolateral side to be exported to the blood capillaries trough ferroportin. However, Mendiburo et al. observed that a fraction of haem iron can be transported out of the cells intact (Mendiburo et al., 2011). Supporting this hypothesis, a recent study in a rat model showed a differential tissue utilization of haem and non-haem iron, suggesting that some haem may be exported into the circulation in a form different from that of non-haem iron, i. e., as haem iron intact (Cao et al., 2014).

The haem transport could be performed by a cell-surface membrane protein named Feline Leukaemia Virus subgroup C Receptor (FLVCR) (Quigley et al., 2004). This haem-efflux protein is expressed in different cells and tissues, including the intestine, where it

appears to function as basolateral haem exporter to prevent toxicity within the enterocytes (Quigley et al., 2004).

1.1.2 Iron transport

Non-haem iron transport

Transferrin

Ferrous iron released into the circulation from enterocytes is oxidised to ferric iron by ferroxidases, such as, hephaestin and/or ceruloplasmin. In this process, ceruloplasmin, captures and oxidises two ferric ions. Following, ceruloplasmin binds to transferrin forming the ceruloplasmin–transferrin adduct. With transferrin and in the formed ceruloplasmin–transferrin adduct, two ferrous ions are transferred from ceruloplasmin to two C-lobes of two transferrins (Eid et al., 2014).

Transferrin is the major extracellular iron binding protein, and the principal source of iron for tissues. It is a single chain bilobal protein with a molecular weight of approximately 80 kDa (Mizutani et al., 2012). Each lobe (termed N- and C-lobes) also has a single iron-binding site with an extremely high affinity for ferric iron (Kd = 10^{-23}M) (Aisen et al., 1978). Despite this, the binding is reversible. The binding of ferric iron to transferrin requires carbonate as a synergistic anion (Harris, 2012) and causes the protein to undergo a conformation change from the "open" apotransferrin form to the "closed" holotransferrin form in which the ferric ions are buried deep within the each lobe (Mizutani et al., 2012). This acts to keep the iron in a soluble but redox inactive state so that it can be safely transported around the body.

Transferrin is produced predominantly by the liver and is one of the most abundant proteins in the plasma, being present at 2–4mg/mL in humans (Anderson and Vulpe, 2009). Under normal conditions, essentially all of the iron found in the circulation is bound to this protein, although it is typically only 30% saturated with iron (El Hage Chahine et al., 2012). This is possible because transferrin exists as four molecular forms: apotransferrin, monoferric A transferrin, monoferric B transferrin, and diferric

transferrin; but all transferrin bound iron can be considered physiologically as a single homogeneous pool (Brissot et al., 2012). The additional iron binding capacity is thought to provide a buffer in the event of a sudden influx of iron into the circulation due to the iron absorbed from the diet, the iron recycled and released by reticuloendothelial macrophages, and the iron utilised by the bone marrow and other tissues. Despite this, the amount of iron in the circulation is maintained at a relatively constant level. Whether, circulating iron levels exceed the binding capacity of transferrin, highly toxic non-transferrin bound iron (NTBI) can be formed.

Non-transferrin bound iron

Iron in the circulation exists in complex with three major proteins, transferrin, haem, and ferritin. Besides these plasma iron forms, another iron species, termed NTBI was originally identified by Hershko et al. (Hershko et al., 1978). NTBI is a heterogeneous group of potentially toxic iron complexes in plasma. The main form of NTBI could be ferric citrate, but albumin, which is present in high concentration in the plasma (close to 40g/L), contains many negatively charged sites on its surface suitable for ferric iron binding (Brissot et al., 2012). Another special form of NTBI in plasma has been identified and it is defined by its ability to engage in redox cycling. This form has been termed labile plasma iron (LPI) (Cabantchik et al., 2005). Thereby, the exact nature of plasma NTBI remains to be determined. It is likely that different forms of plasma NTBI coexist and vary according to the degree of iron excess, to its duration, and to the aetiology.

It is thought that NTBI play a major role in various pathological conditions that are dominated by iron overload. In humans, values of transferrin saturation >45% are consistent with iron overload (Hentze et al., 2010), which may even exceed 60%. Under these conditions, the levels of NTBI increase dramatically (up to 10–15 μM or higher) (Gkouvatsos et al., 2012). The liver is the most prominent site of NTBI deposition, although considerable amounts of NTBI accumulate in further tissues, such as pancreas and heart, leading to organ damage (Gkouvatsos et al., 2012). Cellular uptake of NTBI is mediated by several organ-specific transporters and receptors. NTBI-induced toxicity is the result of oxidative damage to various macromolecules by ROS (Brissot et al., 2012).

Introduction

Haem iron transport

Haemopexin

Haem iron released into the portal circulation from enterocytes is likely caught by haemopexin in an equimolar way. This is a 57-kDa acute phase plasma glycoprotein produced by the liver with an extraordinarily high haem binding affinity (Kd 10^{-9} M). In plasma, haemopexin targets haem to the liver parenchymal cells for haem catabolism, iron storage, and re-distribution (Tolosano and Altruda, 2002), to the spleen (Cao et al., 2014) and macrophages via the low density lipoprotein receptor-related protein/CD91 (LRP/CD91) (Hvidberg et al., 2005). Its function is essential in iron homeostasis by recycling haem iron and against the deleterious effects of haem too. Thereby, free haem excess can cause cell damage and tissue injury since haem catalyses the formation of reactive oxygen species (ROS), resulting in oxidative stress (Chiabrando et al., 2014), and may also drive or amplify tissue inflammation (Lin et al., 2015). Both conditions might be suppressed by haemopexin, either through direct sequestering of free haem, or possibly indirectly through activation of HO-1 (Lin et al., 2015), since it is known that haemopexin mediates haem uptake and its degradation, through HO-1, to CO, bilirubin and iron (Sung et al., 2000).

Albumin and other transporters

In addition to haemopexin, haem can be attached with especially strong affinity to the most abundant protein in the blood serum (60% of the total protein content), albumin, allowing to store the haem in excess in blood serum (Guizado et al., 2012). However, during the first seconds after haem appearance in plasma, more than 80% of this powerful oxidiser binds to HDL and LDL, and only the remaining 20% binds to serum albumin and haemopexin. Serum albumin and haemopexin then slowly remove most of the haem from HDL and LDL (Ascenzi et al., 2005). Recently, a new haem binding protein with an affinity to haem comparable to that of albumin was discovered, α1- proteinase inhibitor (Karnaukhova et al., 2012).

Further, under pathological conditions, α1-microglobulin, a member of the superfamily of lipocalins, may cooperate to haem scavenging in extracellular fluid by binding and degrading haem (Ascenzi et al., 2005).

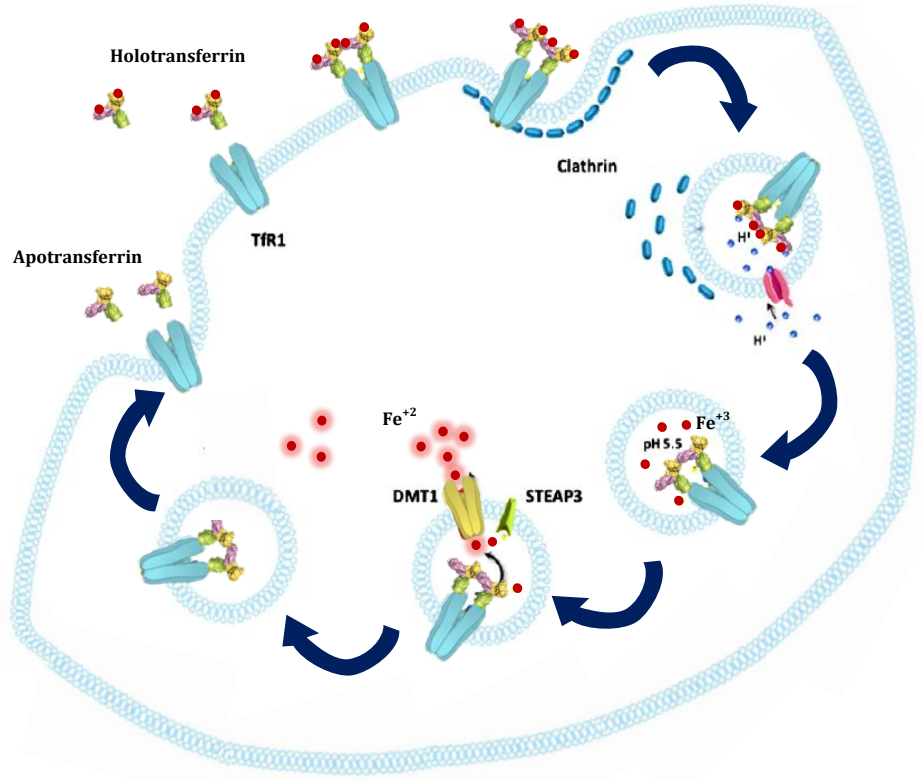
1.1.3 Cellular uptake of iron

Non-haem iron uptake

Transferrin-dependent pathway

Under physiological circumstances, the major iron uptake route utilised by most cells involves transferrin-bound iron and transferrin receptor (TfR) 1 (Hentze et al., 2010). Cells take up transferrin in proportion to the number of TfR1 located at the cell surface. This transmembrane glycoprotein forms a disulfide-bonded homodimer, which can bind one transferrin molecule at each of its subunits (Aisen, 2004). Interestingly, the iron status of transferrin impinges on its affinity for TfR1. Thereby, diferric transferrin binds with 30- and 500-fold higher affinity to TfR1, than monoferric and apotransferrin, respectively (Young et al., 1984). The holotransferrin/TfR1 complex is internalised by receptor-mediated endocytosis via clathrin-coated pits. Acidification of the endosome by a proton pump ATPase to pH 5.5 triggers a conformational change in transferrin resulting in the release of ferric iron (Klausner et al., 1983), while transferrin remains bound to TfR1. Before, ferric iron is reduced to ferrous iron by an endosomal ferrireductase, six transmembrane epithelial antigen of the prostate 3 (STEAP3) (Hentze et al., 2010), or by an novel mechanism involving cellular ascorbate (Lane and Richardson, 2014). Iron is then transported across the endosomal membrane to the cellular cytoplasm by DMT1 (Gunshin et al., 1997) or ZIP14 (Jenkitkasemwong et al., 2012). This nascent cytosolic iron then becomes part of a poorly characterised chelatable or LIP and can be utilised for metabolism, stored in ferritin or released back to the extracellular space (Hentze et al., 2010). Finally, the apotransferrin/TfR1 complex returns to the cell membrane, where apotransferrin is recycled back to the bloodstream, available to recapture iron (see figure 3).

Figure 3. Cellular uptake of transferrin-bound iron.



Adapted from (Gkouvatsos et al., 2012)

Non-transferrin-dependent pathway

In iron overload states, such as hereditary haemochromatosis (HH), NTBI is thought to be a main contributor to tissue iron loading, but the molecular mechanisms remain poorly defined (Brissot et al., 2012). NTBI is accumulated in the circulation and transported to tissue parenchymal cells by transferrin-independent mechanisms (Brissot et al., 2012). Interestingly, as opposed to transferrin-dependent pathway, hepatocyte uptake of NTBI does not appear to be inhibited by increasing levels of liver iron (Baker et al., 1998).

DMT1 was initially proposed to operate as a major NTBI transporter in the liver, which is the principal site of NTBI clearance (Trinder et al., 2000). A study conducted on human

hepatoma cells suggest a role for DMT1 in NTBI uptake (Shindo et al., 2006). Recently, it was proposed that ZIP14 transporter, also known as SLC39A14, has an important role in liver NTBI uptake based on that ZIP14 is most amply expressed in the liver, and that ZIP14 optimal pH is 7.5 (Liuzzi et al., 2006). In addition, a recent study found that in iron-loaded rat liver, ZIP14 levels were up-regulated whereas DMT1 levels were markedly down-regulated, suggesting that ZIP14 plays more of a role than DMT1 in iron uptake (Nam et al., 2013). Interestingly, apart from its NTBI transporting activity, ZIP14 appears to also facilitate cellular assimilation of transferrin-bound iron in cells (Zhao et al., 2010).

This iron uptake process may include cell surface reduction by ferrireductases such as DCYTB (McKie, 2008), or by effluxed reductants, such as ascorbate (Lane and Richardson, 2014). These enzymes reduce ferric NTBI to its ferrous state, which can be imported from the plasma membrane.

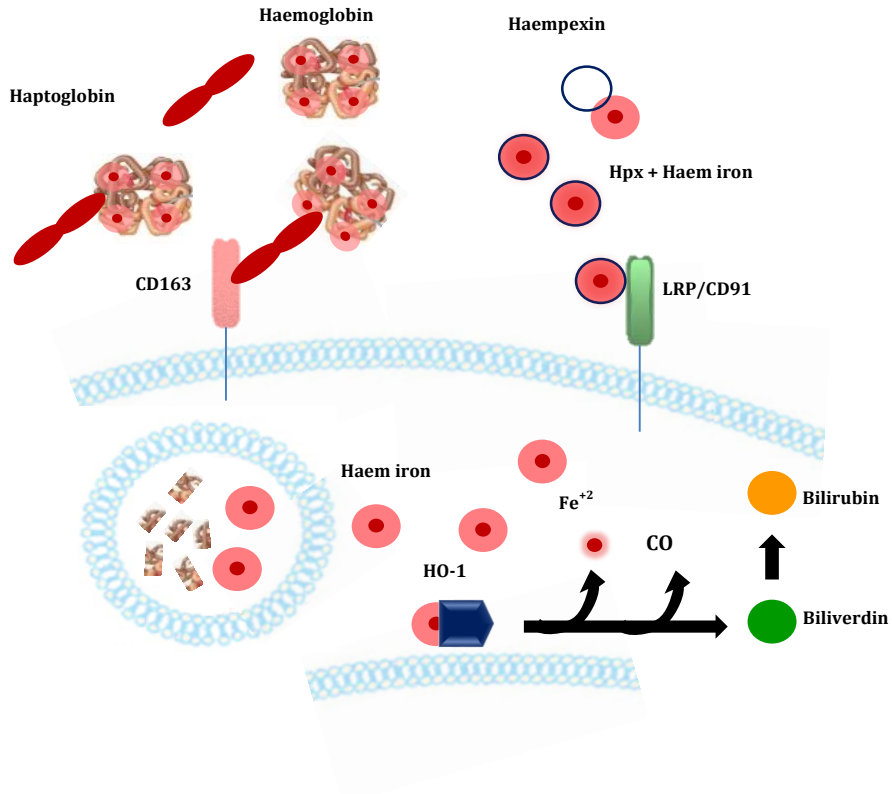
Haem iron uptake

Haemopexin pathway

Free haem iron released into the circulation is caught by haemopexin, which targets haem to the liver parenchymal cells for haem catabolism, iron storage, and re-distribution, and also to the spleen (Cao et al., 2014), and macrophages (Hvidberg et al., 2005). Consistently, it has been found that the receptor responsible for capturing the haemopexin-haem complex, LRP/CD91, is expressed in a wide spectrum of cell types, including hepatocytes, macrophages, adipocytes, neurons and syncytiotrophoblasts (Moestrup et al., 1992). LRP/CD91 leads haemopexin-haem complexes to endocytosis and degradation of the haem iron (see figure 4), mainly in liver (Hvidberg et al., 2005). In this regard, it was shown in rats that more than 90% of intravenous-injected haem-haemopexin was recovered in the liver within two hours (Potter et al., 1993)

Uptake of haemopexin-haem complexes stimulates HO-1 transcription (Hvidberg et al., 2005) in order to degrade haem in biliverdin, CO and free iron. Likely, the liberated iron from the endoplasmic reticulum enzyme HO-1 could be stored inside ferritin molecules.

Figure 4. Pathways for uptake of haemopexin-haem and haptoglobin-haemoglobin complexes.



Adapted from (Hvidberg et al., 2005)

1.1.4 Iron stores

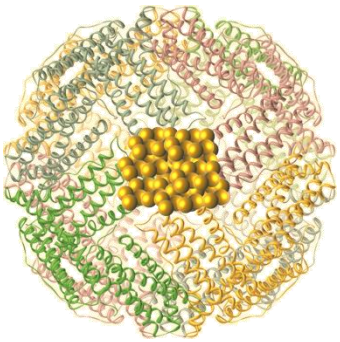
The human body stores iron in the form of ferritin and haemosiderin in liver, spleen, marrow, duodenum, skeletal muscle and other anatomic areas.

Ferritin

Cellular iron in excess is stored as an iron oxide within ferritin core. This protein represents the most common and ancient molecule of iron homeostasis developed by organisms in the three domains of life (Archaea, Bacteria and Eukaryote). Its primary role is in iron sequestration in which it functions as a ferroxidase, converting $Fe^{(+2)}$ to $Fe^{(+3)}$. Thus, iron is internalised and sequestered in the ferritin mineral core, while a controlled release of the metal guarantees its availability for critical cellular processes

while protecting lipids, DNA, and proteins from the potentially toxic effects of iron. Thereby, storage and detoxification of iron represent the main functions of these molecules (Finazzi and Arosio, 2014).

Figure 5. Ferritin structure.



Apoferritin, the iron-free form of the protein, forms a roughly spherical container of about 450 kd. within which ferric iron is stored as a ferrihydrite mineral in the holoferritin form. How iron is delivered to ferritin remains elusive, however, experimental evidence suggests the involvement of the cytosolic iron chaperone Poly(rC)-binding protein 1 (Shi et al., 2008). Channels in the ferritin shell may facilitate its entry and exit. The apoferritin shell in mammals is composed of 24 subunits with two functionally and genetically distinct ferritin types: light-ferritin (L-ferritin) and heavy-ferritin (H-ferritin) (Knovich et al., 2009). In humans, their molecular masses are 19 and 21 kDa, respectively. The heavy subunit is primarily responsible for the ferroxidase activity of the ferritin complex, whereas the light subunit facilitates the storage of iron into the ferritin core. The ratio of these subunits varies widely depending on tissue type, and can be modified by inflammatory and infectious conditions. Tissue ferritins vary from H-subunit rich (found mostly in the heart and kidney) to L-subunit rich (found predominantly in liver and spleen). The efficient storage of iron, up to 4500 iron atoms per ferritin, requires the cooperativity of both ferritin subunits (Alkhateeb and Connor, 2010).

Ferritin is also present extracellularly within the serum, where it serves as an important clinical marker of iron status. Despite its regular use in clinical medicine, the precise

source of serum ferritin (SF) has yet to be determined. It has been suggested that ferritin secretion may provide a mechanism to limit iron storage after a shift from high to low iron, and prior to activation of IRPs (Arosio et al., 2009).

Haemosiderin

Another form of stored iron in the cell is haemosiderin, insoluble degradation product of incomplete lysosomal breakdown of ferritin. In iron overloading conditions haemosiderin becomes the predominant iron storage protein. Under physiological conditions, haemosiderin is not an effective iron donor but plays a protective role. Subject to conditions such as inflammation and hypoxia it could become an iron donor promoting free radical production and tissue damage in iron overloaded cells (Tandara and Salamunic, 2012).

Prolonged iron overload causes an accumulation of haemosiderin in the tissues, clinically manifested as haemosiderosis, and its severe form with damage of tissues haemochromatosis.

1.1.5 Excretion and physiological losses

Unlike most essential nutrients, no active excretory mechanisms exist for iron in humans. However, small amounts, around 1-2mg per day, are lost in healthy individuals via exfoliation of skin and gastrointestinal cells (1mg/day) and sweat (0.2-0.3mg/day), in urine (<0.1mg/day), hair, and bile. The losses are slightly more elevated in women of child-bearing age because of additional losses due to menstruation, pregnancy, and lactation (Geissler and Singh, 2011) (Gulec et al., 2014). Therefore, iron recycling, from senescent red blood cells by the reticuloendothelial cells in the spleen and the Kupffer cells in the liver, accounts for most of the iron homeostasis in human (Waldvogel-Abramowski et al., 2014).

Most iron retained in endothelial ferritin is likely lost via subsequent exfoliation of enterocytes into the gut lumen. Interestingly, a recent study described a protective role for ferritin H in the regulation of intestinal iron absorption during conditions of iron

overload. These authors showed that intestine-specific ferritin H deletion led to a twofold increase in iron absorption in iron-loaded mice, suggesting that ferritin H works to limit iron flux (Vanoaica et al., 2010).

1.1.6 Regulatory proteins of iron metabolism

A revolution occurred during the past decade in the comprehension of the physiology as well as in the pathophysiology of iron metabolism. Many proteins have been identified playing roles in iron homeostasis. Some of the most important are the following:

Hepcidin

Hepcidin (HEPatic bacteriCIDal proteIN) is a liver-derived 25 amino acid peptide identified as the systemic iron-regulatory hormone in 2000 (Krause et al., 2000) (Park et al., 2001). Chromosome 19 contains the hepcidin antimicrobial peptide (HAMP) gene, which codes for hepcidin. It codes for the 84 amino acid precursor, preprohepcidin, which subsequently undergoes two enzymatic cleavages in the hepatocyte cytoplasm and in the blood, to liberate the 25 amino acids containing the biologically active form (hepcidin25) (Kali et al., 2015). Its expression is induced when body iron stores are elevated, and during infection or inflammation, with the net result being lower serum iron levels. On the contrary, it is also known that ID, hypoxia and the erythropoietic demand, reduces hepcidin levels in order to increase iron availability for the production of red blood cells (Evstatiev and Gasche, 2012).

The role of hepcidin in iron homeostasis is to control the level of iron efflux from three key cell-types into the circulation: a) iron efflux into the portal circulation from duodenal enterocytes in dietary iron absorption; b) the release of recycled iron from macrophages; and c) iron release from hepatocytes (Lane et al., 2015). Hepcidin reduces the non-haem iron absorption from the intestinal enterocytes by binding to the only known cellular non-haem iron exporter, FPN (De Domenico et al., 2007), and possibly by inducing the proteasomal-mediated degradation of the brush-border membrane receptor, DMT1 (Brasselagnel et al., 2011). In addition, Cao et al. observed that hepcidin expression has also an impact in the variation of haem absorption (59%), although had a greater relative

impact on non-haem iron absorption (63%) (Cao et al., 2014). The hepcidin-ferroportin interaction also explains the regulation of reticuloendothelial recycling of iron, by decreasing iron efflux into the plasma following the phagocytic turnover of effete erythrocytes, and the iron release from hepatocytes (Lane et al., 2015).

Hepcidin expression is regulated by a cohort of proteins, including the HH protein called HFE, TfR2, and hemojuvelin (HJV), that can sense the circulating levels of iron and relay these messages through signal transduction pathways to the nucleus to regulate hepcidin transcription. Disruption of any one of these proteins results in inappropriate regulation of hepcidin expression and consequently causes either iron overload or ID (Zhang, 2010). In recent years, the bone morphogenetic protein-transcription factors SMAD (BMP–SMAD) pathway has been demonstrated as the predominant pathway responsible for regulating HAMP in response to iron (Rishi et al., 2015). It has been seen that in hepatocytes, BMP6 levels correlate with iron stores (Corradini et al., 2011), but a more complicated regulation of BMP6 was suggested (Enns et al., 2013) (Feng et al., 2012). In fact, the 'iron sensing' role of the liver could be a function of multiple cell types, especially the liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSCs) but also hepatocytes, which may sense the iron levels and respond by releasing BMP6 (Enns et al., 2013). This initiates a cascade of events in hepatocytes leading to the production of hepcidin. This intercellular crosstalk seems to be one of the mechanisms mediating BMP6 upregulation in response to iron, but the actual molecular pathways underlying the production of BMP6 in response to either serum iron or liver iron stores still remain to be identified (Rishi et al., 2015).

HFE

The HFE gene was discovered in 1996 and belongs to the major histocompatibility complex 1 family (Feder et al., 1996). The most common form of HH, type I or also called classical haemochromatosis, arises from mutations in HFE. This gene encodes the HFE protein, which is believed could interact with TfR2 in order to sense the iron levels.

Early studies linked the HFE protein to iron metabolism by identifying an interaction with TFR1 (Feder et al., 1998). With increasing levels of holotransferrin, HFE is displaced

from the HFE–TFR1 complex, a step thought to be important for initiating a signalling cascade that results hepcidin transcription (Schmidt et al., 2008). According to this model, HFE association with TfR1 is inversely proportional to transferrin-iron saturation. If serum holotransferrin levels increase, HFE is displaced from TfR1 to allow its interaction with TfR2, linkage stabilised by holotransferrin binding. The holotransferrin/HFE/TfR2 complex then stimulates hepcidin expression through an incompletely understood pathway, which requires functional HJV (D'Alessio et al., 2012). It has been proposed that the complex potentiates BMP and/or Mitogen-activated protein kinases (MAPK) pathway signalling (Gao et al., 2009) (Ramey et al., 2009) but further work is needed to delineate the molecular interactions involved in this process (Ganz and Nemeth, 2012).

Transferrin receptor 2

TfR2 is a type II transmembrane glycoprotein, member of the TfR family and homologous to TFR1. Its expression is restricted to the hepatocytes and erythroid precursors. The stabilization of TfR2 by holotransferrin and its ability to bind HFE led to the current model in which liver TfR2, in conjunction with HFE, represents a sensor of circulating iron and activates hepcidin in response to elevated transferrin saturation (Silvestri et al., 2014).

In humans inactivating mutations of TfR2 lead to haemochromatosis type 3, a rare recessive disorder characterised by iron overload and low hepcidin levels (Nemeth et al., 2005).

Hemojuvelin

Hemojuvelin, encoded by the HJV gene and also called HFE2, is a membrane protein responsible for the iron overload condition known as juvenile haemochromatosis, a severe form of hereditary disease (Silvestri et al., 2007). One insight into the pathogenesis of juvenile haemochromatosis is that patients have low to undetectable urinary hepcidin levels, suggesting that HJV is a positive regulator of hepcidin (Dunn et al., 2007).

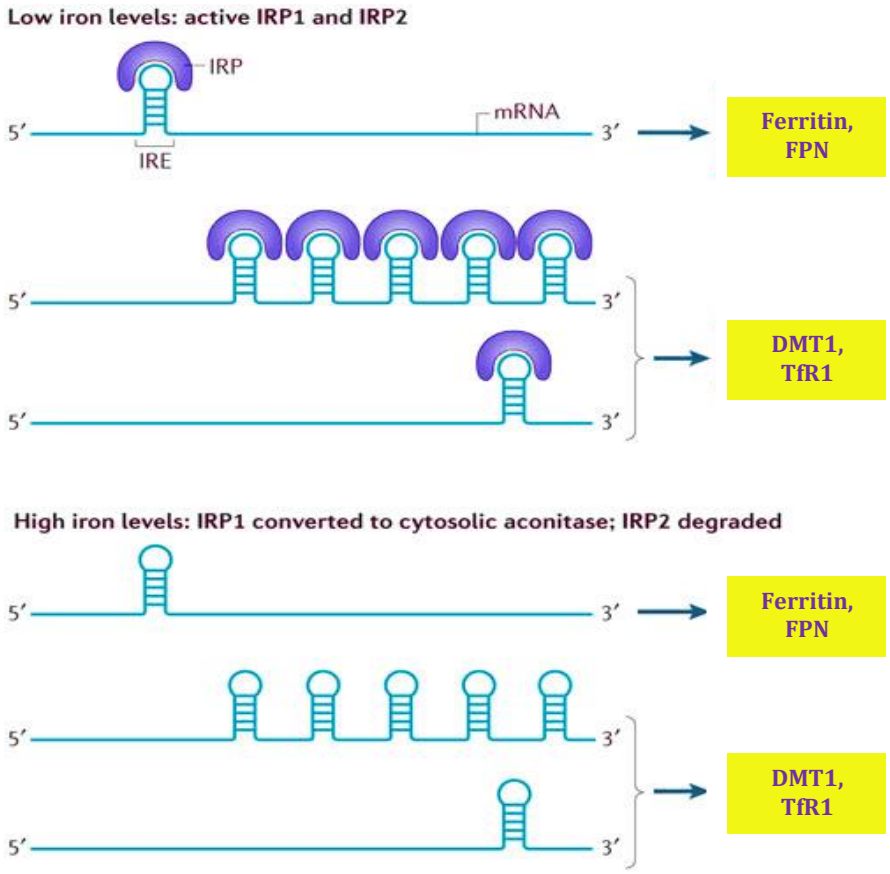
It has been reported that HFE, HJV, TfR2 and HAMP genes are closely related, but the precise underlying mechanisms were not revealed (Steele et al., 2005). A recent study found a direct biochemical evidence that HJV, TfR2 and HFE form a protein complex, and that HJV competes for HFE/TfR1 interaction with similar efficiency as TfR2. These data also directly implicate the BMP co-receptor, HJV, and possibly HJV/BMP/SMAD signalling in HFE/TfR2-dependent hepcidin regulation (D'Alessio et al., 2012).

Proteins involved in the intracellular iron balance

Cellular iron metabolism is controlled by iron regulatory proteins (IRP) 1 and IRP2 (Anderson et al., 2012). In iron-deficient cells, both IRPs interact with iron-responsive elements (IREs), a stem-loop structure, in the 5'-untranslated region (UTR) of the ferritin H- and L-chain, as well as FPN, transcripts in order to inhibit their translation. In contrast to ferritin and FPN, IRPs binding to the IRE in the 3'-UTR region of the TfR1 and DMT1 mRNAs prevent their degradation, and, thereby, cells increase the iron uptake. In cells with elevated iron levels, the translation of ferritin and FPN increase, while TfR1 and DMT1 translation is reduced (see figure 6), in order to decrease free iron levels within the cell (Anderson et al., 2012).

The humoral regulation by hepcidin and cell-autonomous local control by the IRP/IRE systems intersect at the level of enterocytes, and both must function properly for adequate regulation of key iron absorption molecules in the duodenum (Anderson et al., 2012).

Figure 6. The role of iron regulatory proteins in iron homeostasis.



Adapted from (Torti and Torti, 2013)

One obvious point of intersection between the cellular IRP/IRE system and the systemic regulator hepcidin is FPN. The presence of an IRE in the 5'-UTR of the mRNA encoding the hepcidin target, FPN, would predict FPN translation to be inhibited when enterocytes are iron deprived, which seems inconsistent with the observed augmentation of duodenal FPN levels in ID (McKie et al., 2000). This apparent contradiction has been explained by the expression of intestinal FPN mRNA isoforms that lack the 5'-UTR IRE and thus evade translational repression by the IRPs (Zhang et al., 2009). In contrast to FPN, IRPs would exert a positive effect on DMT1 mRNA expression via its 3'-UTR IRE. Inhibition of FPN-mediated iron export by hepcidin would increase iron levels within

enterocytes, reducing IRP activity, and thus decrease DMT1 mRNA levels, and thereby, intestinal iron absorption. However, a study in mice with intestinal IRP deficiency found that they retain their ability to upregulate DMT1 upon stimulation of erythroid iron requirements (Anderson et al., 2012). Further research is warranted in order to elucidate this topic.

Moreover, HIF2α expression in enterocytes mediates the transcriptional upregulation of DMT1 in the duodenum of mice fed an iron-deficient diet (Taylor et al., 2011). How exactly changes in body iron needs are conveyed to the HIF2α pathway in intestinal epithelial cells remains incompletely understood. Additional research is also required in order to clarify this issue.

1.1.7 Dietary factors

The composition of a diet has important effects on iron bioavailability, particularly with regard to non-haem iron. A recent systematic review and meta-analysis showed a large variation in non-haem iron absorption (0.7-22.9%) according to the iron status, but also according to the dietary content of enhancers and inhibitors of iron absorption (Collings et al., 2013).

It is well known that haem-iron absorption is relatively unaffected by other dietary factors that are common inhibitors of non-haem iron and other mineral absorption, such as phytates. However, recent evidence suggests that certain dietary factors may play an important role on haem-iron bioavailability.

1.1.7.1 Enhancers of iron absorption

Ascorbic acid/ascorbate/vitamin C

Accumulating evidence strongly suggests an ability of dietary ascorbate to enhance non-haem iron absorption in the gut in a dose-dependent manner (Cook and Reddy, 2001). The enhancing effect is largely due to its ability to reduce ferric to ferrous iron, but is also due to the possibility of forming a soluble complex with ferric ion (López and Martos,

2004). Ascorbic acid will overcome the negative effects on iron absorption of all inhibitors, which include phytate (Hallberg et al., 1989), polyphenols (Siegenberg et al., 1991) (Kim et al., 2011b), and dairy calcium and proteins (Stekel et al., 1986). Cooking, industrial processing, and storage degrade ascorbic acid and remove its enhancing effect on iron absorption (Teucher et al., 2004).

In addition to the known ability of dietary ascorbic acid to enhance non-haem iron absorption in the gut, ascorbic acid can also modulate cellular iron metabolism; iron transport, by increasing transferrin-dependent and non-transferrin-dependent iron uptake; and iron stores, stimulating ferritin synthesis, inhibiting lysosomal ferritin degradation, and decreasing cellular iron efflux (Lane and Richardson, 2014).

Besides of non-haem iron, a recent experimental study in Caco-2 cells found that ascorbic acid markedly enhanced haem iron transport across the cell monolayer without altering the apical uptake of haem, indicating that ascorbic acid may affect cellular or basolateral events that increase haem-derived free iron export (Ma et al., 2011). This study also showed that the addition of 100μmol/L ascorbic acid completely reversed the inhibitory action of dietary polyphenols on haem iron absorption when the polyphenolic compounds were added at 0.46mg/L concentrations. When the concentrations of polyphenols were increased above 4.6mg/L, ascorbic acid was not able to counteract the inhibitory action of polyphenols on haem iron absorption, although the inhibition was reduced. However, ascorbic acid failed to have any positive effect on haem iron absorption when the polyphenols were added at a high (but still within physiological) level of 46mg/L. These results imply that, while the inhibitory effect of low concentrations of bioactive polyphenols on haem iron absorption can be easily counteracted by ascorbic acid, the inhibitory action of high concentrations of polyphenolic compounds cannot be offset by regular consumption of dietary ascorbic acid (Ma et al., 2011). Further research is required to confirm these observations.

“Meat factor”

The enhancing effect of meat and fish on non-haem iron absorption is well documented. For instance, in a 5 days study an intake of 60g of pork meat resulted in an increased

fractional non-haem iron absorption. Total iron absorption was significantly increased from meat diets compared to the non-meat diets, resulting in a 50–70% increase in total iron absorption (Bach Kristensen et al., 2005).

However, the identification of the meat component, ''meat factor'', involved in this effect remains elusive. Several candidates have been proposed such as some amino acids (Martinez-Torres and Layrisse, 1970), peptides (Hurrell et al., 2006), the L-a-glycerophosphocholine (Armah et al., 2008), and some glycosaminoglycans (Huh et al., 2004) (Laparra et al., 2009).

Alcohol

Although alcohol consumption does not directly affect iron absorption, it has been shown its indirect action. Consumption of up to two alcoholic drinks/day or more was associated with an increase in the risk of iron overload (Ioannou et al., 2004). In this sense, low amounts of alcohol consumption increased serum iron and ferritin levels and, therefore, body iron stores (Whitfield et al., 2001). It seems that the acute or chronic alcohol exposure suppresses the expression of hepcidin in the liver, leading to a higher iron absorption (Bridle et al., 2006) (Harrison-Findik et al., 2006) (Ohtake et al., 2007) (Flanagan et al., 2007) (Harrison-Findik, 2007) (Harrison-Findik et al., 2007) (Harrison-Findik, 2009). Furthermore, it has been found that the intestinal iron absorption increases two-fold in chronic alcoholics, possibly secondary to changes in the intestinal permeability (Duane et al., 1992).

Other enhancers of iron absorption

Organic acids

Although less well studied than ascorbic acid, several other organic acids, such as citric, malic and tartaric acids, appear to have comparable enhancing effects on non-haem iron absorption (Lynch, 1997).

Vitamin A/β-carotenes

The influence of vitamin A on non-haem iron absorption remains largely unknown. While some earlier reports found that vitamin A improved iron absorption (García-Casal et al., 1998), subsequent studies did not confirm this findings (García-Casal et al., 2000) (Walczyk et al., 2003) (Chen et al., 2014).

Regarding β-carotenes, some reports observed an increased non-haem iron absorption in presence of β-carotene (García-Casal et al., 1998) (García-Casal et al., 2000) (García-Casal and Leets, 2014), by a mechanism not yet elucidated.

Sugars

It has been suggested that sugars, such as fructose, could increase iron bioavailability in human (Christides and Sharp, 2013). These results are consistent with a previous works conducted in rodent animal models in which iron-fructose solutions increased gut iron absorption (Pollack et al., 1964). Further studies are warranted to examine these issues.

1.1.7.2 Inhibitors of iron absorption

Phytate/phytic acid

Phytate, myo-inositol hexakisphosphate, is a component of cereals that inhibits iron absorption (Andrews et al., 2014). The negative effect of phytate on iron absorption has been shown to be dose dependent and starts at very low concentrations of 2–10mg/meal (Hallberg et al., 1989). Heat treatment, milling, soaking, germination, and fermentation, can remove or degrade phytate and thereby improve iron absorption (Hurrell, 2004).

Fibre

Traditionally a negative effect on mineral absorption has been attributed to fibre due to its capacity to bind cations. In this sense, an in vitro study has demonstrated that soluble fibre, such as pectin, carrageenan and xanthan gum, presented a great response for

binding cations (Debon and Tester, 2001). However, other experiments with pectin and guar gum in human and animal models (Torre et al., 1991) have concluded that soluble dietary fibre does not have a significant effect on mineral bioavailability. These discrepancies in the role of fibre on mineral availability have been also reported by recent papers. Thus, an experimental study showed that soluble fibre pectin did not cause a reduction in intestinal iron absorption (Feltrin et al., 2009). However, a subsequent published study found a slight decrease in iron uptake (Andrews et al., 2014).

Otherwise, fructo-oligosaccharides, a component of the dietary fibre fraction found in trace amounts as natural components in fruits, vegetables, barley, garlic, honey, onion, and chicory, effectively enhances the mineral absorption rate, such as iron, and counteracts the deleterious effects of phytic acid (Wang et al., 2010b).

Calcium

Dietary calcium is known to reduce the bioavailability of non-haem iron. Many studies observed a reduction of 50-60% on iron absorption at doses of 300-600mg calcium (Hallberg et al., 1991) (Benkhedda et al., 2010). This effect seems to have a direct dose-dependent relationship (Hallberg et al., 1991), although the molecular basis of this interaction is not completely understood. Calcium is a low-affinity non-competitive inhibitor of DMT1, explaining in part the effect of high dietary calcium on non-haem iron bioavailability (Shawki and Mackenzie, 2010). In addition, it was seen that calcium reduces iron bioavailability by decreasing DMT1 expression at the apical cell membrane, thereby downregulating non-haem iron transport into the cell (Thompson et al., 2010).

Other divalent minerals, such as zinc (Olivares et al., 2007a) (Olivares et al., 2012) and magnesium (Wallace et al., 1998), may decrease iron absorption through the DMT1. However, this data was not confirmed in subsequent studies (Snyder and Clark, 1999) (Olivares et al., 2007b).

Calcium also has been shown to have negative effects on haem iron absorption, in addition to non-haem (Hallberg et al., 1991) (Hallberg et al., 1993). The same amount of calcium, around 165 g, also significantly reduced about 50-60% haem iron absorption.

The observed marked inhibitory effect on iron absorption of calcium in amounts frequently encountered in normal meals has important nutritional implications (Hallberg et al., 1991).

Polyphenols

Other dose-dependent common inhibitors of non-haem iron absorption are polyphenols (Morock et al., 1983) (Kim et al., 2011b). Epidemiological studies showed that polyphenolic-containing beverages such as black tea, coffee and cocoa, are potent inhibitors of non-haem iron absorption (Hurrell et al., 1999). Hurrell et al. report that the polyphenolic content typical in a cup of instant coffee reduces iron absorption from a test meal by 60% to 90% (Hurrell et al., 1999). Among elderly participants in the Framingham Heart Study, each 236mL/wk (1 cup per week) of coffee consumed was associated with 1% lower SF concentrations (Fleming et al., 1998).

It was observed that polyphenols, such as chlorogenic acid (Hurrell et al., 1999), the main phenolic compound in coffee; tannic acid (Jaramillo et al., 2015), present also in coffee, tea, cocoa and wine; quercetin (Lesjak et al., 2014), the most abundant dietary flavonoid in onions, tea and apples; and other, decreases the fasting bioavailability of non-haem iron. In addition, experiments in the Caco-2 cells (derived from colon cancer cells) have shown that a molar ratio 1:0.1 of iron:tannic acid produced a 92% inhibition of iron uptake, indicating that tannic acid may be a potent inhibitor (Glahn et al., 2002).

The exact mechanism implicated in the polyphenol dependent iron bioavailability remains elusive, but authors suggested that these compounds enhance apical iron uptake partially by reducing ferric to ferrous ion and by increasing the uptake of polyphenol-iron complexes via an energy-independent pathway, and decrease the basolateral iron release (Kim et al., 2011b).

Besides of non-haem iron, an early study indicated that haem iron absorption decreased due to the consumption of tea in humans (Disler et al., 1975), and this finding was confirmed by Ma et al. in a subsequent study (Ma et al., 2010). The same research group found that green tea catechin, (-)-epigallocatechin-3-gallate and grape seed extract,

inhibit haem iron absorption in a dose-dependent manner in human intestinal cells (Ma et al., 2011). They suggested that haem iron inhibition affects transport across the enterocyte by decreasing basolateral iron export, possibly by forming non-transportable complexes with iron in the cell. However, the precise mechanism by which bioactive dietary polyphenolic compounds inhibit haem iron absorption remains to be elucidated.

Animal and vegetal protein

In vitro model (Caco-2 cell line) found that animal and vegetable protein in general decreased haem iron uptake (Villarroel et al., 2011). This study showed that intact haem uptake was higher than haem plus albumin or digested haem plus albumin, but lower than digested haem. In addition, haem iron uptake decreased in the presence of all legume extracts. Previously, casein in milk has already been shown to reduce haem iron bioavailability (Hallberg et al., 1993). However, the results were not consistent, since pure animal (collagen and casein) and vegetable (zein and glutelin) proteins increased hemin iron uptake, a by-product from haem.

1.1.8 Biological factors

Body iron levels are principally controlled by modulation of iron absorption in the duodenum and proximal jejunum, which allows absorption to be precisely matched to body iron needs. In addition to body iron levels, absorption is also accurately regulated by hypoxia, erythropoiesis, pregnancy, and in the suckling period (Gulec et al., 2014).

Stores regulator

Iron absorption is stimulated when body iron stores decrease, and conversely, at high iron levels absorption is reduced. In this sense, a study observed that both iron status and modifiers of dietary iron absorption have a large effect on percentage absorption, with absorption values ranging from 13.9% to 23.0% when SF concentrations were 6µg/L. However, with higher iron status, absorption was very much reduced, ranging from 1.8% to 3.0% with a SF concentration of 100mg/L (Collings et al., 2013).

Erythroid regulator

Although overall physiological requirements for iron have influence in iron absorption, iron demand to support haemoglobin production in developing erythrocytes in the bone marrow is the strongest stimulator of this process, since it requires large amounts of iron. Iron absorption thus increases when the erythropoietic rate is high, resulting from blood loss or acute haemolysis, and decreases in the reverse situations (Gulec et al., 2014).

Hypoxia

Iron absorption also increases in response to tissue hypoxia. While in part this may relate to changes in the erythropoietic rate, a component of the response relates specifically to oxygen levels (Raja et al., 1988). In this regard, it has been found that iron absorption increased during hypoxia previously to increase of erythrocytes production (Hathorn, 1971), demonstrating that hypoxia exerts a direct effect on the gut. Consistent with this observation, iron regulatory molecules in the liver and genes encoding iron transporters in the duodenum respond directly to hypoxia (Peyssonnaux et al., 2007).

Pregnancy and suckling period

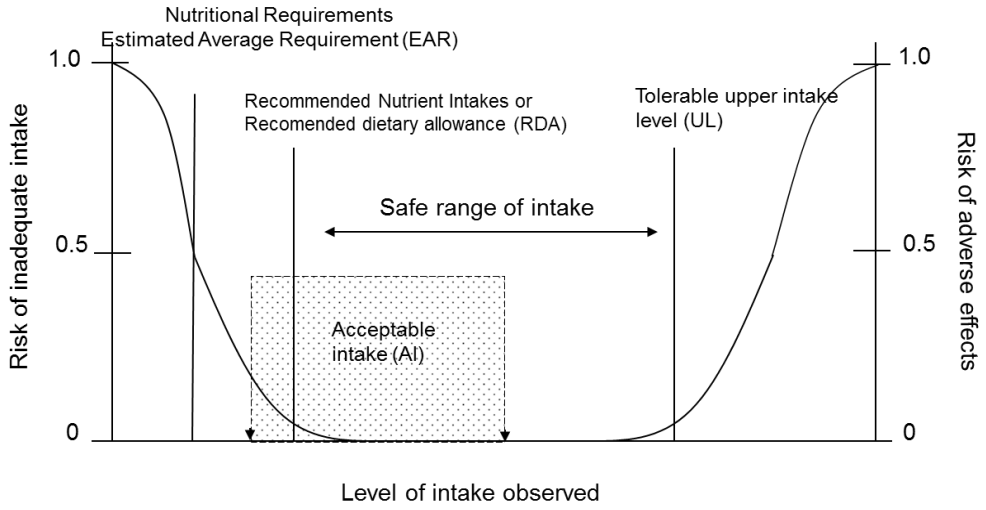
Iron absorption also increases during pregnancy. During gestation, iron requirements are high due to expansion of the maternal erythroid mass and the iron needed by the developing foetus. The cause probably relates to both a reduction in maternal iron stores and a relative tissue hypoxia. Moreover, during the perinatal and neonatal periods, iron requirements of humans are high, and iron absorption from breast milk is very efficient. The high iron absorption of neonates appears to be predominantly due to active transport mechanisms, as occur in adults, but the relative permeability of the neonatal epithelium, which allows passive absorption of solutes, likely contributes to greater absorption of iron (Gulec et al., 2014).

1.2 IRON INTAKE AND IRON STATUS IN POPULATION

1.2.1 Dietary iron intake recommendations

Intake recommendations for iron are provided in the Dietary Reference Intakes (DRIs) developed by the Food and Nutrition Board (FNB) at the Institute of Medicine (IOM) of the National Academies (Institute of Medicine et al., 2001). DRI is the general term for a set of reference values used for planning and assessing nutrient intakes of healthy people (see figure 7). These values, which vary by age and gender, include:

Figure 7. Relationship between individual intake and risk of adverse effects due to insufficient or excessive intake.



Dietary reference intakes include four distinct concepts: a) the average of the nutritional needs of the population group, b) nutritional recommendations located two standard deviations of the average needs, except the recommendations of energy, c) the acceptable nutrient intake when there is insufficient data to estimate the recommendations but adequate information to make this dietary advice is available, and d) the tolerable upper intake levels, above which there can exist a health risk.

Adapted from (Arija et al., 2015)

- Recommended Dietary Allowance (RDA): average daily level of intake sufficient to meet the nutrient requirements of nearly all (97%–98%) healthy individuals.

- Adequate Intake (AI): established when evidence is insufficient to develop an RDA; intake at this level is assumed to ensure nutritional adequacy.

- Estimated Average Requirement (EAR): average daily level of intake estimated to meet the requirements of 50% of healthy individuals. It is usually used to assess the adequacy of nutrient intakes in population groups but not individuals.

- Tolerable Upper Intake Level (UL): maximum daily intake unlikely to cause adverse health effects.

Table 2. Dietary reference intakes (DRIs) for iron by life stage group (mg/day).

	EAR[a]		RDA[b]		AI[c]	UL[d]
	Males	*Females*	*Males*	*Females*		
0 - 6 months					0.27	40
7 - 12 months	6.9	6.9	11	11		40
1 - 3 years	3.0	3.0	7	7		40
4 - 8 years	4.1	4.1	10	10		40
9- 13 years	5.9	5.7	8	8		40
14 - 18 years	7.7	7.9	11	15		45
19 - 30 years	6.0	8.1	8	18		45
31 - 50 years	6.0	8.1	8	18		45
51 - 70 years	6.0	5.0	8	8		45
> 70 years	6.0	5.0	8	8		45
Pregnancy						
≤ 18 years		23		27		45
19 - 50 years		22		27		45
Lactation						
≤ 18 years		7		10		45
19 - 50 years		6.5		9		45

[a]EAR: Estimated Average Requirement

[b]RDA: Recommended Dietary Allowance

[c]AI: Adequate Intake

[d]UL: Tolerable Upper Intake Level. Unless otherwise specified, the UL represents total intake from food, water and supplements

Source: Institute of Medicine 2001

Introduction

When adequate information is available, each nutrient has a set of DRIs. A nutrient has either an EAR and a RDA or an AI. When an EAR for the nutrient cannot be determined (therefore, neither can the RDA), then an AI is set for the nutrient. In addition, many nutrients have a UL (Institute of Medicine et al., 2001).

In order to prevent ID, RDAs were taken as a reference. Table 2 lists the current iron RDAs for non-vegetarians. The RDAs for vegetarians are 1.8 times higher than non-vegetarian people who eat meat, due to lower iron bioavailability in plant based food regarding animal foods.

In 1985, Moreiras et al. established the first values of recommended intakes for Spanish population, and its last update was in 2009 (see table 3) (Moreiras et al., 2009). Recommended Intakes (RIs) are reference standards for intake of energy and nutrients such as iron in order to maintain the health of virtually all healthy individuals in population. They are estimated for groups of age, gender, and physiological condition of pregnancy and lactation.

Table 3. Reference Intakes (RIs) for Iron in Spanish Population (mg/day).

	RDA[a]	
	Males	*Females*
Age Group		
0 - 5 months	7	7
6 months - 1 year	7	7
2 - 3 years	7	7
4 - 5 years	9	9
6- 9 years	9	9
10 - 12 years	12	18
13 - 15 years	15	18
16 - 19 years	15	18
20 - 39 years	10	18
40 - 49 years	10	18
50 - 59 years	10	10
> 60 years	10	10
Pregnancy (2nd half)		18
Lactation		18

[a]RDA: Recommended Dietary Allowance

Source: Moreiras et al. 2009

Iron recommendations in children, men and women are similar in both, Spanish RIs and DRIs of IOM but there is an important difference in pregnancy, and perinatal stage. Thus, iron DRIs for pregnant women are 27mg/day, much higher than IRs (18mg/day). Regarding lactation, iron DRIs are between 9 and 10mg/day, while IRs are 18mg/day. In addition, DRIs for the first months of new-born are 0.27mg/day versus 7mg/day in RIs.

1.2.2 Methods for assessing dietary intake

Several methods for assessing dietary intake allow examining the food consumption, energy and nutrient intake in collective or individual level. These methods provide us with important information about the frequency of food consumption and/or the quantity of food, energy and nutrient intake. Therefore, it enables us to identify inadequate diets and nutritional status, to assess and monitor nutritional health, and to examine trends and changes in dietary patterns of the population. Dietary food information could be obtained in three levels (table 4) (del Pozo de la Calle et al., 2015):

Table 4. Methods for assessing dietary intake.

	Method	Find out differences between
National surveys	Food Balance Sheets	Countries and regions worldwide
Family surveys	Household budget surveys Household surveys: shopping basket	Country, locality, seasonal, household
Individual surveys	The weighing method Food frequency questionnaire 24-hour recall	Geographics, seasonal and demographics subgroup and individuals
	Diet history	Temporary and demographics subgroup and individuals

Source: del Pozo de la Calle et al. 2015

- National level: using the Food Balance Sheet based on data provided by the Ministry of Agriculture. This method allows knowing the food availability in a country.

- Familiar level: by household budget surveys or family record or diary.

- Individual level: using dietary survey from which cross-section information is obtained to assess dietary intake (Gibson, 2005). A wide variety of dietary survey methods exists, the most used are: food frequency questionnaire (FFQ), 24-hour recall, food record, diet history, screener and brief assessment methods.

Food Frequency Questionnaire

FFQ is a dietary assessment tool that is highly used in epidemiological studies to examine the relation between dietary intake and disease or health outcomes. It is a retrospective and direct method to estimated food consumption by which global dietary information is obtained from a certain period of time (i.e. last 3 months or last year). Briefly, this method consists in asking how often and how much food items are consumed over a reference period (Martin and Gorgojo, 2007). This method enables to classify the participants that show a high or low consumption of certain food (Martin and Gorgojo, 2007). Actually, FFQs may be designed to focus on whole diet or on particular group of foods or nutrients such as iron. Also, sometimes they are designed to be administered to specific population groups such as elderly (Huybrechts et al., 2007).

This method can be self-administered, on paper or web-based, or interview administered (face-to-face or by telephone). More complete data may be collected if the FFQ is administered by an interviewer; although self-administered questionnaires may reduce respondent bias (Pérez Rodrigo et al., 2015).

There are three types of FFQ:

- Qualitative FFQs are those that only ask about frequency of food consumption, not about the size of consumed portions.

- Semi-quantitative FFQs include standard portions or reference portion sizes for each item and respondents are asked how often they consume the specified portion of a particular food item.

- Quantitative FFQs ask respondents to estimate either in grams or household measures the size of the portions consumed (Pérez Rodrigo et al., 2015).

The FFQ shows several strengths. They assess food consumption over a wide period and enable us to estimate the usual intake. These questionnaires are highly cost-effective, easy and fast administration since they have a standard format; therefore, they are widespread used in large epidemiological cohort studies. This method implies low respondent load compared to other methods, and this increases the cooperation and participation. Moreover, being a retrospective method, the habitual consumption is not influenced. They show a considerable validity and accuracy to estimate the dietary intake (Arija, 2014). Furthermore, FFQ requires less nutrition knowledge in data entry compared to other food consumption assessment methods and therefore do not require trained professionals (Pérez Rodrigo et al., 2015).

Despite these advantages, several drawbacks exist. Firstly, there is high complexity in designing these questionnaires or their validation, which involve systematic errors and biases in dietary intake estimates (Pérez Rodrigo et al., 2015). It could produce inaccuracies in the result from an incomplete listing of all possible foods and from errors in frequency and usual portion size estimations. However, a comprehensive and complete list of all foods cannot be included since the length of questionnaire influence on accuracy of the dietary report, for instance, over-estimation increases. Other limitation is the inaccuracies in the dietary report due to respondent memory. In addition, respondent should have a relatively high degree of literacy and numeracy skills are required if self-administered.

Introduction

24-hour dietary recall

The 24-hour dietary recall method is one of the most widely used methods in nutrition epidemiology. It is an open interview, retrospective and quantitative method that examines the food consumption of the previous 24 hours. This method consists in a direct interview and currently can also be self-administered using computer programmes (Salvador et al., 2015). The estimated average interview time is about 20 to 30 minutes.

The method consists of describing and quantifying the consumption of foods and beverages consumed in the previous 24 hours, from the first intake in the morning until the last intakes at night (Beaton et al., 1983). The information should describe the type of food and its characteristics, the amount consumed, method of preparation, sauces used, dressings or condiments to add, or accompanied food, as well as the time and place of consumption.

The method requires several support tools such as examples of dishes, volumes and household measures, drawings or photographic models or three dimensional representations. Contribution of novel technologies could be helpful to obtain an accurate assessment of food consumption (Salvador et al., 2015). This method involves professional trained interviewers who should have dietetic and nutrition knowledge and be familiarised with the eating habit of the study population to be able to estimate and record accurate information of daily food consumption. Also, the interviewer should attempt not to influence on the interviewee answers (Salvador et al., 2015). Once the food consumption information is recorded, this should be analysed with a database to obtain grams of food, nutrients and energy intake per day using food composition tables.

One single 24-hour dietary recall does not typically estimate usual intake. A minimum of two or five days of 24-hour dietary recalls is needed to examine usual dietary intake. In common practice two or three days of 24-hour recall are used and they must be carried out on non-consecutive days including a weekend day (Serra-Majem et al., 2006) (Martin and Gorgojo, 2007). This period of time involved acute dietary information without diminishing the participation. Otherwise, 24-hour recall during more days (i.e. 7 days)

could reduce the participation. Also, it is better to administer in different periods of time of the year so as to examine seasonal variation (Arija, 2014).

The 24-hour recall is easy and quick method with low cost and high precision. Response rate is high since its administration does not require so much time and could be administered to low literacy population. Moreover, the habitual food consumption of the participant is not altered since it is a retrospective method. Serial recalls can estimate the usual intake at the individual as well as the community levels (Serra-Majem et al., 2006)(Martin and Gorgojo, 2007) (Shim et al., 2014).

Nevertheless, this method show several limitations. Thus, it depends on the recent memory of the interviewee. Therefore, 24-hour recall is not recommended for the elderly or children less than 12 years. In addition, the accuracy of this method is influenced by the capacity of interviewee to refer food information. For instance, women and individuals who follow a diet tend to specify more exactly the dietary information than men or individuals who not follow a diet. Moreover, the "Flat slope syndrome" is described as the tendency to overestimate low intakes and underestimate high intakes. Underestimated intake is often in the elderly, children, and obese people or with unhealthy eating habits (for instance alcohol or fat excess intake) (Salvador et al., 2015).

Dietary record

The dietary record is a prospective and quantitative method in which the subject records all the foods and beverages consumed and its amounts over a specific period of time, usually between 3-7 days (Thompson and Byers, 1994).

This method usually record detailed information about portion size, food preparation methods, ingredients of mixed dishes and recipes, and even the brand name of commercial products. Therefore, the participant should be specifically trained to be able to describe adequately these items. At the end of the recorded period, a trained interviewer should review the dietary record with the participant, in order to clarify any doubts or ask by possible forgotten foods consumed (Ortega et al., 2015). There are two

main types: dietary record by household measures or by estimation, and dietary record by weight.

In dietary record by household measures or by estimation, the participants have to record all food consumption. The portion sizes could be estimated by household measures (plates, spoon, bowls, cups, and glasses), in reference to standard household measures, using three-dimensional food models or photographs. This is an easy, cost-effective method and it represents a little load to the participant. Therefore, it obtains a high degree of adherence and participation (Ortega et al., 2015).

In weighed dietary record, the amount of food consumed should be precisely measured by a kitchen weighing scale standardised, in order to diminish the bias. The food consumed should be weighted before eating and after eating (the food rest) and the participant should estimate the food eat-out-home. Thus, it is obtained the real quantity food consumed. Moreover, this methods show two derivations:

- Weighed dietary record with interviewer in which the interviewer is who weights and records food consumption. This method is useful for institutionalised population or low literacy population. It could be combined with a 24 hour dietary recall to know the out-of-home dietary intake. Both methods, by weight and by weight with interviewer, show a high accuracy. However, they require a high level of cooperation from the participants, which could diminish the participation.

- Weighted dietary record with chemical analysis in which the methodology is similar to the method of record by weight, but the food composition is obtained chemically. This method requires that respondent keeps up a portion of food which will be chemically analysed. This method presents the highest validity and accuracy, for this reason, it is considered the gold standard method in empirical researches. However, the limitations are the high complexity of the technic, high economic cost and high level of participation of the respondent (Arija, 2014).

Generally, the main advantage of all these dietary record methods are their potential to collect accurate quantitative information. Because of the quality of its data, it is

considered the gold standard of the dietary methods. In addition, this method does not depend on the memory of the participant and is more accurate, since the amount of consumed food is recorded when eaten. The optimal number of days to collect data that are more reliable depends largely on the nutrient or the sample size. Traditionally the most common dietary record monitors the diet for seven consecutive days. This period allows to collect information about the diet minimizing bias related to the day of the week. Ideally, it is needed a long enough period of time to provide accuracy information of dietary (a minimum of 3 days is required) but without diminishing the participation and compliance (periods of no more than 4 consecutive days) (Arija, 2014) (Ortega et al., 2015).

In addition, other limitations are that these methods require that interviewers and participants are well trained and a high cooperation and literate of the participants. This could influence on the participation of some population groups (people with low literacy, immigrants with low language skills, children, elderly and people with writing difficulties) (Ortega et al., 2015). Another limitation is that this method can alter food consumption of the participants, as participants are more conscious about the food and the amount they consume, since their diet will be analysed (Kristjansdottir et al., 2006).

Diet history

The Diet History is a retrospective and quantitative method to describe food and usual nutrient intake during a relatively long period. It consists in a long interview that can take from one to two hours and requires a highly qualified interviewer in nutrition. The diet history method assesses quantitatively the global food intake of an individual for a certain period of time, habits in relation to food consumption, distribution and usual composition of meals throughout the day (Nelson, M, Bingham, 1997) (Martin and Gorgojo, 2007). Some authors considered that the complete method usually consists of:

- An interview recalls estimating the habitual food consumption in the different eating occasions in a day. Often a 24-hour recall is included.

- A FFQ to verify information to assess the overall pattern of food intake.

- A three day dietary record with estimated portion sizes of the foods and beverages consumed.

Nevertheless, the method has several limitations that should be considered. For instance, this method entails a great effort of memory, a high participation and cooperation of the participant and a large duration to implement it. Furthermore, diet history method tends to overestimate intake. Otherwise, as it is focused on evaluation of usual patterns, exceptional intakes are underestimated. The diet history is a complex, large and costly method that require highly well trained personnel, and there is not a standard protocol of complete diet history, for this reason, it is not applicable in large scale population studies. Therefore, currently the main application of the diet history method is in clinical practice (Morán Fagúndez et al., 2015).

Screener and brief assessment methods

Brief assessment tools are easy, self-administered and qualitative questionnaires. From the brief questionnaire, scores are obtained and are usually categorised according to levels. Usually, these tools ask about frequency of consumption or about dietary habits, thus they are useful to identify individuals with a very low or high intake and identify risk of malnutrition or inadequate consumption patterns for specific food groups (Pérez-Rodrigo et al., 2015).

These methods do not require a trained professional nor depend on the memory of the participant (Green and Watson, 2005), thereby, there is a high participation rate and cooperation. Recently these new brief assessment and self-evaluation tools are used by health professionals in primary health care, in community intervention to early screening and health promotion (Pérez-Rodrigo et al., 2015).

A literature review conducted in 2005 identified 71 brief dietary assessment tools (Green and Watson, 2005) used in adults. Most of them included some anthropometry and sociocultural aspects related to food behaviour or biomarkers, and more complex anthropometrical measurements. Some specific instruments for adults are Dietary Risk

Assessment, Mediterranean Diet Adherence Screener (MEDAS), Dietary Intervention Care (DINE), Determine, Mini-Nutritional Assessment (MNA).

Valid dietary assessment tools are needed to facilitate dietary counselling in high-risk populations. Nutrition advice for adults and underserved groups often occurs in clinical settings with time and resource limitations, dietary assessment tools for this setting should be brief (Gans et al., 2003). The Dietary Risk Assessment is a tool developed for non–dietetics-trained Health professionals, such as nurses and physicians, who provide dietary advice to underserved patients. Dietary Risk Assessment response options are arranged in three columns. The left column indicates the most healthful dietary practices, whereas responses in the right or middle columns indicate less healthful practices (Richardson et al., 2011).

A more standardised protocol about application procedures and advice to provide users according to the scoring. Many have not assessed the validity and reliability of the method and often not investigated the sensitivity, specificity and acceptability of these tools. In the PREvención con DIeta MEDiterránea (PREDIMED) trial 14-point Mediterranean Diet Adherence Screener (MEDAS) was validated and used to identify subjects' adherence to the intervention diet, a Mediterranean dietary pattern (Schröder et al., 2011).

Dietary Intervention in Primary Care (DINE) is a 19-item questionnaire developed for use in interview-administered. It measures an individual's intake of total fat and dietary fibre, categorised as low, medium or high. Specific foods are included which account for 70% of the fat and fibre in a typical United Kingdom diet. The tool has been validated with good correlation with a validated four-day semi-weighed food diary. An experienced interviewer can complete it in 5–10 minutes (Richardson et al., 2011).

Web-based tailored interventions aimed to modify food behaviour, physical activity and other lifestyles in adults have also used screeners of brief instruments (Broekhuizen et al., 2012).

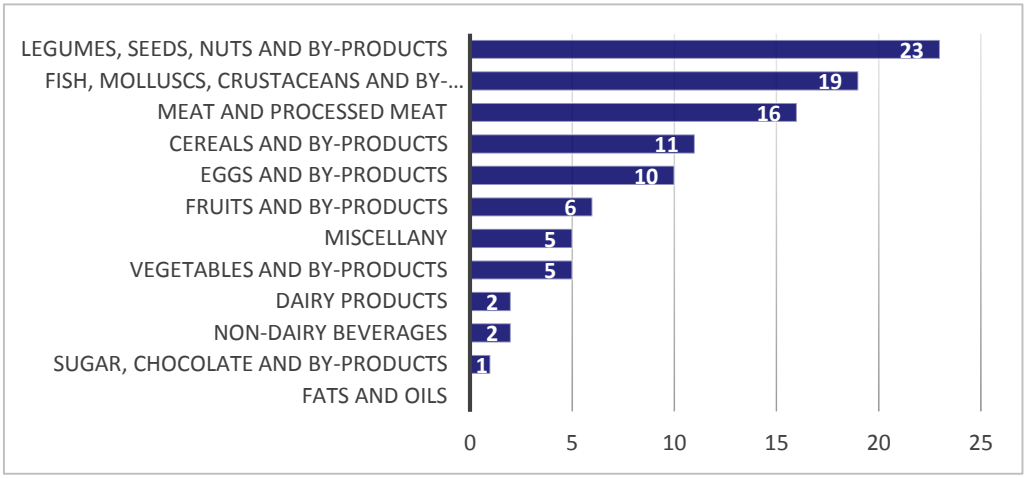
Several screening tools focused on malnutrition targeted to be used in old adults have been validated. These vary depending on the type of population in question, the availability of personnel trained in nutrition, and the possibility of automation, etc. The most commonly used include the Malnutrition Screening Tool (MST), the Nutritional Risk Screening (NRS-2002) method, the Malnutrition Universal screening Tool (MUST), and the first part of the Mini-Nutritional Assessment (MNA) method. Nutritional screening allows the identification of subjects at risk of under-nutrition, as well as those who need a more exhaustive study and a nutritional diagnosis (Campos del Portillo et al., 2015).

1.2.3 Dietary iron intake in population: Epidemiological studies

The Spanish National Survey of Dietary Intake (ENIDE) was the first survey done in throughout the Spanish territory based on a methodology of analysis of individual consumption, through surveys (AECOSAN, 2011). It was conducted by The Spanish Agency for Consumer Affairs, Food Safety and Nutrition (AECOSAN) in a sample of 3000 (1,500 women and 1,500 men) representative adult subjects between 2009 and 2010. The overall aim was to know, through a national study, the food consumption in Spain, and more specifically: to determine patterns of dietary intake in the Spanish population by age and gender; to obtain data to help complete the assessment of nutritional status; and to assess the intake of macro and micronutrients. The estimated micronutrient intake was based on the BEDCA Spanish Database Food Composition.

The dietary iron of Spanish population is provided, to a greater extent, by the group of legumes, seeds, nuts and by-products (23%), followed by fish (19%) and meats (16%). It is also important the contribution of cereals and by-products (11%), and eggs and by-products (10%) to the dietary iron intake (AECOSAN, 2011) (see figure 8).

Figure 8. Contribution (%) to daily iron intake of different food groups.



Adapted from ENIDE 2011

Data showed higher iron intake in men (16.1 ± 6.45mg/day) than in women (13.7 ± 6.21mg/day), in agreement with previous surveys carried out in different regions of Spain, such as, Catalonia (14.5 ± 4.5 vs. 13.5 ± 3.8mg/day, P <0.001) (Bondia-Pons et al., 2007), Comunidad de Madrid (15.6 ± 6.1 vs. 13.7 ± 5.6mg/day, P <0.001) (ENUCAM, 2014) or Communidad Valenciana (18.0 ± 6.5 vs. 14.9 ± 7.0mg/day, P <0.001) (Vioque, 2003). According with this, women of child-bearing age, with increased iron demand, do not reach the recommended values for this mineral (less than 80% of RDAs) established by Moreiras et al. for Spanish population (Moreiras et al., 2009). However, men consume an average of 60% over the RDAs, and postmenopausal women reach the recommendations in ENIDE sample (AECOSAN, 2011) (see table 5). In agreement with ENIDE data, the Food Consumption Survey, conducted for over 20 years by the Spanish Ministry of Agriculture, Food and Environment (MAGRAMA), showed that men aged 20 to 59 years have a better compliance with the recommendations for iron than women. Data since 2000 to 2008 showed that men consume between 35 and 60 over the RDAs (Moreiras et al., 2009), while women do not reach the recommended values for iron (between 25 and 10% below the RDAs) (del Pozo de Calle et al., 2012).

Introduction

The ENIDE survey observed (AECOSAN, 2011) that more than 95% of men older than 20 years had an elevated intake of iron (5th percentile = 12mg/day) regarding Spanish RIs (10mg/day) (Moreiras et al., 2009) and RDAs of IOM (8mg/day) (Institute of Medicine et al., 2001). However, less than 25% of adult women of child-bearing age (75th percentile = 16-17mg/day) complied with the Spanish (Moreiras et al., 2009) and IOM (Institute of Medicine et al., 2001) recommendations (see table 6).

Table 5. Daily intake of total iron by gender and age.

Age (years)	Total Iron Intake[a] (mg)	RI's (Morerias 2011)	% adequacy
MEN			
18-24 (n = 300)	15.9 ± 7.0	10	164
25-44 (n = 656)	16.1 ± 6.3	10	161
45-64 (n = 663)	16.2 ± 6.4	10[b]	163
Total (n = 1,589)	16.1 ± 6.5		
WOMEN			
18-24 (n = 324)	12.5 ± 4.7	18	72
25-44 (n = 731)	14.1 ± 7.1	18	78
45-64 (n = 674)	13.8 ± 5.9	10[b]	139
Total (n = 1,734)	13.7 ± 6.2		

RI's (Recommended Intakes) based on RDA (Recommended Dietary Allowance)

[a] Mean ± standard deviation

[b] RDA for adults older than 50

Adapted from ENIDE 2011

In addition to the ENIDE survey (AECOSAN, 2011), the Health Department of the Autonomous Government of Catalonia has included as one of their objectives in the Health Policy for Catalonia, the making of periodic nutritional surveys for monitoring the nutritional status in a representative sample aged 10 to 75 years of the Catalan population (ENCAT). Serra-Majem et al. analysed the trends in energy and nutrient intakes derived from the last two Nutritional Surveys carried out in 1992-93 (ENCAT 1992-93) and 2002-03 (ENCAT 2002-03) (Serra-Majem et al., 1996) (Serra Majem et al., 2006).

Table 6. Total iron intake in Spanish population between 18 and 64 years.

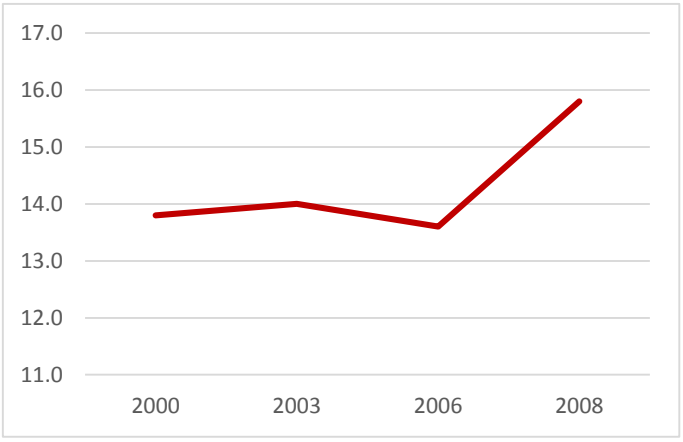
Age (years)	18-24		25-44		45-64	
	Males (n= 260)	Females (n= 260)	Males (n= 619)	Females (n= 620)	Males (n= 619)	Females (n= 620)
	IRON INTAKE (mg/day)					
P5	12	11	12	11	12	11
P25	14	13	14	13	14	13
P50	16	14	16	15	16	15
P75	18	16	18	17	18	16
P95	21	18	21	20	21	19
EAR (IOM 1997)	6	8.1	6	8.1	6	5
Percentage of subjects having intakes below the EAR values	0	0	0	0.2	0	0

EAR: average daily level of intake estimated to meet the requirements of 50% of healthy individuals

Adapted from ENIDE 2011

Regarding iron consumption, a decrease was observed in its daily intake from 1992-93 to 2002-03 (Serra-Majem et al., 1996) (Serra Majem et al., 2006). This decline was due to the reduction in the consumption of meat, and fish. Therefore, the risk of inadequate iron intake increased in the ENCAT-2002-03 in the group of women. Consistently, data from ENIDE survey showed that women of child-bearing age did not reach the recommended values, although the iron intake was higher in both, men and women groups (see table 6), than the last ENCAT survey in 2002-03.

Figure 9. Evolution of daily intake of iron (mg/day) in Spanish population.



Adapted from the Spanish Ministry of Agriculture, Food and Environment (MAGRAMA)(del Pozo de Calle et al., 2012)

1.2.4 Biochemical methods for assessing iron status

Chemical analysis of a liver biopsy is the "gold standard" for quantifying iron stores. However, the need for a relatively large volume of tissue (4 mg net weight) as well as risk of this invasive procedure (haemorrhage occurs in about 0.5% of cases) has made this less appealing to most clinicians and patients (Knovich et al., 2009). Moreover, bone-marrow examination is the gold standard for the diagnosis of ID, particularly when performed and reviewed under standardised conditions by experienced investigators. However, marrow examinations are not performed routinely in clinical practice; furthermore, they are expensive, uncomfortable, and require technical expertise (Zimmermann, 2008). Therefore, biochemical parameters related to iron metabolism are usually used in epidemiological studies in order to accurately measured total body iron stores. The most used of them are the following:

Ferritin

The iron storage protein, ferritin, is mainly found in the liver and spleen and other tissues, but also extracellularly within the serum at trace levels. It is suggested that ferritin may enter the circulation either via secretion of ferritin by cells or through the release of ferritin from damaged cells (Worwood, 1990), in proportion to the intracellular levels. Both mechanisms probably contribute to plasma levels, which serve as an important clinical marker of iron status. Actually, in the absence of inflammation and/or chronic disease, SF is considered as the best single indicator in assessing body iron stores, except liver or bone marrow biopsy as we noted above, due to its less invasive assessment and high specificity (Ren and Walczyk, 2014).

The acute phase response is a systemic reaction to local or systemic infection, inflammation, tissue damage, cancer and in general, immune disturbances (Wang et al., 2010a). Ferritin is widely recognised as an acute phase reactant, and an important role for ferritin during these conditions is to restrict the availability of iron by sequestration into the cavity of the ferritin protein shell. This function is presumed to have developed as a defence mechanism to restrict serum iron from utilization by pathogens and tumours (Ganz and Nemeth, 2009) (Weinberg and Miklossy, 2008).

In healthy individuals, SF is directly proportional to iron stores, thereby, 1µg/L of SF corresponds to 8–10 mg body iron or 120 mg storage iron/kg body weight (Cook, 2005). Normal ranges set by the World Health Organization (WHO) are 15–150mg/L for adult women and 15–200 mg/L for adult males (World Health Organization and Centers for Disease Control and Prevention, 2004). While SF concentration below the normal range (<12–15µg/L) is highly specific for diagnosis of ID (Worwood, 1979), high SF concentration is not necessarily indicative of iron overload, because its serum concentration increases sharply under inflammatory conditions, as discussed previously (Wang et al., 2010a). Here, the serum concentration of the soluble transferrin receptor (sTfR), its ratio relative to SF concentration, or a combination of both is of higher diagnostic value. Anyway, ferritin outperforms other biomarkers of iron status, such as transferrin saturation, mean cell volume, and red cell zinc protoporphyrin levels in terms of sensitivity and specificity at any level (Zähringer et al., 1975).

sTfR

The transferrin receptor (TfR) is a transmembrane glycoprotein key for iron metabolism, because it is involved in the process of cellular uptake of iron. Its expression is determined by the cellular demand for iron. Its truncated form, sTfR, is located in serum in proportion to the transmembrane protein and, therefore, to cell iron demands (Speeckaert et al., 2010). In addition, sTfR represents a significant advanced in the evaluation of body iron status, especially since inflammation processes do not alter it. These are the reasons because it is believed that represents a good biomarker in the evaluation of body-iron status (Désidéri-Vaillant et al., 2011),

Further, sTfR concentration appears to be a specific indicator of iron-deficient erythropoiesis, since around 80 % of TfR in the body is found on erythroid precursors. Therefore, in conditions such as normal expansion of the erythroid mass during growth (Olivares et al., 2000), as well as diseases common in developing countries, including thalassemia, megaloblastic anaemia due to folate deficiency, or haemolysis due to malaria, may increase erythropoiesis and sTfR independent of iron status (Worwood, 2002).

Introduction

sTfR/ferritin ratio

Cook et al. have demonstrated that in healthy subjects the concentration of stored iron may be estimated from the ratio of sTfR/ferritin (Cook et al., 2003). The logarithm of this ratio is directly proportional to the amount of stored iron in iron-replete subjects and the tissue iron deficit in ID. Thereby, sTfR/ferritin ratio appears to be a good marker of iron stores (Malope et al., 2001), especially useful in population studies since it is sensitive not only to elevated, but also decreased, levels of iron stores (Skikne, 2008). For instance, in elderly subjects, the ratio may be more sensitive than other laboratory tests for ID (Rimon et al., 2002). Although, it has been indicated that sTfR/ferritin ratio has no clear advantage over SF alone (Mei et al., 2005), a study assessing ID in the US population (NHANES) (Cogswell et al., 2009) reported that it appeared to be less affected by inflammation than ferritin alone.

Serum iron

The normal plasma iron concentration ranges between 50–120μg/dL. The thresholds used to classify individuals as iron deficient typically range from 50–60μg/dL (World Health Organization and Centers for Disease Control and Prevention, 2004), however natural variation in measurements may lead to misclassification. Serum iron has very limited value in assessing ID or chronic excess in population studies because concentrations within an individual are affected by other influences such as diurnal and post-prandial variations, and concentrations are rapidly reduced following infection or inflammation as part of the acute phase reaction (World Health Organization and Centers for Disease Control and Prevention, 2004).

Transferrin

The globular protein, transferrin, is the specific transport protein for iron in the plasma pool. The concentration of transferrin increases during ID and decreases with protein deficiency, so it is sensitive to several factors. The concentration of this transport protein reflects iron status only when iron stores are exhausted and when the plasma iron concentration is <40–60μg/dl, so it does not diagnose ID prior to ineffective

erythropoiesis (World Health Organization and Centers for Disease Control and Prevention, 2004).

Total iron binding capacity

A proxy measure of transferrin is the measurement of the total iron binding capacity (TIBC). The TIBC is not as subject to rapid changes in concentration as the plasma iron concentration, so it is inherently more stable as an indicator of iron status. The TIBC by itself is not often used as a measure of iron status because it appears not to change until iron stores are depleted (World Health Organization and Centers for Disease Control and Prevention, 2004).

Transferrin saturation

Transferrin saturation is a widely used screening test for ID, and is calculated by dividing the serum iron by the TIBC. Although relatively inexpensive, its use is limited by diurnal variation in serum iron and the many clinical disorders that influence transferrin levels (Cook, 2005). Thereby, in addition to ferritin, transferrin saturation also has some acute-phase reactivity insofar as transferrin may be elevated in the setting of inflammation, which would lower the transferrin saturation if circulating iron is constant (Wish, 2006).

Clinical studies have demonstrated that a transferrin saturation of <15% is insufficient to meet normal daily requirements for erythropoiesis (World Health Organization and Centers for Disease Control and Prevention, 2004). Otherwise, transferrin saturation values higher than 50% appear in iron overload conditions (Wish, 2006).

Haemoglobin

Haemoglobin is a widely used screening test for ID, but used alone has low specificity and sensitivity. Its sensitivity is low because individuals with baseline haemoglobin values in the upper range of normal need to lose 20–30% of their body iron before their haemoglobin falls below the cut-off for anaemia (Cook, 2005). Its specificity is low because there are many causes of anaemia other than ID. Cut-off criteria differ with the

age and gender of the individual, between laboratories, and there are ethnic differences in normal haemoglobin (Johnson-Spear and Yip, 1994).

Mean corpuscular volume and reticulocyte Haemoglobin content

The mean corpuscular volume (MCV) is a reliable, but relatively late indicator of ID. However, false normal values can occur when the MCV is increased or in thalassemia (Zimmermann, 2008).

The reticulocyte haemoglobin content has been proposed as a sensitive indicator that falls within days of the onset of iron-deficient erythropoiesis (Mast et al., 2002). For both reticulocyte haemoglobin content and MCV, low specificity limits their clinical utility (Thomas and Thomas, 2002).

Red cell zinc protoporphyrin, erythrocyte protoporphyrin and zinc protoporphyrin

The absence of iron in the bone-marrow causes that zinc replaces it during formation of the protoporphyrin ring, previous step to the synthesis of haemoglobin. The normal ratio of iron to zinc in protoporphyrin is about 30,000:1, but a lack of iron available to ferrochetalase during the early stages of iron deficient erythropoiesis results in a measurable increase in the concentration of zinc protoporphyrin (World Health Organization and Centers for Disease Control and Prevention, 2004). When there is enough iron the reactions are as follows:

Protoporphyrin + Ferrous iron → Ferrous protoporphyrin

Ferrous protoporphyrin + Globin → Haemoglobin

When there is a lack of iron then zinc replaces iron in a very small but measurable proportion of molecules:

Protoporphyrin + Zinc → Zinc protoporphyrin

Zinc protoporphyrin + Globin → Zinc protoporphyrin-globin

Typically free erythrocyte protoporphyrin is less than 5% of the total. Its measurements are nearly identical to the zinc protoporphyrin. In turn, both erythrocyte protoporphyrin and zinc protoporphyrin should be interchangeable with red cell zinc protoporphyrin (World Health Organization and Centers for Disease Control and Prevention, 2004).

Zinc protoporphyrin has a high sensitivity in diagnosing ID (Labbé et al., 1999) (Labbé and Dewanji, 2004). A healthy normal individual will have an erythrocyte protoporphyrin concentration of less than 40–50μg/dL of red cells. Coincident with a fall in transferrin saturation below 15%, the concentration of erythrocyte protoporphyrin increases rapidly to more than 70–100μg/dL. With a prolonged or severe deficit in iron, the erythrocyte protoporphyrin concentration may reach as high as 200μg/dL (World Health Organization and Centers for Disease Control and Prevention, 2004). However, the specificity of zinc protoporphyrin in identifying ID may be limited, because it can be increased by lead poisoning, anaemia of chronic disease, chronic infections and inflammation, haemolytic anaemias, or haemoglobinopathies (Labbé and Dewanji, 2004) (Graham et al., 1996) (Hastka et al., 1993). Direct comparisons between studies of zinc protoporphyrin are difficult because of interassay variation. Zinc protoporphyrin is a useful screening test in field surveys, particularly in children, where uncomplicated ID is the primary cause of anaemia. Because of the difficulty in automating the assay, zinc protoporphyrin has been not widely adopted by clinical laboratories (Zimmermann, 2008).

1.2.5 Iron status in population: Epidemiological studies

Adequate iron status implies presence of normal erythropoiesis, and iron dependent functions that are not limited by iron supply, as well as a small contingency reserve of iron storage to supply other physiological functions.

Iron deficiency

ID is a state in which there is insufficient iron to maintain the normal physiological function of tissues such as blood, brain, and muscles (World Health Organization, 2008). Haemoglobin determination has been the most widely used screening method for the

diagnosis of ID, but when used as the only laboratory measurement it has serious limitations because of its low specificity and sensitivity. The low haemoglobin specificity for identifying ID is due to the numerous causes of anaemia other than ID that are seen both clinically and in population surveys (Cook, 2005). For the diagnosis of ID bone-marrow examination is the gold standard. However, marrow examinations are not performed routinely in clinical practice, since they are expensive, uncomfortable, and require technical expertise (Zimmermann, 2008). SF is considered a useful indicator of iron stores, despite its concentration rises during inflammation. Thus, the customary thresholds to indicate ID is of <15ng/mL, and <30ng/mL in the presence of infection (World Health Organization, 2011a).

In addition to ID, iron depletion is the state in which iron storage is absent or nearly absent but the tissues that need iron are able to maintain normal physiological functions. Moreover, a functional iron deficiency appears when iron stores are present, but the normal physiological systems for transporting iron to target tissues are impaired. This occurs most commonly because of cytokines released during inflammation states and infectious diseases, and appears to be mediated by hepcidin (World Health Organization, 2008).

Anaemia

Anaemia is a public health problem that affects populations in developed and developing countries. Further, it is one of the most prevalent nutritional deficiency diseases worldwide. Although the primary cause is ID, it is seldom present in isolation. Anaemia frequently coexists with a number of other causes, such as malaria, parasitic infection, nutritional deficiencies, and haemoglobinopathies. WHO defines anaemia as blood haemoglobin values of less than 7.7mmol/L (13g/dL) in men and 7.4mmol/L (12g/dL) in women, and estimates that about two billion people are anaemic (World Health Organization, 2008). The severity of anaemia is based on the patient's haemoglobin/haematocrit level (Johnson-Wimbley and Graham, 2011).

Iron deficiency anaemia

Iron deficiency anaemia (IDA) is a form of anaemia due to the lack of sufficient iron to form normal red blood cells. The diagnosis of IDA requires evidence that iron stores are fully depleted. Isolated or uncomplicated IDA in the absence of other diseases that influence measurements of iron status is seen most often in infants and preschool children (due to rapid growth), in pregnant women (due to the iron demands of the foetus), and in patients with excessive uterine or gastrointestinal blood loss. The key laboratory measurement for its identification is the SF; a concentration below 30mg/L is diagnostic of IDA in patients with anaemia (Cook, 2005). As noted above, ID is the most common cause of anaemia in the world.

IDA is characterised by microcytic, hypochromic erythrocytes and low iron stores. The MCV is the measure of the average red blood cell volume and mean corpuscular haemoglobin concentration is the measure of the concentration of haemoglobin in a given volume of packed red blood cells. The normal reference ranges for MCV is 80-100fL and mean corpuscular haemoglobin concentration is 320-360g/L (Johnson-Wimbley and Graham, 2011).

Although ID is a problem mainly in developing countries, it is also well known to affect large fractions of populations in the industrialised world. In 2009, Sánchez et al. found that the risk of ID, among men and women between 25 to 60 years old in southern Spain, was 12.7%, and 2.1% for IDA (Sánchez et al., 2009). In this study, individuals were considered to have ID if at least two of the following indicators were abnormal: plasma iron, TIBC, transferrin saturation and MCV; and iron-deficient anaemia if they had below-normal values for haemoglobin, MCV, mean cell hemoglobin and mean corpuscular haemoglobin concentration. The percentage of women with ID and IDA was higher than the corresponding percentages in men, according to the highest iron intake observed in men (16.1 ± 6.45mg/day) regarding women (13.7 ± 6.21mg/day) in ENIDE survey (AECOSAN, 2011). Data only for women younger than 45 years (women of child-bearing age) showed that the prevalence of anaemia increased to 13.8%. This prevalence is higher than in other European countries, such as, Northern Ireland (13.5%), United Kingdom (9.0%), Sweden (6.6 - 7.4%), France (4.4%), Norway (4.1%) and Denmark

(2.8%) (Hercberg et al., 2001), but lower than the global prevalence showed by WHO in 2011 in the same group of women of child-bearing age (World Health Organization, 2011b).

Iron overload

Iron overload (see section 1.3) occurs when excess iron accumulates in the body. It can be caused by increased absorption of dietary iron or by parenteral iron loading (repeated blood transfusions). The thresholds suggested for a SF concentration during iron overload have varied widely. WHO concluded that thresholds of >200µg/L for men and >150µg/L for women were appropriate (World Health Organization, 2001).

A study carried out by Altés et al. in 2004 in the northwest of Spain, showed high iron overload prevalence (9.3%) in the whole sample, 14.7% in men and 3.8% in women between 18 and 74 years old (Altés et al., 2004). In that study, iron overload was defined as ferritin levels above 200mg/L in women and 300mg/L in men. They also observed higher prevalence of iron overload in elderly subjects. Thus, 11.7% of young men (<50 years) and 20.4% of those over 50 years presented iron overload. In women, it is noteworthy that only 1.2% of young women had iron overload, but the prevalence increased until 9.4% among women above 50 years. Interestingly, 1.6% of the general population, 2.5% of young men and 3.9% of those over 50 years had ferritin levels greater than 500mg/L (Altés et al., 2004). A previous study carried out in southern Spain among 176 postmenopausal women and 125 men between 40 and 93 years, and without evidence of diseases related to iron metabolism, also found higher prevalence of iron overload in men than in women (Vidal Miñana and Farré Rovira, 2002). Using the same criteria to determine iron overload, a study conducted in 1757 blood donors from Mexico City found a lower prevalence in men (12%) and higher in women (4.8%) than previous Spanish population, and as noted Altés et al., the prevalence increased in parallel with increasing age (from 15.6 to 29.9% for men and from 3.5 to 9.6%, for women) (Baptista-González et al., 2005).

The percentage of subjects with iron overload in a Danish male general population (13.5%) was slightly lower than that from northwest of Spain. Moreover, the Danish

study showed that the percentage of men having elevated SF ≥300μg/L increased gradually with age (Pedersen and Milman, 2009) as noted in earlier studies. Previous Danish studies reported a significantly increase in the prevalence of iron overload (from 11.3% to 18.9%) in men (Milman et al., 2002) and in postmenopausal women (from 2.4 to 5.5%), but not in premenopausal women (Milman et al., 2003), from 1984 to 1994. In addition, they also observed a significant rise in the prevalence of iron overload with age.

Recently, Aranda et al. conducted a study in order to describe iron genetic mutations, dietetic and lifestyle factors, as well as, iron status in a general Mediterranean population. A total of 815 healthy Caucasian subjects (425 female, 390 males) between 18 and 75 years were randomly selected from the local government census of three communities in the Mediterranean region of Tarragona (Spain). Results showed an important percentage of subjects (26.7% of males and 8.7% of females) with elevated iron status, measured as transferrin saturation >45%. When the biomarker used was SF, and according with iron overload criteria of WHO, i.e. >200ng/mL in males and >150ng/mL in females (World Health Organization, 2001), the percentage of subjects with elevated iron status was reduced to 19.7% in males and 4.0% in females (Aranda et al., 2010). Women with SF >200ng/mL represented 2.4% of the sample, quite lower than previous study carried out by Altés et al. in northwest Spanish population (3.8%) (Altés et al., 2004). The transferrin saturation mean of this population was 39.3 ± 18.5%, and SF was 106.9 ± 2.1ng/mL.

Iron overload may be originated by various pathological conditions. The main causes found in a sample of Spanish population with iron overload were severe alterations of the HFE gene, hepatitis C virus infection, and dysmetabolic syndrome with iron overload (Altes et al., 2003).

1.3 IRON OVERLOAD

As it was reported previously, iron overload occurs when excess iron accumulates in the body. WHO suggested that a thresholds of SF >200μg/L for men and >150μg/L for women, were appropriate for iron overload (World Health Organization, 2001). However, there are other clinical conditions, in which SF levels reach 1,000μg/L.

1.3.1 Causal factors for iron overload

Some genetic (primary) and acquired (secondary) disorders can cause iron overload. Haemochromatosis is one of the most common genetic disorders that results in iron overload. It is caused by a series of mutations that affect multiple regulatory proteins at various points along the pathway of hepcidin and iron regulation due to mutations in the HFE and non-HFE (FPN, TfR, HJV) genes (Ekanayake et al., 2015). Normal HFE is required for iron stimulation of hepcidin. The HFE alteration results in a reduced expression of hepcidin and hence iron absorption is inappropriately high for a given body iron load (Nemeth et al., 2005).

1.3.1.1 Primary iron overload

Haemochromatosis type I, also named classical haemochromatosis (see table 7), is an autosomal recessive disorder that occurs in approximately five per 1,000 Caucasians of northern European descent (Pietrangelo, 2010). Most commonly, the disease occurs due to two missense mutations (C282Y and H63D) in the haemochromatosis gene (HFE) located on the short arm (p) of chromosome 6 (Yun and Vincelette, 2015). The prevalence of the most common mutations in HH, C282Y homozygote and C282Y/wild type heterozygote, are 1 in 150–300 and 1 in 10 in general population, respectively (Allen et al., 2008). Of note is that genetic disorders, such as HH, in southern Europe are quite different from those in the USA, China and northern Europe. Thereby, while in Northern Europe the prevalence of the C282Y polymorphism in HFE gene is 5-10% and of H63D polymorphism is 10-20%, in southern Europe the prevalence is 1-5% and >20%, respectively (Kucinskas et al., 2012). In Spain, the prevalence of the main mutation (H63D, C282Y and S65C) reaches 46% in certain regions (Aranda et al., 2007).

Haemochromatosis type IIA, juvenile haemochromatosis, is the most severe form of HH. This form of haemochromatosis is due to mutations in genes encoding HJV protein (Papanikolaou et al., 2004), which results in a reduced activation of hepcidin (Camaschella and Poggiali, 2009). *Haemochromatosis type IIB* is due to mutations of hepcidin, affecting the cysteine fingers or producing a null gene product (Camaschella and Poggiali, 2009). Mutations in TfR2 cause the form of *hereditary haemochromatosis*

type III. Although the function of TfR2 is not completely understood, it is believed to bind to sense transferrin in hepatocytes by binding to HFE. Therefore, dysfunction of TfR2 results in reduced hepcidin production (Camaschella and Poggiali, 2009).

Haemochromatosis type IV is the only autosomal dominant form of the disease interfering with FPN function (Camaschella and Poggiali, 2009). As expected, the phenotype in affected patients is similar to that in patients with classic HH, but with normal or elevated, rather than low, hepcidin levels (Papanikolaou et al., 2005). This can result in IDA due to a reduced circulating iron and is associated with reduced end-organ damage and reduced need for venesection (Camaschella and Poggiali, 2009).

There are other genetic disorders more rare, such as aceruloplasminaemia, atransferrinaemia or neonatal iron overload, which have also been associated with iron overload (Siah et al., 2006).

Table 7. Characteristics of hereditary haemochromatosis.

Types	Gene affected	Protein affected	Prevalence	Penetrance	Function	Resulting phenotype	Severity
Haemochromatosis type I (Classical haemochromatosis)	HFE	HFE	Most common form worldwide. Varies by race	Autosomal recessive: 2–28 % penetrance	Hepcidin regulation	Parenchymal iron overload, liver disease	Mild
Haemochromatosis type IIA	HJV	Hemojuvelin	Rare	Autosomal recessive	Hepcidin regulation	Severe parenchymal iron overload	Severe
Haemochromatosis type IIB	HAMP	Hepcidin	Very rare	Autosomal recessive	Regulating the export of iron from enterocytes, macrophages, and hepatocytes	Severe parenchymal iron overload	Severe
Haemochromatosis type III	TfR2	Transferrin receptor 2	Rare	Autosomal recessive	Hepcidin regulation	Parenchymal iron overload, liver disease	Intermediate
Haemochromatosis type IV	SLC40A1	Ferroportin	Rare	Autosomal dominant	Exporter of non-haem iron	Parenchymal and reticuloendothelial iron overload in the liver	Mild

HJV (HemoJuVelin), HAMP (Hepcidin AntiMicrobial Peptide), TfR2 (Transferrin Receptor 2).

Adapted from (Yun and Vincelette, 2015)

1.3.1.2 Secondary iron overload

Most of secondary iron overload disorders are characterised by some degree of ineffective erythropoiesis, that is, apoptosis of certain erythroid precursors, failure of erythroid maturation, and secondary expansion of erythropoiesis. Hepcidin is down-regulated by signalling molecules associated with these events, and the consequent anaemia, hypoxia, or both. Therefore, the down-regulation of hepcidin persists despite iron overload (Nemeth and Ganz, 2006). Erythrocyte transfusions contribute substantially to the iron burden in patients with these disorders (Brittenham, 2011).

Transfusional iron overload: Thalassemia

Around 15 million people have clinically apparent α- or β-thalassemia worldwide. In patients with severe forms of thalassemia, iron overload is a major cause of illness, whether or not they receive regular transfusions (Hershko, 2010). Therapeutic option in thalassemia is recurrent blood transfusion. Therefore, these patients requires continuous periodic assessment of iron overload to prevent long term complications. Thalassemia major patients undergoing regular blood transfusion are predisposed to iron overload, which can affect endocrine organs such as heart, pancreas, liver, etc. (L N et al., 2015).

Dietary iron overload

It has been described in sub-Saharan Africa an iron overload as a result from the ingestion of large amounts of iron derived from traditional home brewed beer fermented in non-galvanised steel drums. However, only a small percentage of these beer drinkers get this condition. Evidence suggests that genetic predispositions, other than HFE gene, may be involved, but the exact putative locus has not been identified (Siah et al., 2006).

Currently, it is not believed that a high dietary iron intake could cause iron overload without coexisting genetic disorder.

Other causes of iron overload

Congenital sideroblastics anaemias are heterogeneous disorders of haem synthesis. In these conditions, iron accumulates in mitochondria, producing the characteristic ring sideroblasts. The treatment of secondary iron overload consists in phlebotomy, chelation, or both (Fleming and Ponka, 2012).

Myelodysplastic syndromes and aplastic anaemias are disorders also associated with iron overload, particularly when exacerbated by multiple erythrocyte transfusions. These conditions are characterised by an ineffective haematopoiesis and peripheral cytopenias (Mahesh et al., 2008).

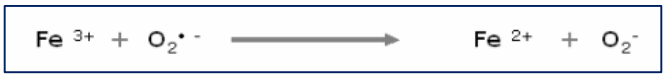
Friedreich's ataxia is an inherited neurodegenerative disease with an alteration in the first intron of the Friedreich's ataxia (FRDA) gene. It is a classic disorder associated with mitochondrial iron accumulation (Rivaud-Pechoux et al., 1998).

Hepatitis C virus infection is associated with an accumulation of iron in the liver parenchyma. Thus, many patients with chronic hepatitis C virus infection often have elevated serum iron, transferrin saturation, and ferritin levels (Boujaoude et al., 2000).

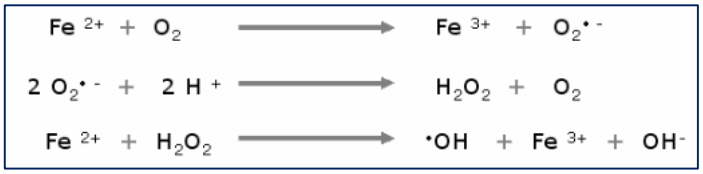
1.3.2 Iron and oxidative stress theory

Iron has the ability to accept and donate electrons easily, rising from an oxidised state (ferric iron) to a reduced state (ferrous iron). This capability makes it physiologically essential, but also biochemically dangerous, since it can participate in redox reactions that lead to generation of ROS. ROS, such as hydroxyl radical, increases the oxidative stress, a condition that it is believed to be involved in numerous pathological disorders (Schulze and Hu, 2005) (Drews et al., 2010).

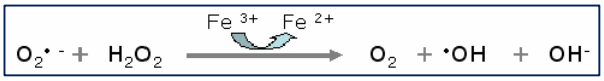
In the Haber-Weiss reaction hydroxyl radicals are generated in the presence of hydrogen peroxide and iron ions (Lipinski, 2011). The first step involves reduction of ferric into ferrous ion:



In the Fenton reaction, ferrous iron fosters the generation of hydroxyl radicals from hydrogen peroxide to generate ferric iron, hydroxide and hydroxyl radicals.



Thereafter, ferric iron is reduced by the superoxide radical to produce ferrous iron. The Haber–Weiss reaction uses iron to catalyse the generation of hydroxyl radicals from hydrogen peroxide and superoxide radical (Papanikolaou and Pantopoulos, 2005).



In addition, iron also reacts directly with organic molecules to generate peroxyl (ROO˙), alkoxyl (RO˙), thiyl (RS˙) or thiyl-peroxyl (RSOO˙) radicals, and ferrous iron can be oxidised by oxygen generating the superoxide radical (Papanikolaou and Pantopoulos, 2005).

Therefore, iron excess can cause damage to various tissues through increasing oxidative stress by the ROS generated, resulting in the onset of disease (Tiedge et al., 1997).

1.3.3 Diseases associated with iron overload

Cancer

It has been suggested that iron is a risk factor for cancer mainly due to its pro-oxidant activity, which can lead to oxidative DNA damage. A recent systematic review and meta-analysis found that elevated haem iron intake is associated with the risk of cancer. This relationship was observed in colorectal, and colon cancer meta-analysis, but not with breast and lung cancer (Fonseca-Nunes et al., 2014). Epidemiological studies show that red and processed meat intake is associated with an increased risk of colorectal cancer. Haem iron, heterocyclic amines, and endogenous N-nitroso compounds are proposed to explain this effect, but their relative contribution is unknown. Recently, a study among rats found that haem iron increased the number of preneoplastic lesions, but dietary heterocyclic amines and N-nitroso compounds had no effect on carcinogenesis. Authors suggested that haem iron could initiate carcinogenesis through lipid peroxidation (Bastide et al., 2015).

Regarding the relationship between body iron status and cancer risk remains inconclusive. A recent meta-analysis showed an unexpected inverse association between iron overload and the risk of developing colorectal cancer (Fonseca-Nunes et al., 2014). Otherwise, in patients with iron overload, iron excess is deposited predominantly in the liver, pancreas, and heart. As the primary site for body iron storage, the liver is usually the first organ affected, which is clinically manifested as fibrosis, cirrhosis, and hepatocellular carcinoma (Batts, 2007).

Neurodegenerative diseases

Accumulation of iron is often detected in the brains of people suffering from neurodegenerative diseases such as Parkinson (Bartzokis et al., 1999), Alzheimer's (Bartzokis et al., 1994), and Huntington's (Jurgens et al., 2010). These disorders can result from both defects in iron metabolism or iron accumulation in specific brain regions (Batista-Nascimento et al., 2012). However, remains to be determined whether iron contributes to the progression of these diseases.

Alzheimer's disease

Alzheimer's disease is the most common cause of age-related neurodegeneration. This disease is characterised by the accumulation of aggregates of insoluble amyloid-β protein, and neurofibrillary tangles consisting of precipitates/aggregates of hyperphosphorylated tau protein, which result in the progressive loss of memory, speech, task performance, and recognition of people and objects (Ross and Poirier, 2004). Iron has been associated with Alzheimer's disease, but the mechanism that mediates this relationship is not well-established. Iron accumulation has been observed in and around the amyloid senile plaques and neurofibrillary tangles (Smith et al., 1997). Other studies have also suggested that high iron toxicity may be due to the propensity of ferrous iron to generate ROS (Jomova and Valko, 2011).

Parkinson's Disease

Parkinson's Disease is a progressive disorder that manifests as tremor at rest, bradykinesia, gait abnormalities, rigidity, postural dysfunction, and loss of balance (Jankovic, 2008). It is the most prevalent neurodegenerative disorder, after Alzheimer's disease, affecting about 2% of people over 65 years old. Parkinson's Disease is characterised by the loss of the substantia nigra dopaminergic neurons (Irizarry et al., 1998) and the deposition of intracellular inclusion bodies known as Lewy bodies. Several studies have confirmed an increase of iron in the substantia nigra of most severe cases of Parkinson's Disease (Hirsch et al., 1991). Anyway, there is a general agreement that total nigral iron levels increase in Parkinson's Disease, possibly leading to nigrostriatal dopamine neuron degeneration as a result of its ability to produce ROS and cause lipid peroxidation (Jomova et al., 2010).

Osteoporosis

Many lines of evidence indicated that iron overload affects bone tissue causing both osteopenia and osteoporosis (Weinberg, 2006), and a high prevalence of fractures (Vogiatzi et al., 2009a). It has been suggested that elevated intracellular iron concentrations has a deleterious effect on differentiation, proliferation, and activity of

osteoblasts, which synthesise osteocalcin (OC) (Yamasaki and Hagiwara, 2009), probably through increased oxidative stress (Zhao et al., 2012).

Cardiovascular disease

Ever since the relationship between iron and cardiovascular disease was proposed by Jerome Sullivan in 1981 (Sullivan, 1981), many studies have attempted to confirm this hypothesis. However, the role of iron stores in CVD remains largely unknown. The disparity in results could be due to different iron biomarkers used in the studies (Muñoz-Bravo et al., 2013). Studies are needed to clarify the true effect of iron status on CVD.

Nevertheless, a recent meta-analysis showed that higher dietary haem iron intake was associated with an increased risk of CVD, whereas no association was found between CVD and non-haem iron intake or total iron intake (Fang et al., 2014).

Metabolic syndrome (MetS)

A recent meta-analysis showed that increased ferritin concentrations was positively associated with MetS (Abril-Ulloa et al., 2014). Oxidative stress could be the potential mechanism explaining iron-induced MetS (Lipinski, 2011).

Type 2 diabetes mellitus

Several prospective (Salonen et al., 1998) (Lee et al., 2004) (Jiang et al., 2004a) (Rajpathak et al., 2006) and cross-sectional (Luan de et al., 2008) studies have found an association between haem iron and risk of T2DM. Moreover, the direct association between haem iron intake before pregnancy and/or during the early period of pregnancy and the risk of gestational diabetes was also reported in the Nurse's Health Study (NHS) II (Bowers et al., 2011) and the U.S. Omega cohort (Qiu et al., 2011). It has been speculated that the increase of iron stores could play a role in the production of ROS, such as hydroxyl radicals, leading tissue damage (Lipinski, 2011).

2. TYPE 2 DIABETES MELLITUS

2.1 GLUCOSE METABOLISM

A fundamental mechanism for the maintenance of glucose homeostasis is the rapid action of insulin to stimulate glucose uptake and metabolism in peripheral tissues. Skeletal muscle is the primary site of glucose disposal in the insulin-stimulated state. The ability of insulin to increase glucose transport in skeletal muscle, and other peripheral tissues, is elicited by the translocation of glucose transporter 4 (GLUT4), the major insulin regulated GLUT, from intracellular vesicles to the plasma membrane and transverse tubules (Choi and Kim, 2010).

In normal subjects, the blood glucose level normally ranges between 90 and 120mg/dL. After a meal, this may go up to 250–300mg/dL. Meanwhile, the fasting insulin level remains at 15μU/mL and after the intake of food, it may go up to 40μU/mL. This increase in insulin secretion is responsible for the post-prandial decrease in glucose, which reaches the normal level after about 2 hours. Removal of this excess glucose by insulin occurs due to glucose uptake and storage in insulin target cells such as skeletal muscle cells, hepatocytes and adipocytes. About 75% of this glucose is stored in skeletal muscle cells; therefore, it is the major target cell of insulin, while the rest is stored in the liver and adipocytes (Bhattacharya et al., 2007).

Pancreatic islet β-cell

Insulin secretion from pancreatic islet β-cells is stimulated by glucose metabolism. When blood glucose is elevated beyond the Km of GLUT2, glucose is rapidly taken up by the β-cell via this transporter. Glucose is phosphorylated via glucokinase, which is the rate-limiting step of β-cell glucose metabolism. Further, glucose degradation leads to formation of pyruvate, which is then guided to the tricarboxylic acid cycle in the mitochondria. The formation of ATP leads to a rise the ATP/ADP ratio, resulting in closure of ATP-sensitive K^+ channels. The closure of the potassium channels will alter the

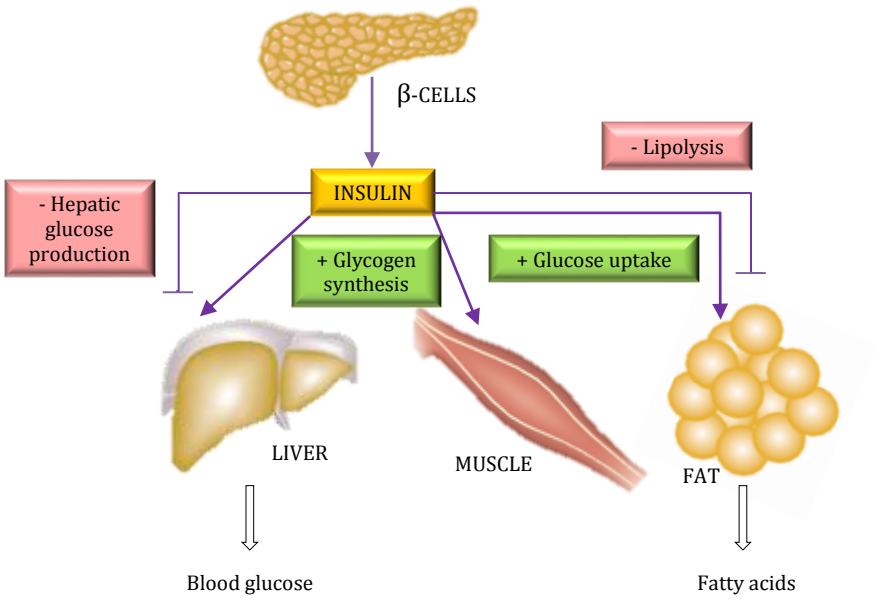
membrane potential and open calcium channels, which triggers the release of preformed insulin-containing granules, plasma membrane depolarization, activation of voltage-gated calcium channels and calcium-mediated stimulation of granule exocytosis (Stumvoll et al., 2005) (Muoio and Newgard, 2008).

Insulin receptor and signalling

Insulin is a potent anabolic hormone (figure 10), which exerts a variety of effects on many types of cells. The main metabolic actions of insulin are, among others (Højlund, 2014):

- to stimulate glucose uptake in skeletal muscle and fat
- to promote glycogen synthesis in skeletal muscle and liver
- to suppress hepatic glucose production
- to inhibit lipolysis in adipocytes

Figure 10. Insulin functions.



Adapted from (Stumvoll et al., 2005)

The action of insulin is initiated through its binding with the target cell surface receptor, the insulin receptor. The insulin receptor is a heterotetramer consisting of two α subunits and two β subunits that are linked by disulphide bonds. Insulin binds to the extracellular α subunit and transduces signals across the plasma membrane, which activates the intracellular tyrosine kinase C terminal domain of the β subunit. Binding of insulin to insulin receptor effects a series of intramolecular transphosphorylation reactions. Although insulin receptor are present on the surface of virtually all cells, their expression in classical insulin target tissues, i.e. muscle, liver and fat, is extremely high (Brunetti et al., 2001). Autophosphorylation of the insulin receptor tyrosine residue stimulates the catalytic activity of receptor tyrosine kinase, which recruits insulin receptor substrate (IRS) proteins (IRS-1 and IRS-2) (Rosen, 1987) (Pessin and Saltiel, 2000). Phosphorylation of IRS1 and IRS2 leads to their association with the phosphatidylinositol 3-kinase (PI3K), which activates Akt/PKB. Akt/PKB plays an important role by linking GLUT4, the insulin-dependent GLUT protein, to the insulin signalling pathway. It activates GLUT4 which moves to the cell surface to transport glucose into the cell (Pessin et al., 1999) (Martin et al., 2000) (Kupriyanova and Kandror, 1999).

2.2 DEFINITION AND CLASSIFICATION OF DIABETES MELLITUS

Diabetes is a chronic disease that occurs when the body cannot produce enough insulin or cannot use insulin effectively (Harris, M Zimmet, 2003). According to the American Diabetes Association (ADA), diabetes mellitus is “a group of metabolic diseases characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels” (American Diabetes Association, 2013). Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction. Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial and

cerebrovascular disease. Also, hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes (American Diabetes Association, 2013).

According to ADA, there are four main groups of diabetes mellitus: type 1 diabetes mellitus (T1DM), T2DM, gestational diabetes mellitus (GDM), and "specific types due to other causes" (American Diabetes Association, 2014).

Type 1 diabetes mellitus

This form of diabetes, previously encompassed by the terms insulin-dependent diabetes or juvenile-onset diabetes, accounts for 5–10% of diabetes. T1DM is caused by an absolute deficiency of insulin secretion, brought about by a cellular-mediated autoimmune destruction of the β-cells of the pancreas (American Diabetes Association, 2015).

Some forms of T1DM, denominated idiopathic diabetes, have unknown aetiologies. These patients have permanent insulinopenia and are prone to ketoacidosis, but have no evidence of autoimmunity. Although only a minority of patients with T1DM fall into this category, of those who do, most are of African or Asian ancestry.

Type 2 diabetes mellitus

T2DM, previously referred to as non–insulin-dependent diabetes or adult onset diabetes, accounts for around 90–95% of all diabetes. The cause of T2DM is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response (American Diabetes Association, 2015).

Although the specific aetiologies are not completely understood, most patients are obese, and obesity itself causes some degree of IR. Autoimmune destruction of β-cells does not occur in this patients, thereby, at least initially, and often throughout their lifetime, these individuals do not need insulin treatment to survive (American Diabetes Association, 2015).

This form of diabetes frequently goes undiagnosed for many years because the hyperglycaemia develops gradually and at earlier stages is often not severe enough for the patient to notice any of the classic symptoms of diabetes (American Diabetes Association, 2015).

Gestational diabetes mellitus

For many years, GDM was defined as any degree of glucose intolerance that was first recognised during pregnancy (Group, 1979), regardless of whether the condition may have predated the pregnancy or persisted after the pregnancy. This definition facilitated a uniform strategy for detection and classification of GDM, but it was limited by imprecision because there are pregnant women with undiagnosed type 2 diabetes. Therefore, ADA proposes that women with diabetes in the first trimester should receive a diagnostic of T2DM. GDM is diabetes diagnosed in the second or third trimester of pregnancy that is not clearly overt diabetes (American Diabetes Association, 2014).

GDM can lead to serious health risks to the mother and her infant and increase the risk for developing type 2 diabetes later in life.

Specific types due to other causes

This group includes monogenic diabetes syndromes, such as neonatal diabetes and maturity-onset diabetes of the young (MODY), diseases of the exocrine pancreas, such as cystic fibrosis, and drug- or chemical-induced diabetes, such as in the treatment of HIV/AIDS or after organ transplantation (American Diabetes Association, 2015).

2.3 PATHOPHYSIOLOGY OF TYPE 2 DIABETES MELLITUS: KEY MECHANISMS IN THE ONSET OF TYPE 2 DIABETES MELLITUS

T2DM is characterised mainly, by a progressive failure of pancreatic β-cell function and/or IR. Once insulin over-secretion can no longer compensate for the degree of IR, hyperglycaemia becomes clinically significant and deterioration of residual β-cell reserve accelerates.

2.3.1 Insulin resistance

The concept of insulin resistance (IR) was proposed as early as 1936 (Himsworth, 1936) to describe diabetic patients requiring high doses of insulin. IR is said to be present when the biological effects of insulin are less than expected for both glucose disposal in skeletal muscle and suppression of endogenous glucose production primarily in the liver (Dinneen et al., 1992). Thus, endogenous glucose production is accelerated in patients with T2DM or impaired fasting glucose (IFG) (Weyer et al., 1999) (Meyer et al., 1998). Because this increase occurs in the presence of hyperinsulinaemia, at least in the early and intermediate disease stages, hepatic insulin resistance is the driving force of hyperglycaemia of T2DM (Stumvoll et al., 2005).

IR is strongly associated with obesity and physical inactivity, and several mechanisms mediating this interaction have been identified. A number of circulating hormones, cytokines, and metabolic fuels, such as non-esterified fatty acids (NEFA) originate in the adipocyte and modulate insulin action. An increased mass of stored triglyceride, especially in visceral or deep subcutaneous adipose depots, leads to large adipocytes that are themselves resistant to the ability of insulin to suppress lipolysis. This results in increased release and circulating levels of NEFA and glycerol, both of which aggravate IR in skeletal muscle and liver (Boden, 1997). Thus, increased intramyocellular lipids are associated with skeletal muscle IR (Machann et al., 2004). In addition to the increased concentrations of NEFA, inflammatory cytokines, such as tumour necrosis factor α (TNFα) and interleukin 6, released by expanded visceral adipose tissue, also adversely affect the insulin signalling cascade (Rajala and Scherer, 2003) (Ravussin and Smith, 2002).

In states of IR, one or more of the following molecular mechanisms to block insulin signalling are likely to be involved. The normal phosphorylation of the receptor and the IRS proteins may be counteracted by dephosphorylation of these by cellular protein-tyrosine phosphatases and by protein phosphorylation on serine and threonine residues, which often occur together (Zick, 2001). In addition, serine/threonine phosphorylation of IRS1 may reduce its ability to act as a substrate for the tyrosine kinase activity of the IR and inhibits its coupling to its major downstream effector systems. Signal downregulation can also occur through internalisation and loss of the insulin receptor from the cell surface and degradation of IRS proteins (Zhande et al., 2002).

2.3.2 Insulin deficiency

β-cell dysfunction is a critical component in the T2DM pathogenesis. Generally, normoglycaemia is maintained by the balanced interplay between insulin action and insulin secretion. Importantly, the normal pancreatic β-cell can adapt to changes in insulin action, i.e., a decrease in insulin action is accompanied by up-regulation of insulin secretion (and vice versa) (Stumvoll et al., 2005). However, at the same time, concentrations of blood glucose at fasting and 2 hours after glucose load will increase mildly (Stumvoll et al., 2003). This increase may well be small, but over time becomes damaging, because of glucose toxicity, and in itself a cause for β-cell dysfunction. Thus, even with unlimited β-cell reserve, IR paves the way for hyperglycaemia and T2DM (Stumvoll et al., 2005).

The notion that hyperglycaemia itself can decrease insulin secretion has led to the concept of glucose toxicity, which implies the development of irreversible damage to cellular components of insulin production over time (Robertson et al., 2003) (Yki-Järvinen, 1992). Indeed, deterioration of insulin secretion over time is the usual course in most patients, and many patients will end with more or less severe insulin deficiency after about 10 years of diabetes (Wallace and Matthews, 2002). In β-cell, oxidative glucose metabolism will always lead to production of ROS, normally detoxified by catalase and superoxide dismutase. β-cell are equipped with a low amount of these proteins and also of the redox-regulating enzyme glutathione peroxidase (Robertson et

al., 2003). Hyperglycaemia has been proposed to lead to large amounts of ROS in β-cell, with subsequent damage to cellular components (Stumvoll et al., 2005).

Lipotoxicity

More recently, the concept of lipotoxicity involving the β-cell has been put forward. Fatty acids lead to enhanced insulin secretion in acute studies, but after 24 hours, they actually inhibit insulin secretion. In the presence of glucose, fatty acid oxidation in β-cell is inhibited and accumulation of long-chain acyl coenzyme A occurs (Robertson et al., 2004). Long-chain acyl coenzyme A itself can diminish the insulin secretory process by opening β-cell potassium channels. A second mechanism might be increased expression of uncoupling protein-2, which would lead to reduced ATP formation and, hence, decreased insulin secretion. A third mechanism might involve apoptosis of β-cell, possibly via fatty acid or triglyceride-induced ceramide synthesis or generation of nitric oxide. (Stumvoll et al., 2005)

2.4 RISK FACTORS FOR TYPE 2 DIABETES MELLITUS

T2DM is a multifactorial disease that increases with age, obesity, and lack of physical activity. It occurs more frequently in women with prior GDM and in individuals with hypertension or dyslipidaemia, and its prevalence varies in different racial/ethnic subgroups. It is often associated with a strong genetic predisposition, more so than is the autoimmune form of T1DM. However, the genetics of this form of diabetes are complex and not fully defined (American Diabetes Association, 2015). The risk factors of T2DM may be classified as modifiable and non-modifiable risk factors, depending on whether it is possible to prevent them or not.

2.4.1 Modifiable risk factors

Overweight and obesity

WHO defines overweight and obesity as an abnormal or excessive fat accumulation that may impair health (World Health Organization, 2014). WHO classifies overweight and obesity in adults through Body mass index (BMI), a simple index of weight-for-height that is commonly used. BMI is defined as a person's weight in kilograms divided by the square of height in meters (kg/m^2). Thereby, BMI ≥25kg/m^2 is defined as overweight, and a BMI ≥30kg/m^2 is defined as obesity. Both, overweight and obesity, affect a large part of adults in developed countries and are increasing rapidly in developing countries.

Overweight and obesity are pushing the global diabetes epidemic. In 2014, more than 1.9 billion adults, over 18 years, were overweight. Of these over 600 million were obese (World Health Organization, 2014). The severity of T2DM is also closely linked with BMI. Obese people have seven times greater risk of T2DM than those of healthy weight, while overweight people have a threefold increase the risk of T2DM (Abdullah et al., 2010). Duration of obesity has also been found to increase risk of developing T2DM, with greater risk in people who have been obese for longer periods of time (Abdullah et al., 2011). In addition, severity of obesity has shown to increase the risk of T2DM. Obese people with BMI ≥40 kg/m^2 were at an even greater risk of T2DM, when compared to obese people with a lower BMI (30- 39.9 kg/m^2) (Vinciguerra et al., 2013). Whilst it is known that body fat distribution is an important determinant of increased risk of diabetes, the precise mechanism of association remains unclear.

Dietary habits

Excessive caloric intake is a major driving force behind growing obesity and T2DM, but diet quality also has independent effects (Hu, 2011). A considerable body of evidence shows the negative effects of an unhealthy diet on T2DM. The NHS showed that the quality of fats and carbohydrates play an important role in the development of diabetes, independent of BMI and other risk factors. Thus, higher dietary glycaemic load and trans fat are associated with increased diabetes risk, whilst greater consumption of cereal fibre

and polyunsaturated fat are associated with decreased risk (Hu et al., 2001). A meta-analysis also showed that a 2 serving/day increment in whole-grain intake was associated with a 21% lower risk of diabetes (de Munter et al., 2007).

Globalization have spurred nutrition transitions in many countries. This nutritional shift involves increased consumption of animal products and energy-dense foods, decreased fibre, and more frequent intake of fast foods. Thus, higher consumption of sugar-sweetened beverages (SSB) increases also the risk T2DM even while taking into account the body weight. Recent meta-analyses found that individuals in the highest SSB intake (most often 1–2 servings/day) had between 18% (Imamura et al., 2015) and 26% (Malik et al., 2010) greater risk of developing the disease than those in the lowest SSB intake, 13% after adjusted by adiposity (Imamura et al., 2015). A high consumption of SSBs, with large quantities of rapidly absorbable carbohydrates, results in a high dietary glycaemic load that drives to quick increases in blood glucose and insulin levels. A high glycaemic load diet may lead to pancreatic β-cell exhaustion in the long run, which has been implicated in increased risk of T2DM (Hu and Willett, 2002). Fructose, an important sugar in SSBs, is preferentially metabolised to lipid in the liver, leading to increased hepatic de novo lipogenesis, dyslipidaemia, IR (Stanhope and Havel, 2010), and it may also promote visceral adiposity (Stanhope et al., 2009), risk factors related to the development of T2DM. Other foods with a high glycaemic index or glycaemic load, such as white rice, has also associated with increased risk of T2DM (Villegas et al., 2007), whereas consumption of brown rice, a whole grain, protected against the disease (Sun et al., 2010).

Moderate alcohol consumption is also associated with reduced risk of diabetes. Thus, a meta-analysis found a U-shaped relationship, with a 30% reduced risk of the disease among those consuming 6-48g/day of alcohol compared with heavy drinkers or abstainers (Koppes et al., 2005). The risk of diabetes among those who consumed three or more drinks/day was similar to that of abstainers. Moderate alcohol consumption improves insulin sensitivity, increases HDL-cholesterol and adiponectin, and the anti-inflammatory effect of alcohol. On the contrary, heavy alcohol intake has multiple deleterious metabolic effects, including excess caloric intake and obesity, increased

triglyceride levels, pancreatitis, disturbance of carbohydrate and glucose metabolism, and impairment of liver function (Koppes et al., 2005).

Other foods, such as fruit and vegetables (Wu et al., 2015), specially green leafy vegetable, (Cooper et al., 2012) caffeinated and decaffeinated coffee (Ding et al., 2014) and tea (Yang et al., 2014) in a dose–response way, among other, have shown their protective effect against the development of T2DM.

In addition, the overall dietary pattern seems to have an even more important role than foods separately regarding disease. Thereby, several dietary patterns have been related to diabetes risk. The western dietary pattern, strongly related to inflammatory markers, is characterised by high intakes of SSBs, refined grains, and red and processed meat, but low consumption of wine, coffee, cruciferous vegetables, and yellow vegetables. As expected, this pro-inflammatory pattern was strongly associated with an increased risk of diabetes (Schulze et al., 2005), suggesting that chronic inflammation may mediate the association between a Western dietary pattern and risk of T2DM. Moreover, Mediterranean diet is akin to the prudent diet, but has a higher total fat content, because it contains sizeable amounts of MUFA in the form of olive oil. In addition, it has alcohol (red wine) in moderation, high amounts of vegetables, fruits, legumes, whole grains, fish, poultry and low-fat dairy products. Because of that, it has emerged as another recognizable and healthy pattern approach to disease prevention (Salas-Salvadó et al., 2011). Thereby, a Mediterranean diet enriched with extra-virgin olive oil but without energy restrictions, has been related to a 40% less risk of T2DM among persons with high cardiovascular risk (Salas-Salvadó et al., 2014). Several other studies have seen this association between Mediterranean pattern and risk of T2DM (Salas-Salvado et al., 2011) (Romaguera et al., 2011).

Sedentary lifestyle

Numerous epidemiologic studies show that increased physical activity reduces risk of diabetes, whereas sedentary behaviours increase risk. Thus, each 2-hour/day increment of time spent watching television (TV) was associated with a 14% increase in diabetes risk (Hu et al., 2003), whilst each 2-hour/day increment of standing or walking around

at home was associated with a 12% reduction in risk. Moreover, each 1-hour/day increment of brisk walking reduced the risk by 34%.

Among sedentary behaviours (TV watching, sitting at work, and other sitting), prolonged TV watching was associated with the highest risk. Watching TV typically takes the place of physical activity, thereby reducing energy expended, and it is also associated with greater food and total energy intake. In addition, people who spend more time watching TV tend to have unhealthy eating patterns characterised by increased consumption of snacks, sugary beverages, and fast foods. (Hu et al., 2003).

Smoking

Cigarette smoking is an independent risk factor for T2DM (Cho et al., 2009). A meta-analysis found a dose-response relationship between the number of cigarettes smoked and the diabetes risk. In addition, current smokers had a 45% increased risk of developing T2DM compared with non-smokers (Willi et al., 2007). Though underlying causes of how cigarette consumption increases the risk of T2DM is not entirely clear, several possible biological mechanisms have been proposed. First, although smokers tend to be leaner than non-smokers, smoking has been associated with increased risk of central obesity or abdominal fat (Barrett-Connor and Khaw, 1989) (Shimokata et al., 1989), an established risk factor for IR and T2DM. The accumulation of visceral adipose tissue among smokers may be because of increased plasma cortisol levels induced by stimulation of sympathetic nervous system activity (Grassi et al., 1992). Moreover, smoking has anti-estrogenic effects in women and decreases plasma testosterone in men (Meikle et al., 1988), which may also promote abdominal fat accumulation and IR (Hu, 2011). Second, nicotine exposure can cause β-cell dysfunction and increase β-cell apoptosis, as it was shown in animal models (Bruin et al., 2008). Smoking showed synergistic interaction with the status of low insulin secretion and high IR for developing diabetes (Cho et al., 2009). Finally, other studies indicated that smoking increases free radical oxidative damage and oxidative stress (Burke and FitzGerald, 2003), contributing to the development of T2DM (Paolisso et al., 1993) (Luo et al., 2015).

Furthermore, a recent meta-analyses showed that passive smoking is a risk factor of T2DM even in those who were not themselves active smokers (Wei et al., 2015).

2.4.2 Non-modifiable risk factors

Family history of diabetes

Family history of diabetes is a well-known risk factor for the development of T2DM. Both genetic and common environmental exposures shared within the family may influence this risk factor of T2DM. In a recent study in adults from Shanghai, individuals with a family history of diabetes were 1.29 times more likely to develop T2DM than those without (Wang et al., 2014). Studies have also observed that maternal history of diabetes showed higher risk of T2DM than paternal history (Tan et al., 2008) (Tam et al., 2014), and sibling history even more risk than parental history (Annis et al., 2005) (Chien et al., 2008) (Zhang et al., 2015).

The pathophysiological mechanism underlying these inter-generational effects remain largely unknown. Some studies reported that maternal history of diabetes is associated with both IR and impaired first-phase insulin secretion (Chen et al., 2012). However, others finding found no difference in insulin sensitivity and secretion in the offspring for groups stratified according to paternal and maternal inheritance (Chien et al., 2008). Epigenetic processes are also suspected to have a key role in the process (Ma et al., 2015).

Genetic factors

Common genetic variants contributing to diabetes susceptibility have been identified in recent years due to advent of genome-wide association studies (GWAS) (McCarthy, 2010). More than 40 genetic loci have been convincingly associated with T2DM, but they do not improve the clinical prediction of diabetes beyond traditional risk factors (Hu, 2011). Moreover, genetic scores based on the number of risk alleles appear to be similar across different ethnic groups, therefore, they do not explain ethnic differences in diabetes risk (Hu, 2011).

Introduction

T2DM is the result of interaction between genetic and environmental factors such as dietary pattern. The Health Professionals' Follow-up Study (HPFS) found significant interaction between a Western dietary pattern and a genetic risk score (GRS) of T2DM susceptibility based on 10 established single nucleotide polymorphisms (P = 0.020) (Qi et al., 2009). Interestingly, the Western dietary pattern was not associated with diabetes risk in those subjects with a lower GRS. Moreover, components which characterise the Western dietary pattern such as processed meat, red meat, and haem iron showed significant interactions with GRS in relation to T2DM risk (P for interaction 0.029, 0.020, and <0.001, respectively). This suggests that the detrimental effects of a Westernised diet may be enhanced by greater genetic susceptibility (Hu, 2011).

Moreover, the "thrifty genotype" hypothesis defends that diseases such as obesity and T2DM are the outcome of efficient genotypes for energy and fat storage, advantageous during long periods of nutrient scarcity (NEEL, 1962). It would be a mismatch between the ancestral genes and modern environment. This hypothesis may explain the high rates of diabetes in some indigenous populations such as Pima Indians. However, the detection of the "thrifty genes" has so far remained elusive.

The "thrifty phenotype" hypothesis would suppose a disarrangement between perinatal and adult life environments (Hales and Barker, 2001). It has been postulated that foetal undernutrition leads to metabolic changes that may increase the risk of disorders such as T2DM in adulthood (Gluckman et al., 2009). The risks of adverse consequences in adulthood are higher in a nutritionally rich than in a scarcity environment.

Both foetal undernutrition and overnutrition are related to an increase future risk of diabetes (Yajnik, 2009), likely, through epigenetics changes (chromatin modification, DNA methylation,…) rather than genetics (Burdge and Lillycrop, 2010).

Increasing age

One of the most prominent, but also non-modifiable, risk factor for T2DM is advancing age. The burden of T2DM has been accelerated in recent years by an increase in the ageing population. IDF Diabetes ATLAS 2013 points that, almost half of all adults with

diabetes are between the ages of 40 and 59 years. This age group will continue to comprise the greatest number of people with diabetes in the coming years. By 2035, it is expected that the number will increase to 264 million. The reasons for this association of age and many metabolic diseases are degenerative processes leading to cellular apoptosis beyond regeneration or repairs (Navarro and Boveris, 2007).

Gender factors

According IDF Diabetes ATLAS 2013, there are differences between men and women in T2DM prevalence (International Diabetes Federation, 2013). Thereby, there are about 14 million more men than women with diabetes (198 million men vs 184 million women) in 2013. However, this difference is expected to increase to 15 million (303 million men vs 288 million women) in 2035 (International Diabetes Federation, 2013).

There are fundamental aspects of the control of metabolic homeostasis that are regulated differently in males and females. This perspective discusses the most fundamental gender differences in metabolic homeostasis, diabetes, and obesity. Together, the role of genetic sex, the programming effect of testosterone in the prenatal period in males, and the activational role of sex hormones at puberty produce two different biological systems in males and females that need to be studied separately. These gender-specific differences in energy homeostasis and metabolic dysfunction represent a source of factors that can be justify the onset of diabetes, and other metabolic disease (Mauvais-Jarvis, 2015).

Ethnicity

T2DM prevalence is increasing worldwide, particularly among people from non-white ethnic groups (Venkataraman et al., 2004) (Brancati et al., 2000) (Tillin et al., 2013). It has been seen that T2DM is up to six times more common in people of South Asian descent and up to three times more common among people of African and African-Caribbean origin than white populations (Ntuk et al., 2014) (Tillin et al., 2013) (Bennet et al., 2014). Thus, south Asians seem to have the highest prevalence of diabetes, followed by Chinese and black participants, with whites having the lowest prevalence (Ntuk et al.,

2014). Obesity is a risk factor in all ethnic groups, but it has observed, in the UK Biobank study that the risk associated with obesity was two- to fourfold higher in non-white participants. Even, in non-white groups, the prevalence of diabetes was equivalent to that in white populations at much lower levels of BMI and waist circumference (Ntuk et al., 2014).

It has been postulated that ethnic differences may be explained by a higher IR among Asian and black populations that would increase the visceral and liver fat depots at a lower BMI. In addition, the "thrifty gene" inherited from Asian ancestors enabled to store energy more efficiently during long periods of nutrient scarcity, but predisposes to overweight and obesity in the current obesogenic environment (Bhopal, 2013), and therefore to an increase in the risk of T2DM. Lower birth weight, deficient physical activity, and other physiological differences such as reduced capacity for fat oxidation have also been proposed as related factors (Hall et al., 2010) (Bhopal, 2013).

Gestational diabetes mellitus

GDM is a significant and growing problem in healthcare worldwide. It has been estimated that the prevalence of GDM is of around 1-14% of all pregnant women, depending on the population studied and the diagnostic test employed (American Diabetes Association, 2013). As noted above, it is known that women who have had GDM are at a higher risk of developing T2DM later in life (Bellamy et al., 2009).

Moreover, there is no question that poorly controlled hyperglycaemia during pregnancy is harmful for the foetus and can cause significant morbidity. In this sense, it has been suggested that the offspring or mothers with GDM are at an increased risk of developing diabetes themselves. However, existing data do not support this hypothesis (Donovan and Cundy, 2015).

2.5 DIAGNOSTIC CRITERIA FOR PREDIABETES AND TYPE 2 DIABETES MELLITUS

Initially, diabetes has been diagnosed, according to the National Diabetes Data Group criteria, by the presence of the classic signs and symptoms of diabetes and unequivocally elevated blood glucose levels, by fasting plasma glucose (FPG) ≥140mg/dl, or by venous plasma glucose ≥200mg/dL at 2 hours after a 75-g oral glucose test (OGTT) (Group, 1979). Moreover, individuals with intermediate plasma glucose levels between those considered normal and those considered diabetic, were included in a new category named impaired glucose tolerance (IGT) (see table 9). It was proposed to abandon the terms chemical, latent, borderline, subclinical, and asymptomatic diabetes, which have been applied to persons in this class (Group, 1979). Thereafter, the World Health Organization (WHO) Expert Committee on Diabetes in 1980 added this new category in (World Health Organization, 1980).

In 1997, the first Expert Committee on the Diagnosis and Classification of Diabetes Mellitus revised the diagnostic criteria, using the observed association between FPG levels and presence of retinopathy as the key factor with which to identify glucose level threshold (ADA, 1997). These analyses confirmed the long-standing diagnostic 2-hours plasma glucose value of ≥200mg/dL (11.1mmol/L). However, the older FPG diagnostic cut point of 140 mg/dL (7.8mmol/L) was noted to identify far fewer individuals with diabetes than the 2-hours plasma glucose cut point. The FPG diagnostic cut point was reduced to ≥126mg/dL (7.0mmol/L).

Table 8. Evolution of diagnostic criteria for type 2 diabetes mellitus.

	NDDG 1979 OMS 1985	ADA 1997 OMS 1999	ADA 2010	OMS 2011
Fasting blood glucose (mg/dL)[a]	>140 (7.8 mmol/L)	>126 (7.0 mmol/L)		
2-hours plasma glucose (mg/dL)[a]	>200 (11.1 mmol/L)			
Random plasma glucose (mg/dL)[b]	>200 (11.1 mmol/L)			
HbA 1C (%)[a]			>6.5%	

[a] In the absence of unequivocal hyperglycemia, results should be confirmed by repeat testing.

[b] In patient with classic symptoms of hyperglycaemia or hyperglycaemic crisis.

Furthermore, the Expert Committee on Diagnosis and Classification of Diabetes Mellitus (ADA, 1997) established another intermediate group of individuals whose glucose levels do not meet criteria for diabetes, yet are higher than those considered normal, the IFG. It should be noted that the 2003 ADA Expert Committee report reduced the lower FPG cut point to define IFG from 110mg/dL (6.1mmol/L) to 100mg/dL (5.6mmol/L), in part to ensure that prevalence of IFG was similar to that of IGT (Genuth et al., 2003). However, the WHO and many other diabetes organizations did not adopt this change in the definition of IFG. The current criteria for diagnosing both groups of prediabetes, according to this Expert Committee on Diagnosis and Classification of Diabetes Mellitus (Genuth et al., 2003), are the following:

- **Impaired fasting glucose**, defined as having FPG levels 100mg/dL (5.6mmol/L) to 125mg/dL (6.9mmol/L).

- **Impaired glucose tolerance**, with 2-hour values in the OGTT of 140mg/dL (7.8mmol/L) to 199mg/dL (11.0mmol/L).

Individuals with IFG and/or IGT have relatively high risk to develop diabetes in the future. They are not clinical entities in their own right, but rather risk factors for diabetes as well as cardiovascular disease. IFG and IGT are associated with obesity (especially abdominal or visceral obesity), dyslipidaemia with high triglycerides and/or low HDL cholesterol, and hypertension (American Diabetes Association, 2015). Later, in 2010, ADA established an HbA1c range of 5.7% - 6.4% in order to identify individuals with prediabetes (American Diabetes Association, 2010).

Table 9. Evolution of diagnostic criteria for prediabetes, IFG and IGT.

		NDDG 1979 OMS 1985	ADA 1996 OMS 1999	ADA 2003	OMS 2006	ADA 2010	OMS 2011
IFG	Fasting plasma glucose (mg/dL)	≥ 140 (7.8 mmol/L)	110 - 125 (6.1-6.9mmol/L)	100 - 125 (5.6-6.9mmol/L)	110 - 125 (6.1-6.9mmol/L)	100 - 125 (5.6-6.9 mmol/L)	
IGT	2-hours plasma glucose (mg/dL)	140 - 199 (7.8-11.1 mmol/L)					
IFG & IGT	HbA1C					5.7 - 6.4%	

IFG (Impaired Fasting Glucose), IGT (Impaired Glucose Tolerance).

In 2008, an International Expert Committee was designated from the ADA, the European Association for the Study of Diabetes (EASD) and the International Diabetes Federation (IDF) in order to redefine the diagnosis of diabetes using glycated haemoglobin (HbA1c). They propose to use the A1C test to diagnose diabetes, with a threshold of ≥6.5%. The diagnostic A1C cut point of 6.5% is associated with an inflection point for retinopathy prevalence, as are the diagnostic thresholds for FPG and 2-hours plasma glucose (International Expert Committee, 2009). Finally, in 2010 the ADA incorporates for the first time HbA1c as a diagnostic test for diabetes, and defined three breakpoints for HbA1c ≤5.6%, non-diabetic levels; between 5.7% and 6.4%, prediabetic levels; and ≥6.5%, consistent with the diagnosis of diabetes (American Diabetes Association, 2010). In addition, patients with severe hyperglycaemia such as those who present with severe classic hyperglycaemic symptoms (polyuria, polydipsia, weight loss, polyphagia and blurred vision) or hyperglycaemic crisis can continue to be diagnosed when random (or casual) plasma glucose of ≥200mg/dL (11.1mmol/L) is found (see table 8).

2.5.1 Methods for the assessment of insulin resistance and β-cell dysfunction

IR is typically defined as decreased sensitivity or responsiveness to metabolic actions of insulin (Muniyappa et al., 2008). There are several methods to evaluated IR, such as:

Hyperinsulinemic euglycemic glucose clamp

The glucose clamp technique is the reference standard for directly determining metabolic insulin sensitivity in humans. It was originally developed by DeFronzo et al. in 1979 (DeFronzo et al., 1979). The technique consists of insulin infused intravenously at a constant rate that may range from 5 to 120 $\mu U \cdot m^{-2} \cdot min^{-1}$ (dose per body surface area per minute), after an overnight fast. This results in a new steady-state insulin level that is above the fasting level (hyperglycaemia). Glucose disposal is increased in skeletal muscle and adipose tissue, whereas hepatic glucose production is suppressed. In this moment, a bedside glucose analyser is used to frequently monitor blood glucose levels at 5- to 10-min intervals. Meanwhile, 20% dextrose is given intravenously at a variable rate to "clamp" blood glucose concentrations in the normal range (euglycaemia). Finally,

steady-state conditions is achieved for blood glucose, plasma insulin, and the glucose infusion rate. Thereby, the latter rate must be equal to the glucose disposal rate (Muniyappa et al., 2008).

Clearly this is unphysiological, thus the hyperinsulinaemic euglycaemic glucose clamp is not appropriate when an estimation of insulin action and glucose dynamics under normal physiological conditions is required. The main advantage of using the glucose clamp to estimate insulin sensitivity/resistance in humans is that it directly measures whole body glucose disposal at a given level of insulinaemia under steady-state conditions. It is a direct measure of insulin sensitivity (Muniyappa et al., 2008).

Frequently sampled intravenous glucose tolerance test

It provides an indirect measurement of metabolic insulin sensitivity/resistance according to glucose and insulin data obtained during a frequently sampled intravenous glucose tolerance test. After an overnight fast, an intravenous bolus of glucose (0.3g/kg body weight) is infused over 2 minutes starting at time 0. Currently, a modified frequently sampled intravenous glucose tolerance test (FSIVGTT) is used where exogenous insulin (4mU·kg-1·min-1) is also infused over 5min beginning 20min after the intravenous glucose bolus (Saad et al., 1997) (Quon et al., 1994). Blood samples are taken for plasma glucose and insulin measurements at different moments. These data are then subjected to minimal model analysis using the computer program MINMOD to generate an index of insulin sensitivity (Muniyappa et al., 2008).

Owing to the complexity of the hyperinsulinaemic euglycaemic clamp, the FSIVGTT may be preferred as an alternative option and, as such, can be considered the 'silver' standard (Bergman, 2007). The main FSIVGTT techniques available are the standard (classical) technique and the more modern insulin-modified version. Hyperinsulinaemic euglycaemic glucose clamp and FSIVGTT are considered reference techniques (Borai et al., 2011).

Introduction

Homeostasis model assessment

Homeostasis model assessment (HOMA), developed in 1985 (Matthews et al., 1985), is a model of interactions between glucose and insulin dynamics that is then used to predict fasting steady-state glucose and insulin concentrations for a wide range of possible combinations of IR and β-cell function. HOMA assumes a feedback loop between the liver and β-cell (Levy et al., 1998) (Matthews et al., 1985); i.e., glucose concentrations are regulated by insulin-dependent hepatic glucose production, whereas insulin levels depend on the pancreatic β-cell response to glucose concentrations. Thus, deficient β-cell function reflects a diminished response of β-cell to glucose-stimulated insulin secretion. Likewise, IR is reflected by diminished suppressive effect of insulin on hepatic glucose production. HOMA describes this glucose-insulin homeostasis by a set of empirically derived nonlinear equations. The model predicts fasting steady-state levels of plasma glucose and insulin for any given combination of pancreatic β-cell function and insulin sensitivity (Borai et al., 2011).

HOMA is a relatively simple, non-invasive alternative to the clamp technique(Borai et al., 2011), defined as:

$$\text{HOMA-IR} = \text{fasting glucose (mmol/L)} \times \text{fasting insulin (}\mu\text{IU/mL)}/22.5$$

Quantitative insulin sensitivity check index

Quantitative insulin sensitivity check index (QUICKI) is an empirically derived mathematical transformation of fasting blood glucose and plasma insulin concentrations that provides a reliable, reproducible, and accurate index of insulin sensitivity with excellent positive predictive power (Muniyappa et al., 2008).

$$\text{QUICKI} = 1/\ [\log(\text{fasting insulin, IU/mL}) + \log(\text{fasting glucose, mg/dL})].$$

Homeostasis model β assessment

HOMA-β is calculated using steady-state blood concentrations of fasting glucose and insulin to estimate the degree of β-cell deficiency and the target-tissue sensitivity to insulin (Cersosimo et al., 2014). The HOMA-β equation is:

HOMA-β = [20 * fasting insulin (μIU/mL)]/ [fasting glucose (mmol/L) - 3.5]

2.5.2 Biomarkers related to type 2 diabetes mellitus

Adiponectin

Human adiponectin, a 244-amino acid collagen-like protein is solely secreted by adipocytes and acts as a hormone with anti-inflammatory and insulin-sensitizing properties. Adiponectin secretion, in contrast to secretion of other adipokines, is paradoxically decreased in obesity which may be attributable to inhibition of adiponectin gene transcription (Ghoshal and Bhattacharyya, 2015).

Adiponectin exhibits two major mechanisms of action by which it inhibits T2DM, one by increasing insulin sensitivity and the other way is to increase fatty acid oxidation. APPL1, stimulated by adiponectin can interact with adiponectin receptors and can mediate the downstream events such as lipid oxidation and membrane translocation of GLUT4, thus increasing glucose uptake, providing a platform for increased insulin sensitization (Mao et al., 2006). APPL1 modulates the insulin signalling pathway by acting with Akt and PI3K (Manning and Cantley, 2007).

The major form of storing and transporting fatty acids is triglycerides. Adiponectin has been reported to decrease tissue triglyceride content by increasing the expression of CD36, a fatty acid transporter (Yamauchi et al., 2001). Thus, lowering of tissue triglyceride content promotes insulin sensitivity.

In addition, it has been established that adiponectin enhances insulin-stimulated IRS-1 tyrosine and Akt phosphorylation, facilitating GLUT4 translocation. Adiponectin also defeats T2DM by increasing fatty acid oxidation, which also elevates insulin sensitivity.

In summary, there are several mechanisms through which adiponectin may decrease the risk of T2DM, including: suppression of hepatic gluconeogenesis, stimulation of insulin secretion, increase insulin sensitivity, stimulation of fatty acid oxidation. Therefore, adiponectin could be a novel target for the therapeutic approach to treat diabetes mellitus (Ghoshal and Bhattacharyya, 2015).

Osteocalcin

Osteocalcin (OC) is the most prevalent non-collagenous protein, of 49 amino acids in human, produced in bone. It is a small protein (49 amino acids in human) that undergoes post-translational modification by vitamin K-dependent γ-carboxylation of three glutamic acid residues (Motyl et al., 2010). OC is a cell specific molecule secreted by osteoblasts, first produced as a prepropeptide, and its mature form is present in fair concentration in the circulation. Although OC is detected at high concentration in the bone extracellular matrix, it has several characteristic of a hormone. (Ferron and Lacombe, 2014). According to previous animal studies, it is the uncarboxylated form of OC, and not the carboxylated form of OC, that regulates glucose metabolism.

Analysis of the OC knockout mice (Ocn-/-) revealed that they are abnormally fat suggesting that somehow OC might affect glucose metabolism. A comprehensive analysis revealed that insulin secretion, glucose tolerance and insulin sensitivity are all decreased in Ocn-/- mice under normal chow diet. In addition, islets number, β-cell area, β-cell mass and insulin content are reduced in the pancreas of Ocn-/- mice. Importantly, OC appears to transcriptionally regulate insulin biosynthesis (Lee et al., 2007).

OC is also a potent insulin secretagogue due to its ability to increase cytosolic calcium levels (Hinoi et al., 2008). Therefore, osteoblast-derived OC control glucose metabolism by directly affecting pancreatic islets biology as well as insulin synthesis and secretion.

It has seen that OC infusions or injections in wild type mice decreased their fat mass, their serum triglyceride levels and the expression of lipolysis inducing genes (Tgl and Perilipin) in fat (Ferron et al., 2008)(Ferron et al., 2012). The mechanism by which fat mass is influenced by OC is unknown, however OC can directly induce expression of adiponectin and its target genes in white fat (Ferron et al., 2008). While a role for adiponectin in insulin sensitivity in mice fed a high fat diet has been suggested (Yamauchi et al., 2001), a recent study has shown that adiponectin regulates bone mass in mice fed a normal diet without affecting glucose metabolism (Kajimura et al., 2013). It was also reported that OC can directly modulate glucose transport in adipocytes, can suppress the secretion of pro-inflammatory cytokines and induce the secretion of anti-inflammatory cytokines as well as adiponectin (Hill et al., 2014).

In addition, OC infusion in wild-type mice increases the expression of genes involved in thermogenesis (Pgc1a and Ucp1) in brown adipose tissue (Ferron et al., 2008). OC might also be affecting glucose uptake in skeletal muscle since it has been shown to modulate this function in vascular smooth muscle cells (Idelevich et al., 2011). These observations suggested that the protective effect of OC on obesity and IR might be, at least in part, due to its capacity to increase energy expenditure in brown adipose tissue and skeletal muscle.

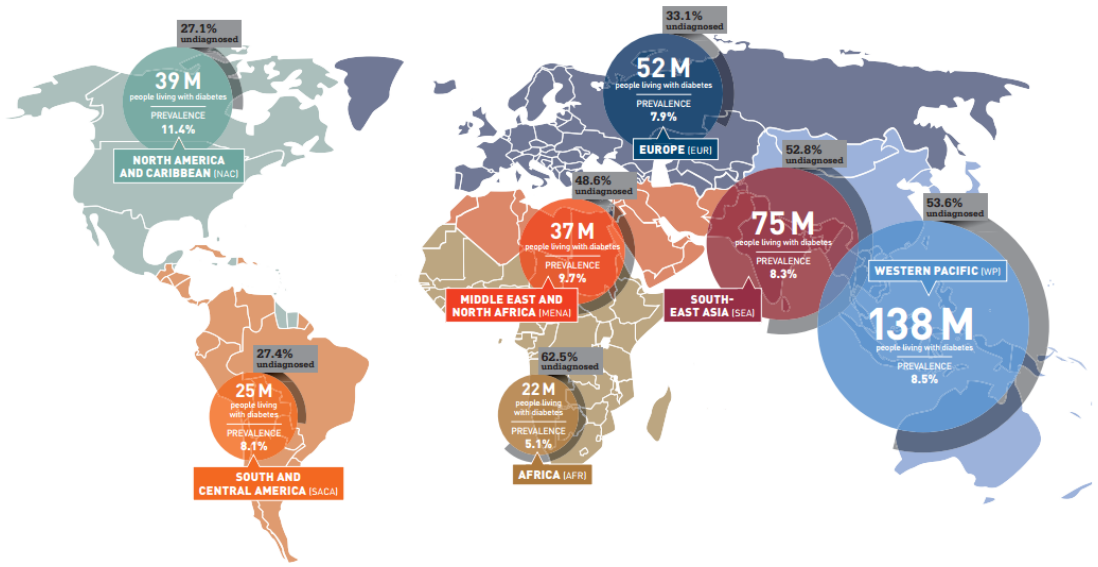
2.6 PREVALENCE AND INCIDENCE OF TYPE 2 DIABETES MELLITUS: EPIDEMIOLOGICAL STUDIES

Diabetes is the most common metabolic disorder worldwide, and its prevalence has been increasing in the last years in developed and developing countries (Souto et al., 2011) (International Diabetes Federation, 2013). It has been estimated that the number of people aged 20 to 79 affected by diabetes will increase from 285 million in 2010 (6.4%) to 439 million in 2030 (7.7%) (Shaw et al., 2010). Meanwhile, WHO notes a global prevalence of diabetes of 5% among adults aged >18 years (World Health Organization, 2015). IDF's most recent estimates indicate that 8.3% (387 million people) of adults in the same age range had diabetes in 2014, and the number is expected to rise beyond 592 million (55% more) in less than 25 years (International Diabetes Federation, 2013)

(International Diabetes Federation, 2014). This rise is associated with economic development, ageing populations, increasing urbanisation, dietary changes, reduced physical activity, and changes in other lifestyle patterns (International Diabetes Federation, 2013).

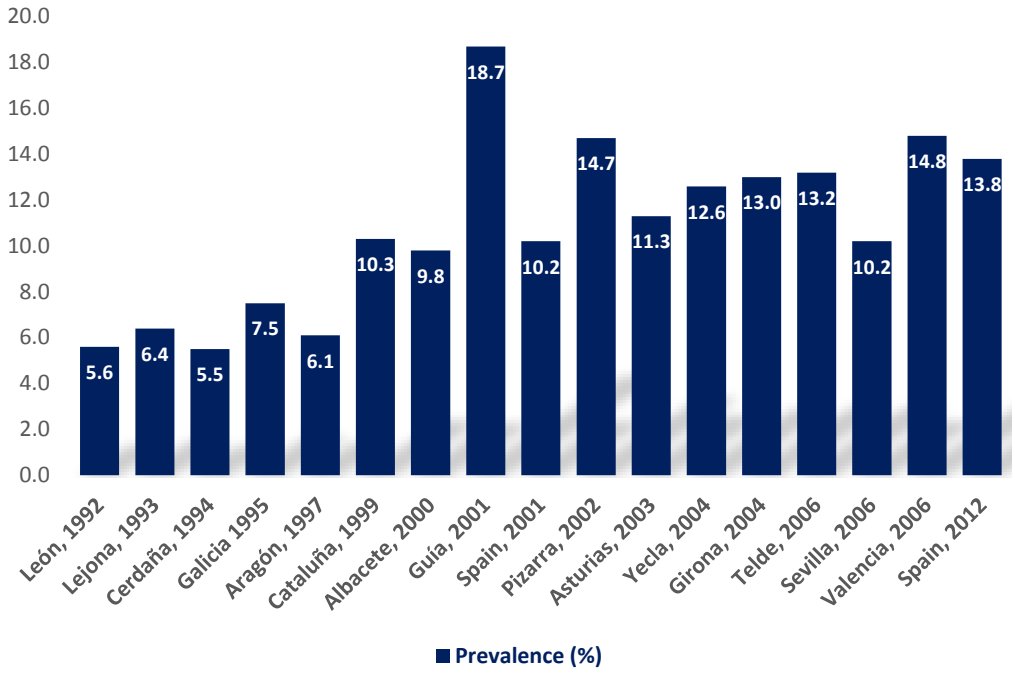
The majority of the 387 million people with diabetes are aged between 40 and 59, and 80% of them live in low- and middle-income countries. With more than 138 million people affected, the Western Pacific has more people with diabetes than any other region. In Europe, the number of people with diabetes is estimated to be 52 million (7.9%) of the adult population (see Figure 11), 37% over 50 years. Turkey has the highest prevalence (14.7%) and Germany has the greatest number of people with diabetes (7.3 million) in Europe, followed by Turkey (7.2 million), Russian Federation (6.8 million), Spain (3.7 million), Italy (3.5 million), France (3.2 million), and the UK (2.5 million) (International Diabetes Federation, 2014).

Figure 11. Number of people with diabetes by International Diabetes Federation Region.



Source: (International Diabetes Federation, 2014)

Figure 12. Evolution of the prevalence of T2DM in the last years in different regions of Spain.



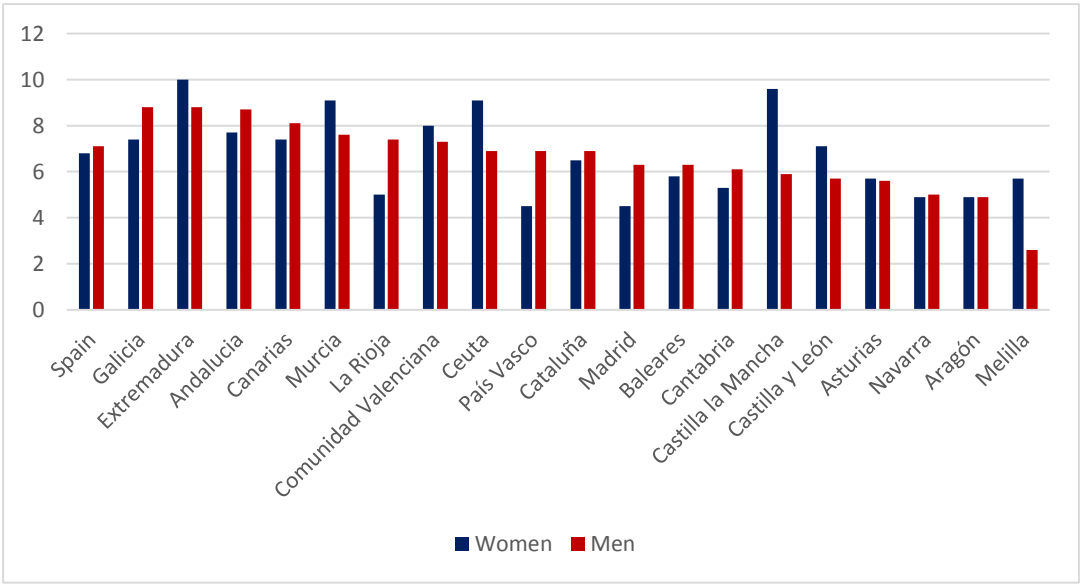
Update from Valdés et al. 2007

In Spain, traditionally, it has been used surveys, especially the National Health Survey (NHSurv) medical records or estimations based on drug consumption in order to determine the prevalence of T2DM. According to data from the NHSurv of Spain in 2013 (figure 13), Galicia, Extremadura, Andalucia and Murcia were the regions with higher prevalence of T2DM (up to 11%) (Ministerio de Sanidad Servicios Sociales e Igualdad, 2013). The last study done in the whole Spanish territory in people over 18 years, the Di@bet.es study, showed a higher prevalence of T2DM (13.8%) (Soriguer et al., 2012) than data from the last NHSurv of Spain (up to 11%) and the IDF Diabetes ATLAS (up to 10.5%) (International Diabetes Federation, 2014). Further, a 6% of the diabetics were unaware of their disease (Soriguer et al., 2012). The highest prevalence of diabetes in Spain has been observed in the Canary Islands (13.2%) in 2006 (Boronat et al., 2006), but we can perceive an increase in the prevalence of T2DM in the whole Spanish population from 1992 to 2012. Figure 12 shows the evolution of the prevalence of T2DM in the last years in different regions of Spain.

Introduction

Diabetes is one of the most common non-communicable diseases. It is the fourth or fifth leading cause of death in most high-income countries and there is substantial evidence that it is epidemic in many economically developing and newly industrialised countries. Approximately 5.1 million people aged 20 to 79 died from diabetes in 2013, represents 8.4% of global all-cause mortality among people in this age group. In Europe, one in 10 deaths in adults can be attributed to diabetes, 619,000 in 2013. The vast majority (90%) of these deaths occurred in people over 50. Diabetes and its complications are major causes of early death worldwide. About half of deaths (48%) are people under 60. Nonetheless, IDF estimates that as many as 175 million people worldwide, or near half of all people with diabetes, are unaware of their disease. Most of these cases are T2DM. Likely, because there are few symptoms during the early years of T2DM, although high blood glucose is silently damaging the body and diabetes complications may be developing (International Diabetes Federation, 2013).

Figure 13. Prevalence of diabetes (%) in adult population according to Spanish region and gender.



Adapted from National Health Survey 2013

The economic burden of this disease is enormous, taking up about USD 548 billion dollars in health spending (11% of the total spent worldwide) in 2013, and it is expected to rise beyond USD 627 billion dollars in 2035. A look at health spending on diabetes by region

reveals huge disparities in responses to the epidemic. Two regions spent more on diabetes than the rest of the regions put together: North America and Caribbean, with an estimated USD 263 billion, equal to nearly half the world's health expenditure on diabetes; and Europe with USD 147 billion. On the contrary, despite their growing diabetes populations, spending in South-East Asia and Africa accounted for less than 1% of all global health expenditure on the disease (International Diabetes Federation, 2013).

Finally, differences between men versus women, and rural versus urban were noted. According IDF Diabetes ATLAS, there are about 14 million more men than women with diabetes (198 million men vs 184 million women). However, this difference is expected to increase to 15 million (303 million men vs 288 million women) by 2035. Further, there are more people with diabetes living in urban (246 million) than in rural (136 million) areas. By 2035, the difference is expected to widen, with 347 million people living in urban areas and 145 million in rural areas (International Diabetes Federation, 2013).

Population-based studies of the incidence of T2DM, is less frequent than those that assess its prevalence. Two prospective, population-based studies have been undertaken, both in northern Spain (Asturias (Vazquez et al., 2000) and País Vasco (Valdés et al., 2007)); the incidence of T2DM was 8 and 10.8 cases per 1000 person-years, respectively. A later study carried out in southern Spain found a greater incidence than previous studies (19.1 cases per 1000 person-years). Probably the high prevalence of obesity and the increase in weight in sample, a risk factor on the onset of T2DM, could have played a role (Soriguer et al., 2008). More recently, a prospective study from Madrid, among subjects over 24 years, showed an incidence of diabetes in 2007 of 9.4 cases per 1000 person-years, although this incidence was reduced to 8.6 cases per 1000 person-years in 2010 (Martín Martínez et al., 2013).

T2DM incidence has also been studied in northern of European countries. Thus, in England, the incidence of T2DM was examined in a population-based longitudinal study (1990–2000) (Forouhi et al., 2007a). The 10-year cumulative incidence of diabetes was 7.3 per 1000 person-years. Curiously, diabetes incidence was lower in normoglycaemic subjects (2.4 per 1000 person-years), than in IFG individuals from 5.6 to 6.0mmol/L (6.2 per 1000 person-years) and IFG subjects from 6.1 to 6.9mmol/L (17.5 per 1000 person-

years). Similarly, the incidence of T2DM in Germany among normoglycaemic subjects was lower (5.5 per 1000 person-years) than in IFG subjects (24.2 per 1000 person-years) and IGT subjects (42.0 per 1000 person-years) (Rathmann et al., 2009), but significantly increased regarding England incidence (Forouhi et al., 2007a). In other European countries, the incidence of T2DM varies widely from Sweden (3.7 and 3.8 cases per 1000 person-years in 1997 and 2003, respectively (Ringborg et al., 2008)), to Italy (7.6 per 1000 person-years) (Bonora et al., 2004).

From USA, the National Center for Health Statistics (NCHS), Centers for Disease Control and Prevention (CDC) conducted continuously since 1957 the National Health Interview Survey (NHIS) in order to estimate the incidence of diagnosed diabetes in adults aged 18-79. From 1980 to 2013, the crude incidence of diagnosed diabetes increased from 3.3 to 7.1 per 1000 person-years (National Center for Health Statistics, 2015).

3. IRON EXCESS AND TYPE 2 DIABETES MELLITUS

3.1 THE ROLE OF IRON IN THE PATHOPHYSIOLOGAL MECHANISMS IMPLICATED IN THE ONSET OF TYPE 2 DIABETES MELLITUS

Iron seems to have a central role in the development of T2DM. Both, dietary iron intake, specially haem iron, and body iron stores have been related to the risk of T2DM, although the exact mechanism implicated is not completely understood.

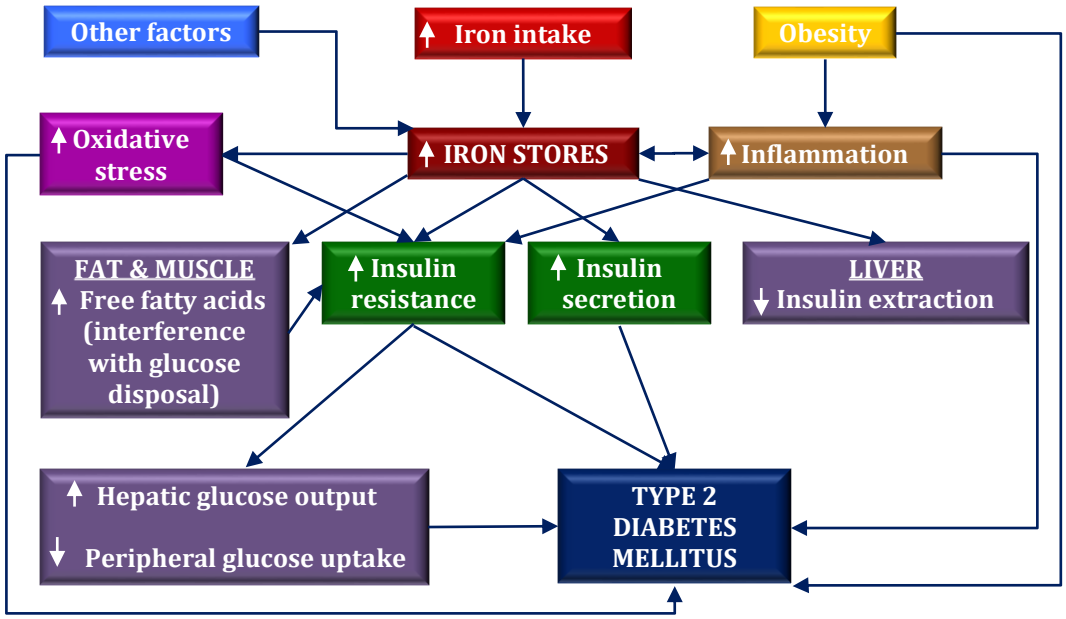
3.1.1 Iron intake and type 2 diabetes mellitus

Despite increasing evidence about the association between high haem iron intake and the risk of T2DM, the exact mechanism is not known. Neither is it fully understood why results vary according to the form of iron intake, non-haem and haem iron. There is strong evidence that supports that non-haem iron absorption in the proximal intestine is accurately regulated by hepcidin to ensure that overall body iron levels are maintained at adequate levels (Gulec et al., 2014). Meanwhile, haem iron absorption seems to be responsive to changes in body iron status, although to a lesser extent than non-haem iron. Recently, Cao et al. observed, in an animal model, that hepcidin has also impact in haem iron absorption (59%), although less than non-haem iron assimilation (63%) (Cao et al., 2014). In addition, it has been shown that elevated haem iron intake is associated with high levels of SF (Vander et al., 2006). It has been speculated that the increase of iron stores could play a role in the production of ROS, such as hydroxyl radicals, inducing tissue damage (Lipinski, 2011). This mechanism could give an explanation why high dietary haem iron intake, in addition to body iron stores (Rajpathak et al., 2009b) (Fonseca-Nunes et al., 2015) (Pourmoghaddas et al., 2014) (Lee et al., 2011) (Sharifi et al., 2010), has been related to T2DM (Rajpathak et al., 2006).

3.1.2 Body iron status and type 2 diabetes mellitus

Iron seems to have a central role in the aetiopathogenesis of T2DM (see figure 14) because of its ease to be reversibly oxidised and reduced. This ability turns iron a strong pro-oxidant agent that catalyses the generation of powerful ROS, such as hydroxyl radical, increasing the oxidative stress (Schulze and Hu, 2005) (Drews et al., 2010). It is believed that oxidants can cause the release of catalytic iron (Schulze and Hu, 2005); and thereby, a vicious cycle is initiated that leads to the production of more ROS. This contributes to tissue damage that may potentially elevate the risk of T2DM. Therefore, oxidative stress could be the mechanism by which iron excess is associated with a higher incidence of T2DM.

Figure 14. Potential mechanisms for the role of iron in aetiology of type 2 diabetes.



Adapted from (Rajpathak et al., 2009a)

Precisely, through its oxidative properties (Schulze and Hu, 2005) (Drews et al., 2010), body iron has been suggested to play a role in IR (Syrovatka et al., 2009) and beta cell dysfunction (Kolberg et al., 2009), which are cardinal features of altered glucose

homeostasis. Another possible mechanism is the peroxidation of lipids, especially free fatty acids, induced by an increased iron stores in muscle, which would reduce insulin sensitivity. Also, iron deposition in the liver may induce hyperinsulinaemia by impeding its capacity for insulin extraction, thereby resulting in impaired suppression of hepatic glucose production (Niederau et al., 1984) (Ferrannini, 2000).

Similar mechanisms have been shown in mouse models of haemochromatosis, in which iron is accumulated specifically in the endocrine pancreas resulting in decreased β-cell function and increased β-cell death (Huang et al., 2011) (Cooksey et al., 2004). Additionally, these models have shown that iron overload had deleterious effects on both the liver and skeletal muscle, and also, increased iron levels in adipocytes were found to be negatively correlated with secretion of adiponectin, an adipocytokine that correlates with insulin sensitivity (Huang et al., 2011) (Gabrielsen et al., 2012).

3.1.2.1 Iron and insulin sensitivity

Accumulating epidemiological studies have reported an association between body iron stores and IR, measured as HOMA-IR (Syrovatka et al., 2009) (Kim et al., 2011a) (Pham et al., 2013) (Aregbesola et al., 2015). In a recent cross-sectional study, a weak and direct association was observed between body iron stores, as assessed by SF quartiles, and IR using HOMA-IR in normoglycaemic subjects. However, a dose-dependent direct association was observed in prediabetes and T2DM individuals (Aregbesola et al., 2015). Likewise, a previous large population study reported that SF concentrations were highest in T2DM, followed by prediabetes, and lowest in normoglycaemia (men: 186, 176 and 156μg/L respectively; women: 85, 75 and 59μg/L respectively) (Kim et al., 2011a).

In addition, in studies among β-thalassemic subjects, insulin sensitivity is significantly decreased (Merkel et al., 1988) (Cario et al., 2003), causing a reduction of 40% in insulin sensitivity (Cavallo-Perin et al., 1995).

A possible mechanism by which iron induces IR is the peroxidation of lipids, especially free fatty acids. The increase in free fatty acids oxidation causes decreased glucose uptake by the muscles, which stimulates gluconeogenesis in the liver and results in

increased IR (Felber et al., 1987) (DeFronzo, 1988). Therefore, both decreased glucose utilization and increased glucose production may occur with higher levels of body iron (Rajpathak et al., 2009a). Also, iron deposition in the liver may induce hyperinsulinaemia by impeding its capacity for insulin extraction (Niederau et al., 1984), thereby resulting in impaired suppression of hepatic glucose production (Ferrannini, 2000). Recently, iron-regulated adiponectin and iron-mediated adipocyte IR have also been suggested (Gabrielsen et al., 2012).

3.1.2.2 Iron and β-cell function

Pancreatic β-cell function has been shown to be associated with body iron stores (Haap et al., 2003) (Aregbesola et al., 2015). In a cross-sectional study the association between body iron stores, measured as SF, and β-cell function, measured by HOMA- β, was assessed in normoglycaemia, prediabetes and T2DM subjects (Aregbesola et al., 2015). The study showed a weak and direct association in normoglycaemia, direct in prediabetes and inverse in T2DM. Authors suggested that this observation could not be evident whether the association is assessed in the general population without separation into the three glycaemic states. It is recognised that accumulation of body iron stores impairs glucose homeostasis by first increasing the IR (Niederau et al., 1984). Then, insulin secretion by β-cells would be enhanced in response to the increasing IR up to a level, and thereafter, the compensatory mechanism would start to fail, resulting in the depreciation of β-cell function.

The iron deposition in pancreatic islets, albeit variable, is preferentially restricted to β-cells (Rahier et al., 1987). This iron accumulated in β-cell would catalyse the formation of hydroxyl radicals, which may attack pancreatic β-cells through increasing oxidative stress, since antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, are less expressed in pancreatic islets than in other tissues. These features makes β-cells particularly susceptible to oxidative damage (Tiedge et al., 1997), which favours apoptosis (Lenzen, 2008) (Utzschneider and Kowdley, 2010), and therefore the impaired insulin synthesis and secretion (Evans et al., 2002).

Similarly, in a mouse model of haemochromatosis, iron excess is accumulated specifically in the endocrine pancreas, resulting in β-cell oxidant stress and decreasing insulin secretory capacity secondary to β-cell apoptosis and desensitization of glucose-induced insulin secretion (Cooksey et al., 2004) (Huang et al., 2011). Another study showed that even at 'normal' levels, iron exerted detrimental effects on pancreatic β-cell function, and that these effects were reversible with dietary restriction or iron-chelation therapy (Cooksey et al., 2010).

3.2 STUDIES RELATING IRON EXCESS AND RISK OF TYPE 2 DIABETES MELLITUS

3.2.1 Studies relating iron intake and risk of type 2 diabetes mellitus

Few years after Salonen et al. had reported a positive association between excess iron deposits and the risk of T2DM in 1998 (Salonen et al., 1998), it became apparent the relationship between haem iron intake and the disease in American cohorts. Thus, Lee et al., in postmenopausal women within the Iowa Women's Health Study (IWHS), and Frank B. Hu research group, in men within the HPFS, found that haem iron intake was positively associated with the incidence of T2DM (Jiang et al., 2004a), especially, in subjects who consumed alcohol (Lee et al., 2004). This link was observed more evidently with haem iron intake from red meat sources than from other sources (Jiang et al., 2004a). It has been suggested that the association between haem iron intake and T2DM was confounded by the food source (Rajpathak et al., 2009a), since the most important source of haem iron in the diet is red meat. It is known, as Loma Linda University's Adventist Health Study had reported for the first in 1985 (Snowdon and Phillips, 1985), that high meat intake is associated with T2DM risk, which it has been consistently observed by several other studies (Pan et al., 2011). However, a subsequent prospective study conducted in another US cohort, the Nurse's Health Study (NHS), confirmed earlier findings in relation to the haem intake, from all sources, and T2DM risk (Rajpathak et al., 2006).

Results from studies carried out among Chinese population are inconsistent (Shi et al., 2006) (Luan de et al., 2008). While Luan et al. also found association between haem iron intake and T2DM (Luan de et al., 2008), as well as in the previous four American cohorts (Jiang et al., 2004a) (Lee et al., 2004) (Song et al., 2004) (Rajpathak et al., 2006); surprisingly, Shi et al., within the Jiangsu Nutrition Study (JIN) cohort, observed a positive relationship between high total iron (Shi et al., 2006) and non-haem iron (Shi and Pan, 2008) intake and risk of T2DM in women, but not in men. However, a subsequent prospective analysis in this cohort showed higher risk of T2DM in those subjects with the highest haem iron intake (Shi et al., 2010), in accordance with previous studies. Finally, one prospective study within the Multi-Ethnic Study of Atherosclerosis (MESA) conducted among U.S. adults found no association between haem iron intake and T2DM incidence (de Oliveira Otto et al., 2012).

Moreover, the direct association between haem iron intake before pregnancy and/or during the early period of pregnancy and the risk of gestational diabetes was also reported in the NHS II (Bowers et al., 2011) and the U.S. Omega cohort (Qiu et al., 2011).

3.2.2 Studies relating iron status and risk of type 2 diabetes mellitus

3.2.2.1 Iron status and type 2 diabetes mellitus in general population

sTfR/ferritin ratio

The relation between iron stores and the risk of T2DM among general population was reported for the first time by Salonen et al. in 1998 in a longitudinal case-control study (Salonen et al., 1998). They observed that elevated iron stores increased the risk of T2DM among Finnish adult men (HR = 2.5, 95% CI: 1.1-6.0; P = 0.040) comparing the lowest with the highest quartile of sTfR/ferritin ratio. These results were prospectively confirmed later within the Nurses' Health Study (RR = 2.4, 95% CI: 1.55-3.71, P-trend = 0.020) (Jiang et al., 2004b), the European Prospective Investigation of Cancer (EPIC) (RR= 0.57, 95% CI: 0.38-0.85, P-trend = 0.005, using the lower quintile as reference) (Montonen et al., 2012), and the Kuopio Ischemic Heart Disease Risk Factor (KIHD)

cohorts (HR = 1.75, 95% CI: 1.06-2.88, P-trend = 0.170) (Aregbesola et al., 2013) in USA, German, and Finish population, respectively.

Ferritin

Ferritin is the most widely used biomarker of body iron stores in epidemiological studies. Positive association has been observed between high levels of this biomarker and the risk of T2DM in different cohorts, such as, the NHS (Jiang et al., 2004b), the EPIC from Norfolk (Forouhi et al., 2007b) and Potsdam (Montonen et al., 2012), the Aerobics Center Longitudinal Study (Le et al., 2008), the Diabetes Prevention Program (DPP) (Rajpathak et al., 2009b), the FINRISK97 and HEALTH 2000 (Salomaa et al., 2010), the Nutrition and Health of Aging Population (NHAP) (Sun et al., 2013), and the KIHD (Aregbesola et al., 2013), in American (Jiang et al., 2004b) (Le et al., 2008) (Rajpathak et al., 2009b), Chinese (Sun et al., 2013), and northern Europe (Forouhi et al., 2007b) (Salomaa et al., 2010) (Montonen et al., 2012) (Aregbesola et al., 2013) population. Only the Atherosclerosis Risk in Communities (ARIC) prospective study (Jehn et al., 2007), conducted on American subjects between 45 and 64 years of age, found no association after adjusting for components of metabolic syndrome (MetS), a strong risk factor of T2DM (Zeng et al., 2011).

In addition, a cross-sectional study found that SF concentration increased from normoglycaemia through prediabetes to T2DM (133.8, 149.1 and 242.2µg/L respectively; P <0.001) (Zheng et al., 2011), suggesting that the gradual increase in ferritin levels would lead to a progressive alteration of glucose metabolism.

sTfR

Few prospective studies evaluating the association between iron status and risk of T2DM have used sTfR as a biomarker (Montonen et al., 2012)(Rajpathak et al., 2009b)(Aregbesola et al., 2013) and this relationship is not clear. Thus, while in the Potsdam EPIC cohort association was not observed (Montonen et al., 2012), in the DPP (Rajpathak et al., 2009b) high levels of sTfR were associated to an increased risk of T2DM. In addition, in the KIHD study, a U-shaped association was observed. Thus, men

with moderate levels of sTfR had a lower risk of T2DM regarding men with low sTfR concentrations (Aregbesola et al., 2013).

Transferrin saturation

Ellervik et al. carried out the first and largest population-based study that consistently demonstrated transferrin saturation as a risk marker of any form of diabetes, and of type 1 and type 2 diabetes separately, in three independent studies. They found that transferrin saturation ≥50% was associated with a twofold increased risk of developing T2DM (Ellervik et al., 2011). However, in recent cross-sectional studies inverse associations with prevalent T2DM were reported in both, population-based Cooperative Health Research in the Region of Augsburg (KORA) F4 study from Germany (Huth et al., 2015) and in men in an Australian study of Caucasian subjects, but no significant association was observed in women (Yeap et al., 2015). Also, no association was found between elevated serum transferrin saturation and the development of diabetes in a retrospective cohort study based on merging the National Health and Nutrition Examination Survey I (NHANES I) with the NHANES I Epidemiologic Follow-up Study (Mainous et al., 2002).

Transferrin

Transferrin has been much less investigated as an iron biomarker of T2DM incidence. This biomarker is synthesised if body iron stores are low, and thus is inversely correlated with ferritin. Nevertheless, studies observed strong positive associations between higher transferrin levels and T2DM (Wlazlo et al., 2012) (Huth et al., 2015). These results suggest that the association between transferrin and T2DM risk could be explained through an iron-independent mechanism. Transferrin may be elevated because of inflammation, therefore it might mask a potential association (Wish, 2006).

Haemoglobin

Although there are different factors associated with low haemoglobin levels, the most common cause of anaemia in developing countries is iron deficiency (World Health Organization, 2001). To the best of our knowledge, only one cross-sectional study has assessed the relationship between haemoglobin levels and the risk of T2DM in Chinese population (Shi et al., 2006). In this study, higher haemoglobin levels were associated with an increased risk of T2DM. In addition, an inverse association between anaemia and T2DM risk was reported. These last results do not support the hypothesis that low iron levels would prevent the development of T2DM.

3.2.2.2 Iron status and type 2 diabetes mellitus in special situations

Haemochromatosis

The first evidence about that systemic iron overload could contribute to abnormal glucose metabolism was initially provided by Trousseau in 1865, from the observation that the prevalence of T2DM increased in classic HH patients (Trousseau, 1865). It was initially referred as "bronze diabetes", due to the pigmentation that occurs with the disease. The prevalence of diabetes among these patients varied between 22% (O'Sullivan et al., 2008) and 63% (Dymock et al., 1972) in different studies over the past half-century.

Transfusional iron overload

β-thalassemia major is a transfusion-dependent chronic haemolytic anaemia and the most common cause of acquired iron overload. The same observation than haemochromatosis was done in this genetic disorder of iron metabolism, revealing that, irrespective of the cause or the gene involved, iron overload results in an increased incidence of T2DM (Swaminathan et al., 2007). The reported prevalence of T2DM in these patients ranges from 6% (Borgna-Pignatti et al., 2004) to 14% (Vogiatzi et al., 2009b), or even 19.5% (Chern et al., 2001). Precisely, a study among β-thalassemic individuals, SF significantly correlated with F2-isoprostanes ($r = 0.57$) and superoxide

dismutase (r = 0.46), two important markers of oxidative stress, suggesting that the relationship between iron overload and T2DM could be mediated by elevated oxidative stress (Rajpathak et al., 2009a).

Until date, β-thalassemia major is the most important example of diabetes due to transfusional iron overload, but there are other causes, like bone marrow transplantation, warranting repeated blood transfusion, that have also reported diabetes as a complication (Baker et al., 2007)

Other causes of iron overload

In porphyria cutanea tarda, a cutaneous condition associated with increased iron overload, up to 87% of patients were glucose intolerant (Franks et al., 1979).

HCV infection is well-known to be associated with an accumulation of iron in the liver parenchyma. Many patients with chronic HCV infection often have elevated serum iron, transferrin saturation and ferritin levels, and a few have severe hepatic iron overload (Boujaoude et al., 2000). This might suggest that iron overload has a role in the pathogenic link between HCV and accelerated end-organ damage in diabetes (Swaminathan et al., 2007).

Friedreich's ataxia, an inherited neurodegenerative disease with an alteration in the first intron of the FRDA gene, is a classic disorder associated with mitochondrial iron accumulation. FRDA is associated with a high incidence of T2DM (Rivaud-Pechoux et al., 1998), suggesting a possible relation between mitochondrial iron accumulation leading to mitochondrial DNA damage and T2DM. Disruption of the FRDA gene in pancreatic β-cells causes T2DM following cellular growth arrest and apoptosis, in parallel to an increase in ROS in islets (Ristow et al., 2003).

3.2.2.3 Blood donation, phlebotomy, and iron-chelating agents and type 2 diabetes mellitus

The best evidence for the causality between iron and T2DM is the improvement in the diabetic state after iron depletion.

Blood donation and phlebotomy

Blood donations and phlebotomy have also been observed to have a positive effect on insulin sensitivity and T2DM risk because they reduce body iron levels. Fernandez-Real et al. observed a decrease in body iron levels associated to and improvement in insulin sensitivity in healthy subjects who made frequent blood donations (at least 2 in the previous 5 years) (Fernández-Real et al., 2005). They also found that the number of donations was positively correlated with insulin sensitivity. The same authors had previously observed that both insulin secretion and sensitivity, and glycosylated haemoglobin levels improved in diabetic patients undergoing phlebotomy (Fernández-Real et al., 2002). These results appear to show that iron reduction has a beneficial effect on the occurrence of T2DM. Interestingly, even in apparently healthy individuals, blood donation, resulting in decreased iron stores, has also been associated with a low incidence of T2DM (Ascherio et al., 2001).

Other pathology associated with increased SF, independently of C-reactive protein and increased liver iron, is insulin resistance hepatic iron overload, or dysmetabolic iron overload disease. In this condition, iron depletion therapy has been shown to increase peripheral and hepatic insulin sensitivity, increase pancreatic insulin sensitivity, reduce HbA1c, and improve liver function test results (Aigner and Datz, 2008).

Nevertheless, Zheng et al. found no significant differences in the risk of T2DM between frequent donors (>8 donations per 2 year) and infrequent donors (1–2 per 2 year) (Zheng et al., 2007). The HPFS study conducted in 33,541 men did not support a link between blood donation and risk of T2DM (Jiang et al., 2004a).

Introduction

Iron-chelating agents

Iron chelation is another treatment modality to reduce body iron levels. In a recent laboratory experiment, iron depletion with deferoxamine was shown to improve insulin sensitivity (upregulated glucose uptake and increased insulin receptor activity and signalling) in hepatocytes (Dongiovanni et al., 2008). In a study among nine T2DM patients without HH, iron chelation therapy with intravenous deferoxamine resulted in a significant improvement in metabolic control as seen by reduction in blood glucose and glycosylated haemoglobin levels (Cutler, 1989). In another study, five T2DM patients with high ferritin levels were treated with intramuscular injections of deferoxamine (Kaye et al., 1993). Two of these patients achieved a normalization of ferritin levels after 12 weeks of deferoxamine injections twice weekly; however, none obtained any significant clinical change in the use of antidiabetic medication, and deferoxamine had no effect on glycaemia. Controlled trials are required to address both the beneficial and potential adverse effects of iron chelation therapy.

UNIVERSITAT ROVIRA I VIRGILI
IRON EXCESS AND RISK OF TYPE 2 DIABETES MELLITUS IN A PROSPECTIVE COHORT OF MEDITERRANEAN POPULATION
José Cándido Fernández Cao

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IRON EXCESS AND RISK OF TYPE 2 DIABETES MELLITUS IN A PROSPECTIVE COHORT OF MEDITERRANEAN POPULATION
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JUSTIFICATION

UNIVERSITAT ROVIRA I VIRGILI
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JUSTIFICATION

Diabetes is the most common metabolic disorder worldwide. Its prevalence in both developed and developing countries has been increasing in the last few years in parallel to the obesity epidemic. It has been estimated that the number of people aged 20 to 79 affected by diabetes will increase from 6.4% in 2010 to 7.7% in 2030 and that this trend will continue into the future. Diabetes has become an important public health problem since it is one of the main causes of morbidity and death. The International Diabetes Federation recently estimated that roughly 5.1 million people aged between 20 and 79 died from diabetes in 2013, accounting for 8.4% of global all-cause mortality among people in this age group. In Europe, one in 10 adult deaths (619,000 in 2013) can be attributed to diabetes. The vast majority of these deaths (90%) were in people over the age of 50. However, diabetes and its complications are also major causes of early death worldwide. The evolution of this illness leads to complications such as cardiovascular disease, retinopathy, nephropathy, neuropathy, leg ulcers and gangrene, These common disorders are costly and shorten life expectancy.

Type 2 diabetes mellitus (T2DM) is a multifactorial disease that increases with age, obesity, and inadequate lifestyles habits such as smoking, an unhealthy diet and a lack of physical activity. An excessive caloric intake is a major driving force behind escalating T2DM epidemics worldwide but dietary quality also has independent effects. Much evidence shows the negative effects of an unhealthy diet on T2DM. Foods such as white rice, with a high glycaemic index; red meat and processed meat have been associated with an increased risk of T2DM. Meat in particular is a source of many components that may elevate the risk of T2DM, such as saturated fatty acids, cholesterol, sodium, advanced glycation end products (AGEs), dioxins, phthalates, bisphenol A, nitrosamines, trimethylamine N-oxide, nitrates, nitrites and haem iron. Haem iron intake has been associated with the risk of T2DM in U.S. and Chinese populations but this relationship has not been studied in the European population.

Justification

Although the potential mechanism that mediates the relationship between haem iron and T2DM is not well-established, iron could play a role in glucose metabolism because of its action as a pro-oxidant. It is speculated that an increase in iron stores may play a role in the production of ROS, such as hydroxyl radicals, thus leading to tissue damage. Specifically, it is suggested that via its oxidative properties, body iron may play a role in IR and β-cell dysfunction, which are two key events in the clinical development of T2DM. New biomarkers, such as osteocalcin (OC), have recently been related to glucose metabolism. Much research has indicated that iron overload affects bone tissue causing both osteopenia and osteoporosis. Interestingly, in vitro studies have shown that ferritin itself can directly inhibit osteogenesis in a dose-responsive manner through its ferroxidase activity. Iron is also reported to have a detrimental effect on the differentiation, proliferation, and activity of osteoblasts, which synthesise OC. It would be interesting, therefore, to investigate the possible relationship between body iron stores and OC concentrations as a potential mechanism for explaining iron-induced T2DM.

As well as to haem iron intake, body iron stores have also been associated with T2DM in U.S., Chinese, and northern European population. To date, however, no studies have been conducted in southern Europe to explore this relationship. Some characteristics of iron metabolism in southern European populations are quite different from those of previously studied populations. For example, the consumption of food items of animal origin is greater in northern Europe than in southern Europe. It would be interesting, therefore, to evaluate the relationship between iron intake and body iron stores and the risk of T2DM in a southern European population.

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HYPOTHESIS AND OBJECTIVES

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HYPOTHESIS

Elevated iron levels could lead to an increase in oxidative stress. This could cause progressive failure of pancreatic β-cell function and a decrease in insulin sensitivity, which are two key events in the pathophysiology of type 2 diabetes mellitus (T2DM). Iron may therefore be a risk factor in the development of T2DM.

In a Mediterranean population, a high iron intake and/or elevated iron status could increase the risk of T2DM.

GENERAL OBJECTIVE

To assess, in a prospective study, the effect of elevated dietary iron intake and body iron status on the risk of T2DM in an adult Mediterranean population and to evaluate the relationship between iron status and serum osteocalcin (OC) as a potential mechanism for explaining iron-induced T2DM.

SPECIFIC OBJECTIVES

1. To compare, at the beginning of the longitudinal study, the socio-demographic and lifestyle variables, anthropometrical measurements, and food and nutrient intake of incident and non-incident diabetic subjects.

2. To evaluate the effect of iron intake, at baseline, on the risk of new-onset T2DM during follow-up, while taking into account other factors that are associated with this relationship, such as socio-demographic, anthropometric, lifestyle, and dietary factors.

3. To analyse the association between iron status and serum OC concentrations as a potential mechanism for explaining iron-induced T2DM in both impaired fasting glucose subjects and normal glucose metabolism subjects, while taking into account other factors that are associated with this relationship, such as socio-demographic, anthropometric, lifestyle, inflammation and oxidation confounding variables.

4. To compare, at the beginning of the longitudinal study, the iron status, fasting glucose and insulin levels, insulin resistance, inflammation, lipid profile, metabolic syndrome (MetS) status and prevalence of related pathologies in incident and non-incident diabetic subjects.

5. To prospectively assess the effect of iron status at baseline, measured as serum ferritin (SF) and soluble transferrin receptor (sTfR), and the risk of new-onset T2DM during follow-up, while taking into account other factors that are associated with this relationship, such as socio-demographic, anthropometric, lifestyle and dietary factors, inflammation, and MetS components.

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MATERIAL AND METHODS

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MATERIAL AND METHODS

The following three studies were included in the present thesis in order to assess the objectives:

Study 1: **Iron intake and type 2 diabetes mellitus**

Study 2: **Body iron status and serum osteocalcin levels**

Study 3: **Body iron status and type 2 diabetes mellitus**

Data collected from Reus-Tarragona, Pamplona and Barcelona centres of the PREDIMED study was used. Men, aged 55 to 80, and women, aged 60 to 80, free of T2DM and CVD at baseline, but at high cardiovascular risk, were enrolled. The protocol, design, aims and methodology of the PREDIMED trial have been described in detail previously (Martínez-González et al., 2012), and is available in http://www.predimed.es. The study protocol was approved by the Clinical Research Ethics Committee of the Hospital Clínic de Barcelona and the other institutions involved. The protocol was in accordance with the Declaration of Helsinki and was registered in the ISRCTN clinical trials register (http://www.controlled-trials.com/ISRCTN35739639).

1. STUDY 1: IRON INTAKE AND TYPE 2 DIABETES MELLITUS

An observational cohort analysis was conducted in order to evaluate the effect of elevated iron intake on the risk of T2DM. At baseline, a subset of 1,073 non-diabetic participants, 460 men aged 55 to 80 years and 613 women aged 60 to 80 years, were included in the analysis from two Spanish centres (Reus-Tarragona and Pamplona). During the follow-up (median: 4.9 ± 1.3 years), a total of 131 incident cases of T2DM were diagnosed using the ADA criteria (American Diabetes Association, 2013).

Material and Methods

Medical information was collected on subjects' medical record of a 47-item questionnaire about education, lifestyle, history of illnesses and medication use (**Appendix II**). Trained personnel measured baseline weight, height and waist circumference as previously reported, as well as blood pressure in triplicate with a validated semiautomatic oscillometer (Omron HEM-705CP, Hoofddorp, Netherlands) (Redón and Coca 2003). Energy expenditure in leisure-time physical activity was estimated using the validated Spanish version of the Minnesota Questionnaire (Elosua et al., 1994) (**Appendix IV**). Sociodemographic and smoking data were also recorded at baseline.

A validated 137-item FFQ (**Appendix III**) was administered (Fernández-Ballart et al., 2010). Consumption of the following food groups was calculated: dairy; meat, fish and eggs; cereals, legumes and nuts; vegetables; fruits; oils and fats; candies and sweetened beverages; alcoholic beverages; tea and coffee. Total energy and nutrient intake were calculated on the basis of Spanish food composition tables (Mataix, 2003) (Moreiras et al., 2009). According to the Monsen model, an average of 40% of total iron in meat, fish and poultry was considered as haem iron (Monsen et al., 1978). Then, we created a categorical variable of haem iron intake: low (<3.173mg), medium (3.173– 4.130mg) and high (>4.130mg) consumers.

Serum levels of total triglycerides, and total and HDL-cholesterol were measured by standard enzymatic methods. LDL-cholesterol was calculated using the Friedewald equation. From these data, hypercholesterolaemia (total cholesterol ≥240mg/dL), and hypertriglyceridaemia (triglycerides ≥200mg/dL) was calculated. These cardiovascular risk factors, besides hypertension, were defined according to the Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) (Expert Panel on Detection, Evaluation, 2001).

In order to assess the association between haem iron intake and T2DM, we used several proportional hazard models. The first model used raw data with unadjusted haem iron intake. The second model used data adjusted for energy intake. And the final model was adjusted for dietary, anthropometric, socio-demographic and lifestyle variables: for

example, age, gender, waist circumference, smoking, education level and intervention group. Some components of foods that can interfere with haem iron absorption were introduced as confounding variables, such as: fruit; meat, fish and eggs [animal protein]; cereals, legumes and nuts [vegetable protein]; calcium; alcohol beverages; tea and coffee (the last two representing the consumption of polyphenols). No interactions were found between gender or age.

The methodological characteristics of this study are more widely discussed in the corresponding article included in the results section.

2. STUDY 2: **BODY IRON STATUS AND SERUM OSTEOCALCIN LEVELS**

A cross-sectional study was performed with the purpose of assess the relationship between iron status and serum concentration of osteocalcin (OC). A sample of 423 non-diabetic subjects, men aged 55 to 80, and women aged 60 to 80, were randomly selected from three Spanish centres (Reus-Tarragona, Navarra and Barcelona).

Medical information was collected on subjects' medical record of a 47-item questionnaire about education, lifestyle, history of illnesses and medication use (**Appendix II**). Trained personnel measured baseline weight, height and waist circumference as previously reported, as well as blood pressure in triplicate with a validated semiautomatic oscillometer (Omron HEM-705CP, Hoofddorp, Netherlands) (Redón and Coca 2003). Energy expenditure in leisure-time physical activity was estimated using the validated Spanish version of the Minnesota Questionnaire (Elosua et al., 1994) (**Appendix IV**). Sociodemographic and smoking data were also recorded.

Serum levels of fasting glucose, total cholesterol, HDL-cholesterol and triglycerides were measured by standard enzymatic automated methods. LDL-cholesterol concentrations were calculated using Friedewald's equation in those patients whose triglyceride levels were <400mg/dL. Plasma fasting insulin concentrations were measured by an ELISA kit for human insulin (Millipore, St. Charles, Missouri, USA). IR was estimated by the HOMA

method using the following equation (Matthews et al., 1985). Altered β-cell function was estimated using HOMA-β (homeostasis model assessment- β-cell) (Matthews et al., 1985).

Serum concentrations of total and uncarboxylated OC were measured by electrochemiluminescence immunoassay (N-mid osteocalcin, Roche, Indianapolis, IN). Serum ferritin (SF) (Ferritin Elecsys, Roche diagnostics, Mannheim, Germany) and soluble transferrin receptor (sTfR) (Access sTfR 0QC, Beckman Coulter, USA) were measured by immunochemiluminescence. Adiponectin levels were measured using an enzymatic immunoassay (Millipore, St. Charles, Missouri, USA). Plasma oxidised LDL (oxLDL) concentrations were also measured by a commercial ELISA (Mercodia Oxidised LDL ELISA, Uppsala, Sweden). Plasma concentrations of high-sensitivity C-reactive protein (hs-CRP) were measured using a highly sensitive immunoassay (Helica Biosystems Inc., Santa Ana, CA, USA).

In order to examine the relationship between markers of iron metabolism with serum concentrations of total and uncarboxylated OC, linear regression models were fitted including gender, age, BMI, smoking status, total energy intake, energy expenditure in leisure-time physical activity, fasting plasma glucose and insulin, and markers of inflammation or oxidation (adiponectin, CRP, oxLDL) as potentially confounding variables in the fully adjusted model. In addition, both regressions analyses with either SF or sTfR as outcomes were mutually adjusted for each other to account for negative confounding because they are both independently associated with IR despite their negative reciprocal correlation (Fumeron et al., 2006) (Vari et al., 2007). Interactions between iron status with gender, and glucose metabolism were tested in the fully adjusted models. For $\log_e$-transformed outcome variables (i.e. serum concentrations of total and uncarboxylated OC) regression coefficients were converted into percentages of relative change in the original variable (percentage decrease in total or uncarboxylated OC per 50ng/mL increase in ferritin or per 1ng/mL of increased sTfR).

The methodological characteristics of this study are more widely discussed in the corresponding article included in the results section.

3. STUDY 3: **BODY IRON STATUS AND TYPE 2 DIABETES MELLITUS**

A prospective nested case-control study was conducted to evaluate the relationship between excess body iron and the risk of T2DM. At baseline, from 1378 Caucasian non-diabetic individuals aged 55 to 80, recruited from three Spanish centres (Reus-Tarragona, Pamplona and Barcelona-Clinic), we selected all the 153 individuals who developed T2DM during a median follow-up of 6.0 years (interquartile range: 3.9-6.5). For every subject who developed T2DM, two controls were matched to the cases by age, gender, BMI, and dietary intervention group, resulting in 306 controls. The 1225 non-diabetic subjects that were candidates for inclusion in the control group were classified according to categories of matching variables: age (≤67 or >67 years), gender, BMI (≤27 or >27kg/m^2), and intervention group (Mediterranean diet supplemented with virgin olive oil, Mediterranean diet supplemented with nuts or control group). In total 24 groups were created (2*2*2*3 = 24 groups). Then, out of each of the 24 groups, two controls were randomly selected for every case belonging to the same group. The effective sample size for statistical analyses was 459 participants.

Medical information was collected on subjects' medical record of a 47-item questionnaire about education, lifestyle, history of illnesses and medication use (**Appendix II**). Trained personnel measured baseline weight, height and waist circumference as previously reported, as well as blood pressure in triplicate with a validated semiautomatic oscillometer (Omron HEM-705CP, Hoofddorp, Netherlands) (Redón and Coca 2003). Energy expenditure in leisure-time physical activity was estimated using the validated Spanish version of the Minnesota Questionnaire (Elosua et al., 1994) (**Appendix IV**). Sociodemographic and smoking data were also recorded. New onset T2DM during follow-up was diagnosed using the criteria of the ADA (American Diabetes Association, 2013).

Serum levels of fasting glucose, total cholesterol, HDL-cholesterol and triglycerides were measured by standard enzymatic automated methods. LDL-cholesterol concentrations were calculated using Friedewald's equation in those patients whose triglyceride levels were <400mg/dL. Plasma fasting insulin concentrations were measured by an ELISA kit

for human insulin (Millipore, St. Charles, Missouri, USA). IR was estimated by the HOMA method using the following equation (Matthews et al., 1985).

Plasma concentrations of high-sensitivity C-reactive protein (hs-CRP) were measured using a highly sensitive immunoassay (Helica Biosystems Inc., Santa Ana, CA, USA). SF (Ferritin Elecsys, Roche diagnostics, Mannheim, Germany) and sTfR (Access sTfR 0QC, Beckman Coulter, USA) were measured by immunochemiluminescence.

In order to analyse the relationship between body iron levels and the incidence of T2DM, participants were categorised into quartiles according to the distributions of SF, sTfR and sTfR/ferritin ratio at baseline in control individuals. Given the documented differences between males and females with respect to body i stores, the independent variables were adjusted for gender. The adjusted variables were categorised to avoid any assumption of linearity, and to evaluate the dose–response relationship in the onset of T2DM. Several conditional logistic regression models were applied. A crude model (without adjustment) was fitted with each independent variable. The model was re-fitted with adjustment for lifestyle variables such as marital status (married/not married), educational level (primary/secondary/tertiary), smoking (current smoker/former smoker/never smoked), alcohol consumption (drinker/non-drinker), physical activity (200 metabolic equivalents (MET)-min/d/ ≥200MET-min/d); family history of T2DM (yes/no). components of the MetS, according to the harmonised criteria proposed by International Diabetes Federation (IDF) and the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI)(26), i.e. blood pressure ≥135/85mmHg, serum TAG ≥1.7mmol/L, HDL-cholesterol ,1.03mmol/L in males and,1.3mmol/L in females, waist circumference ≥102cm in men and ≥88cm in women, and fasting glucose concentration ≥5.6mmol/L; dietary variables categorised by quartiles, such as, energy, Mg, vitamins D and E, dairy products, meat, vegetables and fruits; and hs-CRP (mg/L). The test for linear trend across the quartiles was performed by assigning the median value to each category and introducing these new variables into the conditional logistic regression as continuous variables.

The methodological characteristics of this study are more widely discussed in the corresponding article included in the results section.

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RESULTS

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Heme iron intake and risk of new-onset diabetes in a Mediterranean population at high risk of cardiovascular disease: an observational cohort analysis

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RESEARCH ARTICLE Open Access

Heme iron intake and risk of new-onset diabetes in a Mediterranean population at high risk of cardiovascular disease: an observational cohort analysis

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Abstract

Background: Several epidemiological studies have observed an increased risk of type 2 diabetes mellitus (T2DM) among subjects with a higher consumption of red and processed meat. Heme iron intake has been directly associated with a higher risk of T2DM in healthy adult Chinese and U.S populations. The objective of the present study was to evaluate the association between heme iron intake and the incidence of T2DM in a Mediterranean population at high cardiovascular risk.

Methods: We assessed a subset of participants in the PREDIMED trial as an observational cohort, followed up for a maximum of eight years. We initially included 1073 non-diabetic subjects (57.1% women) aged 67.3 ± 6.0 years, at high cardiovascular risk. Diet was assessed at the study baseline using a validated, semi-quantitative food frequency questionnaire.

Results: During the follow-up period 131 diabetics were newly diagnosed. The risk of developing T2DM was assessed using baseline heme iron intake and proportional hazard models, first unadjusted, then adjusted for energy, and finally adjusted for dietary, anthropometric, socio-demographic and lifestyle variables. Significant direct associations with the incidence of T2DM were found for heme iron (Hazard Ratio [HR] 1.30, 95% confidence interval [CI], 1.02 to 1.66). Secondarily, we have also observed that coffee (HR:0.93, 95% CI, 0.89 to 0.98) and alcoholic beverages (HR: 1.02, 95% CI, 1.01 to 1.04) were also found to reduce and increase the risk of T2DM, respectively.

Conclusion: High dietary intake of heme iron was associated with an increased risk of developing T2DM in a Mediterranean population at high cardiovascular risk.

Trial registration: Identifier: ISRCTN35739639.

Background

In recent years the prevalence of T2DM has increased worldwide. It has been estimated that the number of people aged 20 to 79 years affected by T2DM will increase from 285 million in 2010 (6.4%) to 439 million in 2030 (7.7%) [1].

Diet is one of the components of lifestyle that has most been studied in the prevention of T2DM. A recent meta-analysis reported that red meat, and particularly processed meat [2], is associated with a greater risk of T2DM incidence. In recent years, some studies in healthy adult Chinese [3,4] and U.S populations [5-7] suggest that the component of meat responsible for this association could be the intake of total iron [3] or heme iron [4-7].

The deregulation of iron homeostasis has been implicated in the origin of such pathologies as cancer, Alzheimer's disease, cardiovascular disease and T2DM, among others [8]. Although the exact mechanism by which iron

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induces T2DM is not clear, it has been speculated that, being a pro-oxidant element, it strengthens the formation of hydroxyl radicals [9], thus favouring situations of oxidative stress and contributing to insulin resistance or long-term pancreatic failure [9].

The association between heme iron intake and increased risk of T2DM has been studied in the general population, but not in individuals at high cardiovascular risk. Therefore, the objective of the present study was to evaluate the effect of heme iron intake on the incidence of T2DM in a nondiabetic Mediterranean population at high cardiovascular risk.

Methods

The study design was an observational cohort analysis of a subset of non-diabetic participants in the PREDIMED trial [10], who were followed up between 1 and 8 years (median 4.85 ± 1.28 years). PREDIMED is a large multicentre, randomized, controlled trial that attempts to test the efficacy of the Mediterranean diet (MeDiet) in the primary prevention of cardiovascular disease. Two traditional Mediterranean diets were evaluated – one enriched with extra virgin olive oil and the other with mixed nuts – along with a control low-fat diet normally recommended for cardiovascular disease prevention in a high-risk population. Full details of the PREDIMED trial (http://www.controlled-trials.com/ISRCTN35739639) have been published elsewhere [10].

Study participants

The present study was conducted on 1 073 subjects: 460 men aged 55 to 80 years, and 613 women aged 60 to 80 years from two PREDIMED trial centers (Reus and Pamplona). The subjects included were non-diabetics and had three or more of the following cardiovascular risk factors: current smoker (>1 cigarette/day for the past month), high blood pressure (systolic ≥ 140 mmHg or diastolic ≥ 90 mmHg or taking blood pressure medication), LDL cholesterol ≥ 169 mg/dl or lipid-lowering treatments, HDL cholesterol ≤ 40 mg/dL in men or ≤ 50 mg/dL in women; body mass index (BMI) ≥ 25 kg/m^2; and family history of premature coronary disease (≤ 55 years in men and ≤ 60 years in women). Exclusion criteria were a previous history of cardiovascular disease (coronary heart disease, stroke or peripheral arterial disease); any severe chronic illness; alcohol or drug abuse; BMI > 40 kg/m^2; more than a 5% loss in body weight in the last year; and a history of allergy to nuts [10]. The Clinical Research Ethics Committee of the Hospital Sant Joan de Reus approved the study protocol and all participants provided written informed consent.

During the follow-up a total of 131 incident cases of T2DM were diagnosed using the American Diabetes Association criteria [11] (fasting blood glucose ≥ 7.0 mmol/L or blood glucose ≥ 11.1 mmol/L after an oral dose of 75 g glucose, measured annually). A second test with the same criteria was required to confirm the diagnosis. Incident T2DM cases were confirmed by the PREDIMED study's Clinical Events Committee.

Measurements

Dietary intake was determined at baseline using a validated 137-item, semi-quantitative food frequency questionnaire [12]. For the present analysis, food was grouped according to its nutritional composition. Finally, consumption of the following food groups was calculated: dairy; meat, fish and eggs; cereals, legumes and nuts; vegetables; fruits; oils and fats; candies and sweetened beverages; alcoholic beverages; tea and coffee. Total energy and nutrient intake were calculated on the basis of Spanish food composition tables [13]. Sociodemographic, anthropometric (weight, height, waist circumference) and biochemical data were also recorded at baseline and on each annual visit to identify health problems such as obesity (BMI ≥ 30 kg/m^2), hypercholesterolemia (total cholesterol ≥ 240 mg/dl), hypertriglyceridemia (triglycerides ≥ 200 mg/dl) and hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg). The last three cardiovascular risk factors were defined according to the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) [14].

The participants were divided into three grups: 0 – control group; 1 – Mediterranean Diet supplemented with olive oil; and 2 – Mediterranean Diet supplemented with nuts. Subjects were assessed as smokers or non-smokers. Education was recoded into two categories: high-medium and low. Finally, we created a categorical variable of heme iron intake: 1 – low (< 3.173 mg), 2 – medium (3.173–4.130 mg) and 3 – high (> 4.130 mg) consumers.

Statistical analysis

The data are presented as a percentage or average ± standard deviation. Statistical testing included chi-square tests for qualitative variables and the t-Test for quantitative data.

First, we studied whether there was a linear relationship between heme iron intake and T2DM. Because the association between heme iron intake and T2DM was linear and at the border of significance (P = 0.05), we decided to analyze the association in the total population with several proportional hazard models. The first model used raw data with unadjusted heme iron intake. The second model used data adjusted for energy intake. And the final model was adjusted for dietary, anthropometric, socio-demographic and lifestyle variables: for example, age, sex, waist circumference, smoking, education level and intervention group. Some components of foods that can interfere with heme iron absorption were also adjusted

for: for example, fruit, meat, fish and eggs [animal protein], cereals, legumes and nuts [vegetable protein], calcium, alcohol beverages, tea and coffee (the last two representing the consumption of polyphenols). Fruit was included as an adjustment variable because consumption was significantly higher in the group of non-diabetics. The variable "time to onset of T2DM" was measured in days. No interactions were found between sex and age.

Because the association between heme iron intake and T2DM was linear and at the border of significance, we decided to analyze the association for each tertile of heme iron intake, using the same adjustment variables and the last proportional hazard model.

Significance was set at $P < 0.05$. The data were analysed using version 20.0 of the SPSS statistical package for Windows.

Results

A total of 12.2% of the initial sample developed T2DM with a median follow-up of 4.8 ± 1.3 years. Table 1 shows the general baseline characteristics of non-incident and incident diabetics. At baseline, weight, BMI, waist circumference and smoking prevalence were significantly higher in incident than in non-incident T2DM subjects.

Table 2 shows the average consumption of the different food groups, the total energy intake and the consumption of main nutrients at baseline in non-incident and incident diabetics in total subjects and subjects classified according to tertiles of heme intake. The group of incident T2DM subjects consumed less fruit ($P = 0.010$) and coffee ($P = 0.001$), and more heme iron ($P = 0.036$) than the non-incident group in the total sample but not by tertiles. Only in the second ($P = 0.040$) and third ($P = 0.029$) tertiles was coffee consumption significantly higher in non-incident than incident diabetics.

Table 1 Baseline general characteristics of participants in incident and non-incident diabetics

VARIABLES	TOTAL n = 1073		
	NON INCIDENT	INCIDENT DIABETICS	p
	n = 942 (87.8%)	n = 131 (12.2%)	
Age (years)	67.3 ± 6.0	66.3 ± 5.7	0.073
Weight (kilograms)	75.3 ± 10.8	79.2 ± 10.6	< 0.001
IMC (kg/m2)	29.4 ± 3.1	30.4 ± 3.1	0.001
Waist circumference (cm)	97.2 ± 9.9	101.8 ± 9.3	< 0.001
Women (%)	57.7	52.7	0.271
Obesity (%)	41.5	48.1	0.153
Hypercholesterolemia (%)	35.8	35.7	0.992
Hypertriglyceridemia (%)	16.3	23.8	0.239
Hypertension (%)	65.6	62.8	0.719
Smoking (%)	16.3	25.2	0.012
Medium-high education (%)	26.9	27.9	0.807

Results expressed as mean ± standard deviation or percentages.

To evaluate the association between heme iron intake and various components of the diet with the risk of T2DM, proportional hazard models were applied (Table 3). The first model used raw data with unadjusted heme iron intake (HR = 1.13, 95% CI, 0.99 to 1.30; p = 0.075). The second model used data adjusted for energy intake (HR = 1.20, 95% CI, 1.02 to 1.42; p = 0.029). And the final model was adjusted for dietary, anthropometric, socio-demographic and lifestyle variables and showed that greater heme iron intake (HR = 1.30, 95% CI, 1.02 to 1.66; p = 0.037) was associated with an increased risk of T2DM onset. Although it was not our principal aim, we also observed an association between others components of the diet and the risk of T2DM. Thus, the consumption of alcohol beverages was associated with an increased risk of T2DM (HR = 1.02, 95% CI, 1.01 to 1.04), while coffee was associated with better protection against T2DM incidence (HR = 0.93, 95% CI, 0.89 to 0.98).

Furthermore, when we constructed the last proportional hazard model, by dividing subjects into tertiles of heme iron intake, we observed no significant association in either the first tertile of heme iron intake (HR = 1.58, 95% CI, 0.52 to 4.78; P = 0.175) or the second (HR = 1.59, 95% CI, 0.50 to 5.06; P = 0.013), but there was a significant association in the third tertile and T2DM (HR = 1.57, 95% CI, 1.09 to 2.27; P = 0.003).

Discussion

Several epidemiological studies have linked excess iron deposits and risk of T2DM [3,4,15]. However, few studies have analysed the association between heme iron intake and T2DM [3-7,16]. The present prospective study shows that higher amounts of dietary heme iron are associated with a higher risk of T2DM. This is the first time that this association has been assessed in a large elderly Mediterranean population at high cardiovascular risk.

This association has been evaluated in four long-term prospective studies [5-7,16] and two cross-sectional studies [3,4]. However, most of them were conducted in healthy cohorts of U.S adults (Professionals Follow-up Study, Women's Health Study, Nurse's Health Study and MESA), and showed that the intake of heme iron is associated with an increase in the risk of T2DM [5-7]. Only one study found no association [16]. In the Health Professionals Follow-up Study cohort, heme iron derived from red meat consumption, but not total iron intake, was positively associated with risk of T2DM [5]. In the Iowa Women's Health Study cohort of postmenopausal women, a high intake of heme iron and/or iron supplements was associated with increased incidence of T2DM, especially in subjects who consumed alcohol [7]. Lastly, in the Nurses Health Study, the association between T2DM incidence and iron intake was demonstrated only for heme iron intake, but not for

Table 2 Baseline food, energy and nutrient consumption in incident cases of diabetes and controls

VARIABLES	TOTAL n = 1073		p	FIRST TERTILE		SECOND TERTILE		THIRD TERTILE	
	NON INCIDENT	INCIDENT DIABETICS		NON INCIDENT	INCIDENT DIABETICS	NON INCIDENT	INCIDENT DIABETICS	NON INCIDENT	INCIDENT DIABETICS
	n = 942 (87.8%)	n = 131 (12.2%)		n = 324 (90.8%)	n = 33 (9.2%)	n = 310 (86.6%)	n = 48 (13.4%)	n = 358 (86.0%)	n = 50 (14.0%)
Heme iron (mg/day)	3.7 ± 1.19	3.9 ± 1.3	0.036	2.5 ± 0.3	2.6 ± 0,4	3.6 ± 0.3	3.7 ± 0.3	5.1 ± 0.9	5.1 ± 1.2
Non-heme iron (mg/day)	12.0 ± 3.23	11.8 ± 3.4	0.538	11.1 ± 3.0	10.3 ± 2.7	11.9 ± 2.8	12.0 ± 3.6	13.2 ± 3.5	12.8 ± 3.4
Iron (mg/day)	15.7 ± 3.80	15.8 ± 4.0	0.886	13.6 ± 3.1	12.8 ± 2.8	15.5 ± 2.9	15.7 ± 3.6	18.2 ± 3.9	17.9 ± 3.8
Vitamin C (mg/day)	170.5 ± 69.56	165.0 ± 58.1	0.394	153.5 ± 62.9	140.6 ± 64.9	175.2 ± 68.9	161.6 ± 53.2	183.6 ± 73.4	184.5 ± 52.0
Calcium (mg/day)	978.6 ± 328.73	963.5 ± 368.6	0.628	853.7 ± 289.7	820.4 ± 246.6	997.5 ± 309.9	927.6 ± 313.9	1091.0 ± 341.7	1092.4 ± 440.6
Fiber (g/day)	23.0 ± 6.74	22.2 ± 6.7	0.189	21.9 ± 6.6	20.5 ± 7.3	22.8 ± 5.9	21.9 ± 6.1	24.4 ± 7.4	23.6 ± 6.8
Carbohydrates (g/day)	241.1 ± 72.28	228.3 ± 79.8	0.062	222.5 ± 70.5	188.2 ± 47.9	238.2 ± 63.5	233.3 ± 79.8	263.4 ± 76.5	250.0 ± 87.7
Protein (g/day)	90.1 ± 19.76	90.8 ± 19.5	0.687	75.0 ± 14.0	73.3 ± 9.9	89.2 ± 12.4	89.77 ± 13.6	106.9 ± 17.7	103.4 ± 20.1
Total fat (g/day)	102.7 ± 27.02	104.5 ± 28.1	0.482	89.5 ± 22.3	89.0 ± 26.4	102.3 ± 22.1	103.8 ± 27.0	117.4 ± 28.7	115.4 ± 25.8
Total energy (Kcal/day)	2 328.5 ± 553.55	2 322.9 ± 607.0	0.916	2059.3 ± 484.7	1912 ± 362.9	2305.5 ± 446.8	2344.6 ± 610.3	2634.8 ± 76.5	2573.4 ± 596.9
Dairy (g/day)	369.6 ± 212.0	351.1 ± 233.2	0.357	342.5 ± 201.8	344.2 ± 184.1	382.5 ± 209.8	335.1 ± 200.4	385.1 ± 222.4	371.1 ± 288.1
Meat, fish and eggs (g/day)	231.4 ± 67.1	237.7 ± 60.4	0.303	177.2 ± 41.1	181.4 ± 37.7	229.1 ± 41.9	238.0 ± 40.3	290.6 ± 60.3	237.7 ± 60.4
Cereals, legumes & nuts (g/day)	196.5 ± 91.2	196.1 ± 101.0	0.961	185.3 ± 90.7	164.9 ± 60.2	188.6 ± 82.2	199.5 ± 109.1	216.4 ± 97.1	213.5 ± 110.9
Vegetables (g/day)	279.6 ± 108.8	276.3 ± 105.1	0.741	254.3 ± 96.6	243.6 ±105.8	277.9 ± 103.5	250.0 ± 97.3	308.2 ± 119.1	323.2 ± 96.3
Fruits (g/day)	352.9 ± 181.3	315.4 ± 150.8	0.010	328.8 ± 170.6	278.8 ± 177.2	373.1 ± 186.1	329.5 ± 135.8	357.8 ± 185.0	326.0 ± 144.8
Oils & fats (g/day)	57.9 ± 22.0	60.5 ± 25.0	0.220	53.0 ± 19.9	52.5 ± 19.3	57.5 ± 21.0	62.4 ± 29.9	63.6 ± 23.8	63.9 ± 22.4
Alcoholic beverages (cc/day)	128.5 ± 190.9	17.0 ± 287.4	0.087	106.5 ± 185.2	91.1 ± 151.2	132.1 ± 210.3	174.4 ± 221.3	147.9 ± 173.7	225.7 ± 387.2
Coffee (cc/day)	31.5 ± 47.5	20.5 ± 32.9	0.001	27.2 ± 46.1	16.0 ± 33.4	31.4 ± 43.8	20.6 ± 31.5	36.0 ± 52.2	23.4 ± 34.3
Tea (cc/day)	5.3 ± 23,1	4.5 ± 13.2	0.720	4.6 ± 25.6	3.0 ± 10.9	5.8 ± 24.3	5.1 ± 14.1	5.4 ± 23.1	4.9 ± 13.2

Results expressed as mean ± standard deviation.

Table 3 Cox regression evaluating the association between heme iron intake and the risk of new onset diabetes

VARIABLES	P[1]	HAZARD RATIO	95% Confidence interval for hazard ratio	
			Lower	Higher
Model 1: Heme iron	0.075	1.13	0.99	1.30
Model 2: Heme iron +	0.029	1.20	1.02	1.42
Energy	0.210	0.98	0.94	1.01
Model 3: Heme iron +	0.037	1.30	1.02	1.66
Energy +	0.113	0.95	0.88	1.01
Animal protein +	0.155	0.97	0.93	1.01
Vegetable protein +	0.320	1.02	0.99	1.05
Fruit +	0.339	0.99	0.98	1.01
Alcoholic beverages +	0.005	1.02	1.01	1.04
Coffee +	0.005	0.93	0.89	0.98
Tea +	0.665	0.98	0.89	1.08
Calcium	0.747	1.01	0.95	1.08

Last model adjusted also for age (years), gender, waist circumference (cm) ($P < 0.001$; Exp(B) = 1.05), education level (medium-high, low), intervention group (control, olive oil and nuts) and smoking.
Heme iron (mg/day), energy intake (100 kcal/day), animal protein [meat, fish and eggs] (10 g/day), vegetables protein [cereals, legumes and nuts] (10 g/day), fruit (10 g/day), alcoholic beverages (20 cc/day), coffee (10 cc/day), tea (10 cc/day), calcium (100 mg/day).
[1]P-value for hazard ratio.

total iron, iron supplements, or non-heme iron intake [6]. The association between total iron [3] or heme iron intake [4] and prevalent T2DM was also cross-sectionally observed in healthy adult Chinese populations. Finally, a recent meta-analysis and systematic review suggest that increased heme iron intake is associated with higher risk of T2DM [17]. The association between heme iron intake before pregnancy and/or during the early period of pregnancy and gestational diabetes was also recently reported in the Nurses Health Study II [18] and the U.S. Omega cohort [19].

These findings have been reinforced by the fact that several studies have found an association between elevated levels of iron and the risk of T2DM [3,4,15]. Recently, a study on the Potsdam EPIC cohort found that high ferritin levels – above 110 ng/mL in females and 280 ng/mL in males – were associated with an increased risk of T2DM [15].

Blood donations and phlebotomy have also been observed to have a positive effect on insulin sensitivity and risk of T2DM because they reduce body iron levels. Fernandez-Real et al. observed a decrease in body iron levels and improved insulin sensitivity in healthy subjects who made frequent blood donations (at least 2 in the previous 5 years) [20]. They also found that the number of donations was positively correlated with insulin sensitivity. The same authors had previously observed that both insulin secretion and sensitivity, and glycosylated hemoglobin levels improved in diabetic patients undergoing phlebotomy [21]. These results appear to show that iron reduction has a beneficial effect on the occurrence of T2DM and controls the parameters associated with T2DM. Interventional studies should be carried out to determine whether reducing the heme iron intake diminishes the risk of healthy subjects developing T2DM and improves control and management of patients with T2DM.

Although the exact mechanism by which iron induces T2DM and other diseases is unclear [8], there is increasing evidence to suggest that iron status has a considerable influence on the oxidative damage suffered by biomolecules such as DNA [22]. It has been speculated that this phenomenon can affect the physiopathology of such key events in the onset of T2DM as insulin resistance or deficiency [9]. For example, storage of iron in pancreatic β cells could affect β cell secretion [23], inducing apoptosis mediated by oxidative stress [24].

The bioavailability of heme iron is determined by diet and is probably one of the important factors that explains why heme iron is the component of the diet that has consistently been associated with a risk of T2DM by our study and others. For this reason, the following factors were included in the proportional hazard models: meat, fish and eggs [animal protein]; cereals, legumes and nuts [vegetable protein]; alcoholic beverages; tea and coffee and calcium. This last factor, in conjunction with polyphenols [25], inhibits heme iron absorption [26]. The effects of polyphenols were regarded as being indirectly due to the consumption of tea and coffee, which are rich in phenolic compounds. Alcohol beverages were included because it has been suggested that acute or chronic exposure to alcohol can suppress hepcidin expression in the liver, which increases the intestinal transport of iron into plasma [27].

In our study, the effect of vegetable protein, another new factor that influences heme iron absorption [28], was estimated from the consumption of cereals, legumes and nuts. Another important factor involved in heme iron absorption is animal protein [28]. In the proportional hazard models this is presented as "meats, fish and eggs [animal protein]". Although the variable "meats, fish and eggs [animal protein]" shows collinearity with "heme iron", we decided to include both in the proportional hazard models because they assess different aspects. "Heme iron" is associated with the quantity of iron in the diet and "meats and fish [animal protein]" with facilitating iron absorption. In addition, "meats, fish and eggs [animal protein]" improved the β coefficient of the primary variable, "heme iron", and the general model, both of which support the inclusion of these variables.

The results of our study are in agreement with those of other epidemiological studies [3-7]: a high intake of heme iron seems to be associated with an increased risk of T2DM, also in a Mediterranean population at high cardiovascular risk. This relationship was particularly evident in our study at high doses of heme iron intake. Although it is not fully understood why results vary according to the form of iron intake, heme iron is more bioavailable because its absorption is independent of body iron status and elevated heme iron intake has been associated with high levels of serum ferritin [29], which in turn is related to greater risk of T2DM [4].

On the final proportional hazard model applied, we also observed a positive association between the consumption of alcohol beverages and an increased risk of T2DM. On the other hand, coffee consumption was associated with a reduced risk, as has previously been observed in other studies [30]. The negative relationship between coffee consumption and the risk of T2DM could also be explained by the positive relationship between caffeine and insulin sensitivity, and between decaffeinated coffee and β cell function [31]. Polyphenols, including coffee and tea polyphenols, seem to inhibit the uptake of intestinal hemo iron [25], thus reducing its adverse effects. In the case of alcohol beverages, the presence of alcohol seems to have a detrimental effect on the metabolism of iron. In American postmenopausal women aged 55 to 69 years, an association was observed between higher heme iron or supplemented iron intake and greater risk of developing T2DM, especially in those who also consumed alcohol [7]. High or moderate consumption of alcohol was associated with a significant increase in the risk of iron overload [32]. In this regard, Whitfeld et al. observed that ferritin and iron serum were increased by alcohol consumption [33], and this, in turn, was associated with a risk of TDM2 [34].

Because of the observational nature of our study, we cannot completely establish a cause-effect relationship between heme iron intake and the risk of developing T2DM. However, the covariates in the statistical models minimize the major sources of confounding. One of the strengths of the study was that the diagnosis of T2DM was not self-reported and was verified by a second analytical test, thus making the identification of new incident cases more reliable and accurate. However, some participants did not undergo an OGTT, so T2DM could only be diagnosed by fasting blood sugar ≥ 7.0 mmol/L confirmed by a second test. This might have falsely lowered overall incidence rates. Finally, the dietetic variables used in the analysis were obtained from a semiquantitative food frequency questionnaire that had been validated in the same population so that the possibility of error was reduced [12].

Conclusion

In conclusion, the results of the present study show that an elevated heme iron intake was associated with a significant increase in the risk of T2DM incidence in a Mediterranean population at high cardiovascular risk.

Competing interests

Dr. Jordi Salas-Salvadó is a non-paid member of the Scientific Advisory Board of the International Nut Council. The other authors have no conflict of interest affecting the conduct or reporting of the work submitted.

Authors' contributions

JCF performed the literature searches, contributed to the interpretation of the results and wrote the manuscript. VA supervised the statistical analyses and helped to interpret the results and revise the manuscript. NA and MB contributed to the interpretation of the results and revised the manuscript. JB coordinated the fieldwork and revised the manuscript. MAG conceived the study, participated in its design and revised the manuscript. JD revised the manuscript. JS conceived the study, participated in its design and revised the manuscript. All authors read and approved the final manuscript.

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Association between Serum Ferritin and Osteocalcin as a Potential Mechanism Explaining the Iron-Induced Insulin Resistance

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Abstract

Background: Increased iron stores are associated with increased risk of type 2 diabetes, however, the mechanisms underlying these associations are poorly understood. Because a reduction of circulating osteocalcin levels after iron overload have been demonstrated in cell cultures, and osteocalcin is related to glucose and insulin metabolism, the iron-induced osteocalcin reductions could contribute to explain the role of iron metabolism in the development of type 2 diabetes mellitus.

Objective: To analyzed the associations between serum total and uncarboxylated osteocalcin and adiponectin concentrations with serum ferritin and soluble transferrin receptor (sTfR) in elderly subjects.

Design: We evaluated a total of 423 subjects from the PREDIMED cohort in a population-based cross-sectional analysis. Extensive clinical, nutritional and laboratory measurements, including total and uncarboxylated osteocalcin, adiponectin, ferritin and sTfR were recorded.

Results: Serum ferritin was positively correlated with increased glucose and insulin circulating levels but also with HOMA-IR, and was inversely associated with total osteocalcin and adiponectin. A regression analysis revealed that serum ferritin and transferrin receptor levels were significantly associated with a decrease in total and uncarboxylated osteocalcin. Serum sTfR levels were associated with lower uncarboxylated osteocalcin levels in the whole-study subjects and remained significant only in the IFG (impaired fasting glucose) individuals.

Conclusions: We described, for the first time, an inverse association between serum ferritin and sTfR with osteocalcin and extend previous results on adiponectin, thus supporting that factors related to iron metabolism could contribute to the insulin resistance and the development of type 2 diabetes mellitus.

Trial Registration: Controlled-Trials.com ISRCTN35739639 <http://www.controlled-trials.com/ISRCTN35739639>.

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Introduction

Iron is an essential mineral for humans although is potentially hazardous in excess amounts. Excessive iron stores in patients with hereditary hemochromatosis (HH) have been causally related with the development of type 2 diabetes mellitus (T2DM) [1]. However, moderately increased iron stores are also associated with hyperglycemia and hyperinsulinemia, or an increased risk of type 2 diabetes mellitus in apparently healthy subjects [2–5]. These

findings have been confirmed in a recent meta-analysis of five prospective epidemiologic studies, giving a pooled RR of 1.63 for type 2 diabetes mellitus in subjects with the highest levels of ferritin [6]. Additionally, in a nested case-control study, increased soluble transferrin receptor (sTfR) levels were associated with increased T2DM risk (OR 2.26 [1.37–4.01] [7]. Increased iron stores have also been associated with gestational diabetes [8], prediabetes [9], central adiposity [10], metabolic syndrome [11], cardiovascular disease [12] and osteopenia or osteoporosis [13,14].

Possible mediators linking iron stores and diabetes are still poorly understood. Serum ferritin levels, the commonly used marker for total body iron stores, has been associated with insulin resistance measured by homeostasis model assessment (HOMA IR) or hyperinsulinemic euglycemic clamp [15,16] but not with pancreatic beta-cell function in humans [3], whereas in obese mouse, dietary iron restriction protects from loss of beta cell function [17]. Iron deposition in the muscle decreases glucose uptake due to muscle damage [18] and it has additionally been suggested that iron deposition in pancreatic β-cells impairs insulin secretion in more advanced states of iron overload [19]. Despite that, the effect of iron depots on other insulin-related tissues such as adipose or bone tissues is far from clear. Recent studies conducted in animals or in humans have demonstrated a direct and causal effect of iron stores in circulating levels of adiponectin, independently of other peripheral markers of inflammation [20,21], thus explaining the attenuated association between ferritin and incident type 2 diabetes mellitus observed after adjustment for circulating adiponectin levels [5]. Additionally, a dose-response decreased expression of genes related to the osteoblast phenotype, including osteocalcin, after iron overload have been demonstrated in cell cultures [22–24]. Thus, because osteocalcin (OC) has been related to a decrease in fasting glucose concentrations and to an increase of pancreatic beta-cell proliferation, insulin secretion and sensitivity [25], the iron-induced osteocalcin reductions could contribute to explain the role of iron overload in the development type 2 diabetes mellitus.

To our best knowledge, there are no studies demonstrating a direct association between markers of iron metabolism and osteocalcin concentrations in humans. We therefore conducted the present study to evaluate possible associations between serum total and uncarboxylated osteocalcin concentrations with serum ferritin and (sTfR) in elderly subjects at high cardiovascular risk.

Methods

Study design and population

For the present analysis, non-diabetic participants from three Spanish centers (Reus-Tarragona, Navarra and Barcelona-Clinic) within the framework of the PREDIMED study were randomly selected. The PREDIMED study is a multicenter, randomized clinical trial conducted in Spain to assess the effects of the Mediterranean diet (MedDiet) on the primary prevention of

Table 1. Baseline characteristics of the study subjects.

	Total subjects	NGM	IFG	P
N	423	250	173	
Men/women	202/221	111/139	91/82	0.097
Age (years)	66.3±0.3	66.3±0.4	66.2±0.4	0.902
BMI (kg/m^2)	29.6±0.1	29.4±0.2	29.8±0.2	0.161
Waist circumference (cm)	99.00±0.45	97.94±0.60	100.44±0.70	0.007
Total energy intake (Kcal/day)	2377.75±28.78	2368.37±37.20	2391.21±45.53	0.707
Physical activity (METs-min/day)	273.4±11.6	272.4±15.3	274.9±17.9	0.916
Smoking habit (yes/no)	96/327	59/191	37/136	0.757
Fasting glucose (mg/dL)	97.00 (89.01, 109.01)	90.00 (85.00, 95.29)	111.15 (105.00, 122.75)	<0.001
Total cholesterol (mg/dL)	223.21±1.84	223.07±2.22	223.41±3.17	0.929
HDL cholesterol (mg/dL)	55.29±0.67	56.67±0.95	53.30±0.89	0.014
LDL cholesterol (mg/dL)	139.61±1.57	139.59±2.02	139.63±2.51	0.990
Triglyceride level (mg/dL)	141.82±4.27	133.73±4.51	153.57±8.12	0.022
Fasting plasma insulin (mU/mL)	4.61 (3.12, 7.01)	4.23 (3.06,6.48)	5.04 (3.332,7.61)	0.037
HOMA-IR	1.35±0.04	1.14±0.04	1.68±0.07	<0.001
HOMA-BCF %	63.12±2.88	77.37±4.40	41.93±1.87	<0.001
Total osteocalcin (ng/mL)	8.00 (6.17,10.75)	8.50 (6.36, 11.52)	7.44 (5.78,9.64)	0.005
Uncarboxylated osteocalcin (ng/mL)	4.15 (2.38,6.06)	4.58 (2.59,6.80)	3.56 (2.15,5.46)	0.003
Ratio ucOC/OC	0.58±0.02	0.62±0.03	0.53±0.03	0.077
Adiponectin (ng/mL)	8.30 (5.29,13.22)	9.15 (6.10,14.23)	6.98 (4.68,11.54)	0.001
C-Reactive Protein (ng/mL)	1.32 (0.26,3.58)	1.24 (0.22,3.17)	1.43 (0.31,3.90)	0.612
oxLDL (mU/L)	59.17±1.26	60.58±1.62	57.11±2.01	0.150
Ferritin (ng/mL)	126.20 (67.98, 212.47)	118.85 (66.64, 204.75)	132.70 (75.38, 230.91)	0.104
Transferrin receptor (mg/L)	1.24 (1.07,1.42)	1.25 (1.09,1.43)	1.22 (1.07,1.41)	0.242
Ratio Transferrin/ferritin	0.018±0.0012	0.019±0.0018	0.015±0.027	0.155

Data expressed as mean ± SE, mean (IQR). P* are differences between normal glucose metabolism (NGM) and impaired fasting glucose (IFG) groups.
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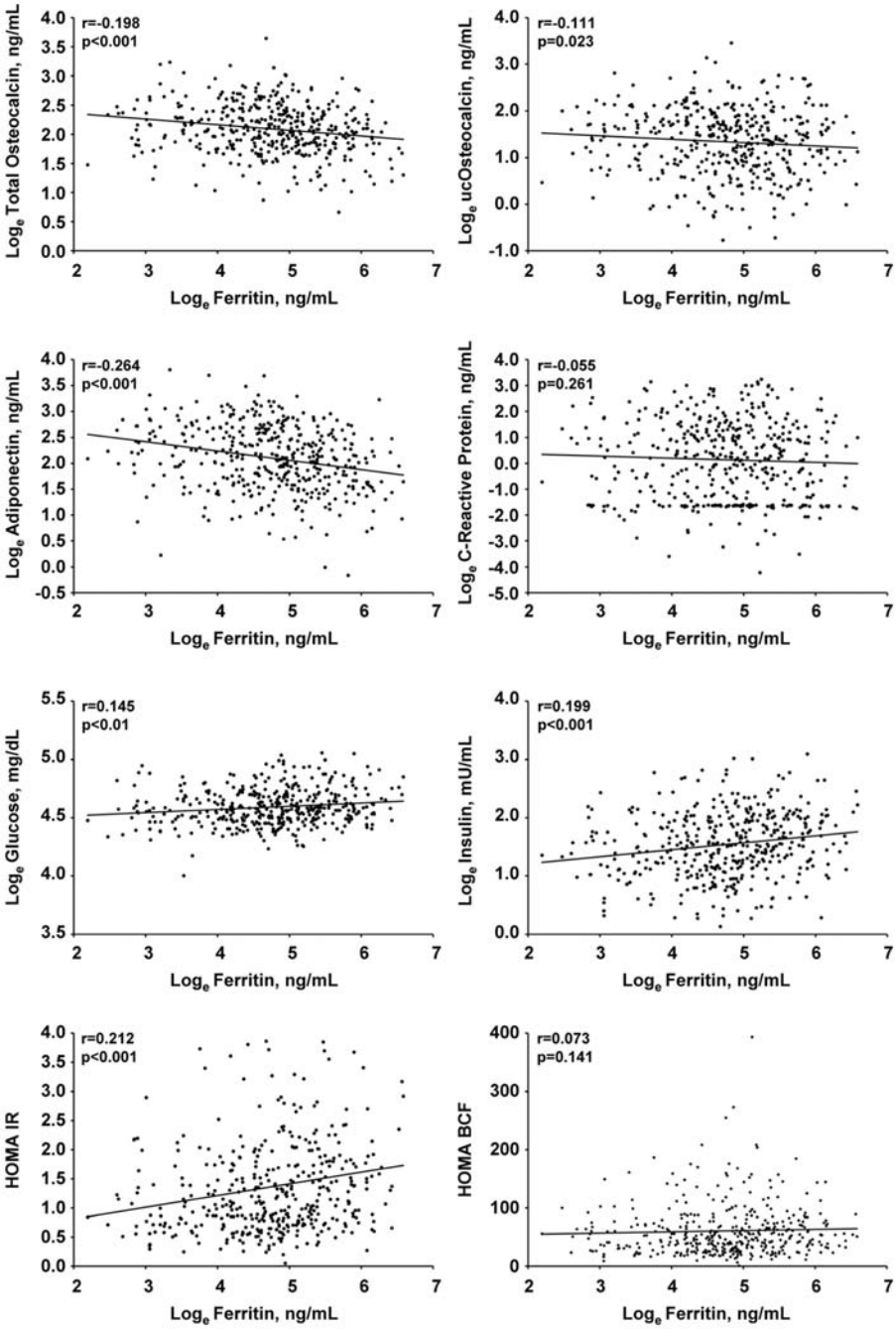

Figure 1. Correlations between serum ferritin levels and selected biochemical parameters. Correlation coefficients are based on $\log_e$-transformed values of markers except HOMA-IR and HOMA-BCF.
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cardiovascular disease. The design of the PREDIMED trial (http://www.controlled-trials.com/ISRCTN35739639) has been reported elsewhere [26,27], and it is available at http://www.predimed.org and www.predimed.es. Subjects were men aged 55 to 80 years and women aged 60 to 80 years without prior cardiovascular disease (CVD) at baseline but with at least three cardiovascular risk factors namely: smoking, hypertension, dyslipidemia, overweight (Body mass index (BMI) $\geq$25 kg/m^2), and a family history of early-onset coronary heart disease (before age 55 years in men or before age 65 years in women) in first-degree relatives [27]. The exclusion criteria for the PREDIMED study were any severe chronic illness, alcohol or drug addiction, history of food allergy to olive oil or nuts, or a low predicted likelihood of changing dietary habits according to Prochaska and DiClemente's stages-of-change model [28].

We performed the current cross-sectional analyses at baseline evaluation of a subsample of 455 subjects of this cohort, after excluding 9 participants with very low values of serum ferritin who were clearly outside the normality levels according to the cut-off of our central laboratory to exclude individuals with ferropenia ($<$10 ng/dL in women and $<$20 ng/dL in men). We also excluded other 23 subjects with missing data on plasma glucose, insulin or some important covariates for the analysis.

The PREDIMED study protocol was approved by the institutional review boards of all the centres involved, and all subjects agreed to participate in the study and gave their written informed consent.

Measurements

Medical information was collected on subjects' medical record of a 47-item questionnaire about education, lifestyle, history of illnesses and medication use. A validated 137-item food frequency questionnaire (FFQ) was administered [29] and dietary energy intake was calculated from Spanish food composition tables [30]. Trained personnel measured baseline weight, height and waist circumference as previously reported, as well as blood pressure in triplicate with a validated semiautomatic oscillometer (Omron HEM-705CP, Hoofddorp, Netherlands). Enegy expenditure in leisure-time physical activity (LTPA) was estimated using the validated Spanish version of the Minnesota Questionnaire.

Blood samples were collected from all participants after an overnight fast and were immediately processed, coded, and shipped to a central laboratory. Serum levels of fasting glucose, total cholesterol, HDL-cholesterol and triglycerides were measured by standard enzymatic automated methods. LDL-cholesterol concentrations were calculated using Friedewald's equation in those patients whose triglyceride levels were $<$400 mg/dL. Plasma fasting insulin concentrations were measured by an ELISA kit for human insulin (Millipore, St. Charles, Missouri, USA). Insulin resistance was estimated by the HOMA method using the following equation [31]: HOMA-IR = [fasting insulin (μIU/mL) x fasting glucose (mmol/L)]/22.5. Serum total and uncarboxylated osteocalcin levels were measured by electrochemiluminescence immunoassay (N-mid osteocalcin, Roche, Indianapolis, IN); the intra- and inter-assay coefficients of variation were $<$3.6% and $<$6.6% respectively). Altered beta-cell function was estimated using HOMA-BCF (homeostasis model assessment-beta cell) as previously described [32]. Serum ferritin and soluble transferrin receptor were measured by a particle-enhanced immunoturbidimetric assay using the Hitachi analyzer and sTfR:ferritin ratio was calculated. C-reactive protein (CRP) concentrations were measured via a highly sensitive immunoassay (Helica Biosystems Inc, Santa Ana, CA). Adiponectin levels were measured using an enzymatic immunoassay (Millipore, St. Charles, Missouri, USA). Plasma oxidized LDL (oxLDL) concentrations were also measured by a commercial ELISA (Mercodia Oxidized LDL ELISA, Uppsala, Sweden).

Statistical analysis

Variables with skewed distribution according to the Kolmogorov–Smirnov tests were $\log_e$-transformed before analysis. Means (SE), percentages (%) or median (IQR) were used for descriptive purposes. Whether the associations differed between sexes were tested by adding the interaction terms sex*ferritin and sex*sTfR to the fully adjusted models. Because no interaction was found, all results are presented for the whole population studied. Although no interaction was observed between markers of iron status and glucose metabolism (IFG*ferritin and IFG*sTfR) it is physiologically plausible that impaired fasting glucose (IFG) (considered when fasting glucose levels were higher than 100 mg/dL) could affect the associations between ferritin and sTfR with total and uncarboxylated osteocalcin or adiponectina [33]. For this reason, results are also presented stratified according to glucose metabolism for two different groups: for subjects with normal glucose metabolism (NGM) and for those with IFG. General characteristics of the study subjects were compared between both sexes using ANOVA. Associations between markers of iron metabolism (ferritin, transferrin receptor and the ratio sTfR:ferritin) and metabolic or inflammatory variables were examined using Spearman's correlations. To examine the relationship between markers of iron metabolism with total or uncarboxylated osteocalcin, linear regression models were fitted including sex, age, BMI, smoking status, total energy intake, energy expenditure in leisure-time physical activity, fasting plasma glucose and insulin, and markers of inflammation or oxidation (adiponectin, CRP, oxLDL) as potentially confounding variables in the fully adjusted model. In addition, both regressions analyses with either serum ferritin or sTfR as outcomes were mutually adjusted for each other to account for negative confounding because they are both independently associated with insulin resistance despite their negative reciprocal correlation [16,34]. For $\log_e$-transformed outcome variables (i.e. total and uncarboxylated osteocalcin) regression coefficients were converted into percentages of relative change in the original variable (percentage decrease in total or uncarboxylated osteocalcin per 50 ng/mL increase in ferritin or per 1 ng/mL of increased sTfR). All analyses were performed using the SPSS 20.0 software (SPSS Inc, Chicago, IL).

Results

General characteristics of the study subjects are reported in **Table 1**. Total and uncarboxylated osteocalcin and adiponectin were significantly lower in subjects with impaired fasting glucose. A tendency to higher ferritin concentrations and a lower ratio of sTfR:ferritin were observed in individuals with impaired fasting glucose in comparison to those with normal glucose metabolism as expected, although these differences didn't reach statistical significance.

In the whole-study population, serum ferritin was positively correlated with increased glucose and insulin circulating levels but also with HOMA-IR, and was inversely related with total and uncarboxylated osteocalcin and adiponectin but not with HOMA-BCF (**Figure 1**). Soluble transferrin receptor concentrations were positively related with plasma oxLDL levels ($r = 0.134$, $p = 0.006$) and non- related with plasma glucose ($r = -0.078$, $p = 0.113$, insulin ($r = 0.003$, $p = 0.946$), HOMAIR ($r = -0.032$, $p = 0.522$) and HOMA-BCF ($r = 0.014$, $p = 0.771$). A non-significant correlation was observed between sTfR and ucOC ($r = -0.088$,

Table 2. Association between markers of iron metabolism and total or uncarboxylated osteocalcin serum levels.

	For each 50ng/mL of increased ferritin			For each 1ng/mL of increased sTfR			For each 0.1 ng/mL of increased ratio sTfRferritin		
Outcome	Regression coefficient	95% CI	P-value	Regression coefficient	95% CI	P-value	Regression coefficient	95% CI	P-value
In the whole population									
Total OC (ng/mL) (% change)									
Crude	−3.82	−5.25, −2.27	<0.001	−8.05	−19.42, 4.91	0.211	9.85	−6.48, 29.04	0.250
Model 1	−2.76	−4.30, −1.09	0.001	−6.76	−17.96, 6.07	0.286	5.02	−10.41, 23.24	0.543
Model 2	−2.46	−4.01, −0.79	0.004	−12.97	−23.73, −0.79	0.038	0.80	−13.92, 18.05	0.921
Uncarboxylated OC (ng/mL) (% change)									
Crude	−1.98	−4.49, 0.70	0.140	−17.05	−33.16, 2.83	0.088	7.03	−17.79, 39.37	0.612
Model 1	−0.29	−2.95, 2.53	0.859	−17.55	−33.30, 2.02	0.075	−2.95	−25.54, 26.49	0.825
Model 2	−0.39	−3.24, 2.42	0.765	−22.11	−37.76, −2.56	0.029	−5.25	−27.45, 23.86	0.694
In the NGM									
Total OC (ng/mL) (% change)									
Crude	−4.11	−6.26, −1.98	<0.001	−14.27	−27.31, 1.00	0.066	0.60	−16.80, 21.65	0.951
Model 1	−2.95	−5.25, −0.59	0.013	−10.05	−23.58, 5.86	0.200	0.002	−17.30, 20.92	0.998
Model 2	−2.95	−5.06, −0.69	0.010	−16.13	−29.03, −0.98	0.038	−4.87	−21.25, 14.91	0.603
Uncarboxylated OC (ng/mL) (% change)									
Crude	−0.99	−4.78, 2.94	0.620	−16.97	−37.12, 9.52	0.186	−1.39	−28.46, 35.79	0.929
Model 1	1.00	−3.05, 5.01	0.644	−14.61	−35.07, 12.29	0.257	−9.87	−34.62, 24.11	0.521
Model 2	0.40	−3.72, 4.81	0.815	−16.13	−37.37, 12.29	0.236	−10.05	−35.20, 24.85	0.542
In the IFG									
Total OC (ng/mL) (% change)									
Crude	−3.14	−5.16, −0.98	0.005	0.90	−18.86, 25.35	0.939	32.04	−2.37, 78.42	0.071
Model 1	−2.37	−4.59, −0.19	0.036	−1.29	−20.30, 22.14	0.901	15.83	−14.78, 57.45	0.345
Model 2	−2.17	−4.49, 0.30	0.080	−7.03	−25.91, 16.64	0.526	14.50	−15.88, 55.89	0.386
Uncarboxylated OC (ng/mL) (% change)									
Crude	−2.27	−5.63, 1.10	0.183	−22.81	−44.95,8.22	0.132	20.44	−25.09, 93.67	0.441
Model 1	−1.39	−4.87, 2.32	0.468	−25.53	−48.31, 1.51	0.061	3.97	−36.36, 69.89	0.875
Model 2	−2.07	−5.82, 1.91	0.308	−34.29	−54.38, −5.35	0.024	3.35	−37.37, 70.57	0.897

β values are unstandardized regression coefficients and represents the change in total and uncarboxylated osteocalcin according to increases in ferritin, soluble transferrin receptor and the ratio of sTfR/ferritin. Model 1: adjusted for sex, age and BMI, smoking status, total energy intake and energy expenditure in leisure-time physical activity. Model 2: additionally adjusted for fasting plasma glucose, insulin and markers of inflammation and oxidative stress (adiponectin, C-reactive protein, oxidized LDL) and ferritin and transferrin receptor adjusted for each other).

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p = 0.071), and between sTfR:ferritin and glucose, insulin and HOMA-IR (r = −0.154, r = −0.177, r = −0.194 p<0.001 for all).

Total OC and adiponectin were negative associated with HOMA-IR (−0.193 and −0.338, p<0.001 respectively). In addition, ferritin and sTfR serum levels were inversely correlated (r = −0.250, p<0.001). In a linear regression model, serum ferritin and transferrin receptor concentrations were significantly associated with a decrease in total OC (p = 0.004) per 50 ng/mL of increased

ferritin, and a decrease in uncarboxylated osteocalcin (ucOC) ($p = 0.038$) per 1 ng/mL of increased sTfR even after adjustment for sex, age, BMI, smoking status, total dietary energy intake, physical activity, fasting plasma glucose, and insulin, and peripheral markers of inflammation and oxidation (adiponectin, CRP, oxLDL), and sTfR or serum ferritin (for models with ferritin or TfR as the outcome, respectively). These associations were apparent both in normoglycemic and in subjects with impaired fasting glucose, although in the last case the association between ferritin and total OC was not statistical significance ($p = 0.080$). Serum sTfR levels were associated with lower ucOC levels in the whole-study subjects and remained significant only in the IGM individuals (**Table 2**). In contrast, sTfR:ferritin ratio was not significantly associated nor with total OC neither with ucOC.

Discussion

The present study confirmed, for the first time, that both serum ferritin and soluble TfR levels, as markers of iron metabolism, are independently and inversely associated with total and uncarboxylated osteocalcin. Because osteocalcin has been related not only with bone metabolism but also with insulin resistance and sensitivity, our results would help to explain one of the possible mechanisms relating iron metabolism, insulin resistance and risk of type 2 diabetes mellitus. We also confirm and extend previous results showing the inverse association between serum ferritin and adiponectin concentrations as a potential mechanism linking iron stores with insulin resistance [21,35].

Serum OC levels were used to evaluate bone metabolism because it has been considered a better sensitive marker of bone formation than serum alkaline phosphatase. However, increasing data supporting extra-skeletal roles of OC have emerged, being widely accepted its hormonal effect on energy metabolism, angiogenesis or insulin metabolism [36]. In this sense, mice lacking osteocalcin show lower beta-cell proliferation, glucose intolerance, and insulin resistance than wild-type mice [15]. Additionally, osteocalcin knockout mice have reduced levels of serum adiponectin, which suggests a potential role for osteocalcin in insulin sensitivity and secretion. In a previous study, our group demonstrated, for the first time, that increased total or uncarboxylated OC serum concentrations were directly associated with HOMA-BCF and inversely with insulin resistance determined by HOMA-IR [25].

Iron overload can damage several important organs such as liver, pancreas and heart. Many lines of evidence indicated that iron overload affects bone tissue causing both osteopenia and osteoporosis [37]. Addition of iron to a culture of human osteoblasts decreased osteocalcin concentrations dose-responsively. This has been attributed to the ferroxidase activity of ferritin rather than its iron sequestering capacity [24]. Furthermore, iron exposure on human osteoblast-like cells reduced the expression of genes involved in bone matrix formation or osteoblast differentiation such as COL1AI, Runx2 or osteocalcin [23,38]. The observed inverse associations of ferritin with total and uncarboxylated osteocalcin observed in our study, independently of other inflammatory markers and glucose metabolism status, extend the results obtained in vitro. Given that ferritin is inversely correlated with sTfR we expected a negative relationship between sTfR uncarboxylated osteocalcin rather than the negative observed in our study. However, our results are in agreement to those reported by Rajpathak and coworkers who observed, in a nested case-control study conducted in the framework of the Diabetes Prevention Program, a positive association between serum sTfR levels and T2DM risk even after adjusting by ferritin levels, thus suggesting another potential mechanism linking sTfR and T2DM unrelated to iron overload [7]. Increased levels of sTfR have been observed in vitro after insulin administration to rats (32) and it cannot be discard sTfR levels as a biomarker of other factors causally related to T2DM [7]. In our study subjects, sTfR levels could be the result of a compensatory mechanism for a reduction of free iron levels secondary to inflammatory or oxidative status common in old subjects at cardiovascular risk. Moreover, in addition to other epidemiological studies, we also showed positive associations of ferritin and sTfR with fasting glucose, insulin and HOMA-IR, but not with HOMA-BCF, suggesting that the contribution of iron metabolism to type 2 diabetes mellitus is basically related to the induction of insulin resistance more than through an effect on beta-cell function [3,15]. In contrast to previous prospective studies suggesting that lower ratios of sTfR: ferritin were associated with increased risk of type 2 diabetes [2,4], no impact on the association of sTfR:ferritin and osteocalcin or adiponectin was observed in our study. However, as expected, we observed a negative tendency in the association between this ratio and HOMA-IR.

Iron stores could also induce insulin resistance through induction of oxidative stress [39] and serum ferritin has been associated with circulating oxidized LDL lipoproteins and advanced oxidation products [40]. Nevertheless, we observed a significant relationship of iron markers and osteocalcin or adiponectin independently of oxidized LDL, suggesting that the associations between iron and insulin resistance could be additionally mediated by other pathways rather than oxidative stress.

One concern of the present study is that ferritin concentrations may reflect other physiological aspects rather than iron storage, especially subclinical systemic inflammation related to insulin resistance. This is more relevant in our subjects because they are old, obese and at high cardiovascular risk. The cross-sectional nature of our assessment also hinders the possibility of a proper ascertainment of the direction of the causal sequence and we acknowledge this limitation. Also, because the associations between iron metabolism and osteocalcin forms observed in our study are weak, we must be cautious to consider iron metabolism markers as predictors of osteocalcin levels. Therefore, further prospective investigations should be designed to confirm these associations.

We tried to minimize the potential confounding by obesity and inflammation controlling for BMI and oxidative and inflammatory status (CRP, adiponectin, oxLDL) in the multivariate models. However, we cannot rule out the possibility that our results may have been influenced by other unmeasured factors. In any case, the high cardiovascular risk of our study subjects is to our advantage because they are a homogeneous population with smaller between-subjects variability in cardiovascular risk factors than a sample of the general population.

In sum, we described an inverse association between serum ferritin and sTfR with total or uncarboxylated osteocalcin and adiponectin in subjects at high cardiovascular risk. These findings suggest that body iron metabolism may contribute to the induction of insulin resistance through the inhibition of adiponectin and osteocalcin thus providing support for the hypothesis that iron metabolism could contribute to the origin of type 2 diabetes mellitus. Further research is warranted to understand the exactly mechanisms by which ferritin and sTfR levels induce insulin resistance.

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Author Contributions

Analyzed the data: MB JSS. Wrote the paper: MB JSS. Designed research: MB JSS MAMG RE MF. Conducted research: MJF. Performed some biochemical analysis: JCF VAV. Had primary responsibility for final content: MB JSS. Read and approved the final manuscript: MJF JCF JSS MAMG RE MF VAV MB.

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Excess body iron and the risk of type 2 diabetes mellitus: a nested case–control in the PREDIMED (PREvention with MEDiterranean Diet) study

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Abstract

A prospective nested case–control study within the PREvention with MEDiterranean Diet (PREDIMED) was conducted to evaluate the relationship between excess body Fe (measured as serum ferritin (SF), soluble transferrin receptor (sTfR) and sTfR:ferritin ratio) and the risk of type 2 diabetes mellitus (T2DM) in a Mediterranean population at a high risk of CVD, without T2DM at the start of the study. The study contained 459 subjects, 153 with incident T2DM (cases) and 306 without incident T2DM (controls). The follow-up period was for 6·0 (interquartile range 3·9–6·5) years. For each incident diabetic subject, two subjects were selected as controls who were matched broadly for age as well as for sex, intervention group and BMI. We observed a relationship between SF values >257 μg/l in males and >139 μg/l in females and the risk of T2DM, following adjustment in the conditional logistic regression model for high-sensitivity C-reactive protein, fasting glucose and other components of the metabolic syndrome (OR 3·62, 95 % CI 1·32, 19·95; $P=0·022$). We also found an association between low sTfR:ferritin ratio levels and the incidence of T2DM (OR 3·02, 95 % CI 1·09, 8·39; $P=0·042$), but no association with sTfR (OR 1·29, 95 % CI 0·51, 3·23; $P=0·722$). Oxidative stress has been hypothesised to contribute to the development of insulin resistance and β-cell dysfunction, the two key events in the clinical development of T2DM. Following adjustment for other risk factors for T2DM, excess body Fe (measured as SF and sTfR:ferritin ratio) was associated with an increased risk of developing T2DM in a Mediterranean population at a high risk of CVD.

Key words: Serum ferritin: Soluble transferrin receptor: Body iron stores: Type 2 diabetes

The worldwide prevalence of diabetes in adults was estimated as 6·4 % in 2010, and has been forecast to increase to 7·7 % by 2030[1]. Recently, excess body Fe has been shown to be a risk factor for type 2 diabetes mellitus (T2DM)[2].

Serum ferritin (SF) is the most widely used biomarker of body Fe stores in epidemiological studies, despite being shown to be affected by inflammation status. Conversely, soluble transferrin receptor (sTfR) is not altered by inflammatory processes[3], and their levels in blood are proportional to the cell requirements for Fe[4].

Several prospective studies[2,5–14] have identified excess Fe as a risk factor for T2DM. Oxidative stress could be the mechanism by which excess Fe is associated with a higher incidence of T2DM. Oxidative stress would mediate in the pathophysiology of several key events related to the onset of T2DM, such as insulin resistance (IR) and β-cell dysfunction[15]. Most of these studies[2,5–12] used SF as a biomarker to estimate body Fe levels. However, in assessing the relationships between SF and the risk of T2DM, few studies had adjusted for fasting glucose levels[8] and other components of the metabolic syndrome (MetS)[5], which are the parameters with a high predictive capacity for T2DM[16]. As such, it is still not clear whether the relationship between SF levels and the risk of T2DM is independent of these risk factors.

Abbreviations: CLR, conditional logistic regression; hs-CRP, high-sensitivity C-reactive protein; IR, insulin resistance; MetS, metabolic syndrome; PREDIMED, PREvention with MEDiterranean Diet; sTfR, soluble transferrin receptor; SF, serum ferritin; T2DM, type 2 diabetes mellitus.

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Few prospective studies evaluating the association between excess body Fe and the risk of T2DM have used sTfR as a biomarker[2,7,11], and despite having found that elevated SF levels increased the risk of T2DM, the relationship between sTfR and T2DM was not clear. Thus, while the Potsdam European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study did not observe any association[2], the Diabetes Prevention Program (DPP) cohort study[7] observed that high levels of sTfR increased the risk of T2DM. Also, the recent Kuopio IHD Risk Factor (KIHD) study observed a U-shaped association[11].

To date, no studies have been conducted in southern Europe exploring the relationship between excess Fe and the risk of T2DM. Of note is that some characteristics of Fe metabolism, such as genetic predisposition and/or dietary intake in southern Europe, are quite different from the populations studied earlier, such as those in the USA[7], China[10] and northern Europe[2]. For example, while in northern Europe, the prevalence of the C282Y polymorphism in the haemochromatosis (HFE) gene is 5–10 % and that of the H63D polymorphism is 10–20 %, in southern Europe, the prevalence is 1–5 % and > 20 %, respectively[17]. In Spain, the prevalence of the H63D mutation reaches 46 % in certain regions[18]. Furthermore, the consumption of food items of animal origin is greater in northern compared with southern Europe[19].

To test the hypothesis that high body Fe stores increase the risk of T2DM in our geographical area, we measured excess body Fe (as SF, sTfR and sTfR:ferritin ratio) in relation to the risk of T2DM in a Mediterranean population at a high risk of CVD, without T2DM at the start of the prospective study.

Experimental methods

Study design

This is a case–control study nested in the PREDIMED (PREvention with MEDiterranean Diet) cohort, followed-up for a median of 6·0 (interquartile range 3·9–6·5) years. The PREDIMED trial[20] was intended to test the effectiveness of the Mediterranean diet on the primary prevention of CVD. The comparisons were between two traditional Mediterranean diets (one enriched with extra virgin olive oil and the other with nuts) *v.* advice alone on a low-fat diet. The present study was registered at ClinicalTrials.gov (registration no. ISRCTN35739639; http://www.controlledtrials.com/ISRCTN35739639).

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Clinical Research Ethics Committee of the Hospital Sant Joan de Reus. Written informed consent was obtained from all subjects.

Subjects

The present study was conducted with 459 Caucasian subjects aged 55–80 years, free of T2DM at baseline, and with three or more CVD risk factors; 153 were incident T2DM (cases) and 306 non-incident (control) individuals. The study population was recruited in the Primary Care Centres of Reus, Barcelona and Pamplona. For every incident diabetic individual identified, two subjects were randomly selected as controls matched for age (≤ 67 *v.* > 67 years), sex, intervention group and BMI (≤ 27 *v.* > 27 kg/m^2), using an incidence density sampling procedure.

Ascertainment of incident type 2 diabetes mellitus

The 153 cases of incident T2DM were diagnosed during the follow-up, according to the American Diabetes Association criteria[21], i.e. fasting plasma glucose concentration ≥ 7·0 mmol/l or plasma glucose concentration ≥ 11·1 mmol/l measured 2 h after a 75 g oral glucose load. A routine glucose test was performed on all participants in the PREDIMED study at least once a year to detect new cases of diabetes. When new-onset T2DM was identified by the physicians of Primary Care Centres, the test was repeated within the next 3 months to confirm the diagnosis. The homeostasis model assessment (HOMA) index was calculated for each individual as follows:

$$\text{HOMA-IR} = \text{fasting insulin (U/l)} \times \text{fasting glucose (mmol/l)}/22{\cdot}5).$$

Biochemical determination

All blood samples were collected after an overnight fast at the beginning of the study. Aliquots of serum and EDTA plasma were immediately processed, coded and shipped to a central laboratory in a portable cooler (−4°C), and stored at −80°C until analysis. The time between blood sampling and freezing was less than 1 h. Serum levels of fasting glucose, total TAG, total and HDL-cholesterol were measured by standard enzymatic methods. LDL-cholesterol was calculated using the Friedewald equation. Fasting plasma insulin concentrations were measured in duplicate by ELISA (Ezhi-14K; Millipore). Plasma concentrations of high-sensitivity C-reactive protein (hs-CRP) were measured using a highly sensitive immunoassay (Helica Biosystems, Inc.). The assay has a sensitivity of 0·2 μg/l, with intra- and inter-assay CV of ≤ 3·7 and < 4·8 %, respectively. SF (Elecsys Ferritin; Roche Diagnostics) and sTfR (Access sTfR 0QC; Beckman Coulter) were measured by immunochemiluminescence. The assay has a sensitivity of 0·05 μg/l for ferritin, and intra- and inter-assay CV of ≤ 2 and < 3·5 %, respectively, for SF. The assay has a sensitivity of 0·05 nmol/l for sTfR, and intra- and inter-assay CV of ≤ 5 and ≤ 8 %, respectively (1 mg/l = 13·55 nmol/l of sTfR).

Other measures

At baseline and at each annual visit, a general questionnaire on sociodemographic and lifestyle characteristics was administered, and anthropometric variables were measured. Also, a semi-quantitative 137-item FFQ that had been previously validated[22] was applied. Nutrients and energy intake were quantified according to the Spanish food composition

tables[23]. Leisure-time physical activity was assessed according to a validated questionnaire[24]. Blood pressure was measured in triplicate using a calibrated semi-automatic oscillometer (Omron HEM-705CP; Omron Healthcare Europe BV)[25].

Statistical methods

Variables showing a non-normality of distribution were log-transformed to normalise the distributions. Qualitative variables were compared using the χ^2 test. Quantitative variables were compared using the Student's *t* test or the Mann–Whitney test. Data are presented as percentages, means or geometric means and standard deviations, and medians and interquartile ranges in the case of variables being non-normally distributed.

Partial correlation coefficients of SF, sTfR and the sTfR:ferritin ratio adjusted for sex, age and BMI, as well as for several T2DM risk factors such as MetS components, fasting insulin, HOMA-IR and hs-CRP were calculated in the overall study sample, controls and cases (Table 2). A multiple linear regression analysis was applied to evaluate the influence of SF on IR.

To analyse the relationship between body Fe levels and the incidence of T2DM, participants were categorised into quartiles according to the distributions of SF, sTfR and sTfR:ferritin ratio at baseline in control individuals. Given the documented differences between males and females with respect to body Fe stores, the independent variables were adjusted for sex. The adjusted variables were categorised to avoid any assumption of linearity, and to evaluate the dose–response relationship in the onset of T2DM. Several conditional logistic regression (CLR) models were applied. A crude model (without adjustment) was fitted with each independent variable. The model was re-fitted with adjustment for lifestyle variables including the following: marital status (married/not married); educational level (primary/secondary/tertiary); smoking (current smoker/former smoker/never smoked); alcohol consumption (drinker/non-drinker); physical activity (200 metabolic equivalents (MET)-min/d/≥200 MET-min/d); family history of T2DM (yes/no). Adjustment also included four diagnostic criteria of the MetS, according to the harmonised criteria proposed by International Diabetes Federation (IDF) and the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI)[26], i.e. blood pressure ≥135/85 mmHg, serum TAG ≥1·7 mmol/l, HDL-cholesterol <1·03 mmol/l in males and <1·3 mmol/l in females, and waist circumference ≥102 cm in men and ≥88 cm in women. Dietary variables measured in relation to the risk of T2DM were categorised by quartiles, and included energy, Mg, vitamins D and E, dairy products, meat, vegetables and fruits[27]. Then, hs-CRP (mg/l) was introduced in model 1. Finally, fasting glucose concentration ≥5·6 mmol/l was introduced into the model. The test for linear trend across the quartiles was performed by assigning the median value to each category and introducing these new variables into the CLR as continuous variables.

The effects of SF and fasting glucose on the risk of T2DM were evaluated in another CLR model adjusted as 'enter' mode for all the variables included in model 1, except SF and fasting glucose, which were introduced as 'conditional' mode (CLR-conditional). We performed the same analysis for sTfR and the sTfR:ferritin ratio.

All data analyses were performed with the SPSS package for Windows (version 20.0; SPSS, Inc.). A value of $P<0·05$ was considered statistically significant.

Results

Of the 459 subjects, two individuals were removed from the study for not having the values of SF and sTfR recorded, and another two for having extreme values of SF. The final analysis contained 455 individuals.

There were no significant interactions between SF, sTfR and the sTfR:ferritin ratio *v.* sex ($P>0·05$). These interactions were assessed in the same CLR models in which we studied the association between high SF, sTfR and the sTfR:ferritin ratio levels and the risk of T2DM.

Table 1 summarises the baseline characteristics of the overall group, as well as the case and control groups separately. Of the participants, 71% had abdominal obesity, 42% the MetS, 34% hypertriacylglycerolaemia, 97% hypertension and 25% IR. Additionally, prevalences of the MetS and IR were higher in the cases than in the controls. Compared with the controls, patients with incident diabetes had greater waist circumference and higher levels of fasting glucose, insulin, TAG and IR. Also, SF levels were higher in the cases than in the controls, while sTfR levels were similar in both groups.

Table 2 summarises the partial correlation coefficients adjusted for sex, age and BMI between SF, sTfR and the sTfR:ferritin ratio together and several T2DM risk factors such as MetS components, fasting insulin, HOMA-IR and hs-CRP in the overall study sample, and in the controls as well as cases. SF and sTfR:ferritin ratio, but not sTfR levels, correlated significantly with fasting glucose, fasting insulin and HOMA-IR in the overall study sample. Furthermore, both biomarkers of body Fe stores significantly correlated with fasting insulin and HOMA-IR in non-incident diabetic individuals, and with SF alone in incident diabetic subjects. Conversely, SF was not correlated with hs-CRP, while sTfR was positively correlated with this biomarker of inflammation. SF and the sTfR:ferritin ratio were poorly correlated with the components of the MetS. Nevertheless, components such as blood pressure, TAG and HDL-cholesterol were significantly correlated with sTfR in the overall non-incident diabetic group.

To evaluate the association between the levels of SF and the risk of T2DM, several CLR models were applied (Table 3). The OR for the crude model (without adjustment for any variable) was 1·99 (95% CI 1·12, 3·52; $P=0·022$). Model 1 adjusted for lifestyle variables, family history of T2DM, and four components of the MetS showed an improvement in the trend (OR 2·39, 95% CI 1·11, 5·16; $P=0·030$). This trend did not substantially change when hs-CRP was included in the multivariable model (OR 2·38, 95% CI 1·10, 5·14; $P=0·031$). Finally, adjusting the model for fasting glucose showed an improvement in the trend (OR 3·62, 95% CI 1·32, 9·95; $P=0·017$).

The same models were employed in evaluating the relationship between the sTfR:ferritin ratio and the risk of T2DM.

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Table 1. Baseline characteristics of the overall study participants, and segregated by cases and controls (Mean values and standard deviations; medians and interquartile ranges (IQR))

	All (*n* 455)		Non-incident diabetics (*n* 302)		Incident diabetics (*n* 153)		
	Mean	SD	Mean	SD	Mean	SD	*P*
Sociodemographic characteristics							
Men (%)	47·0		47·1		47·2		1·000
Age (years)	66·30	6·04	66·29	6·12	66·33	5·89	0·940
Married (%)	78·50		79·50		76·50		0·462
Low educational level (%)	69·70		69·90		69·30		0·898
Family history of diabetes (%)	28·10		24·20		35·90		0·008
Lifestyle							
Never smoked (%)	55·20		57·90		49·70		
Current smoker (%)	22·90		21·20		26·10		0·239
Former smoker (%)	22·00		20·90		24·20		
Alcohol consumer (%)	67·30		66·60		68·60		0·657
Sedentary behaviour (%)	44·20		45·70		41·20		0·359
Anthropometry							
Weight (kg)	77·27	10·97	76·65	10·79	78·46	11·25	0·091
BMI (kg/m^2)	29·91	3·06	29·77	2·99	30·20	3·17	0·157
Waist circumference (cm)	99·59	9·79	98·50	9·79	101·73	9·46	0·001
Obesity (%)	45·90		45·00		47·70		0·588
Diet							
Energy (kJ/d)	9923·77	2463·45	9884·81	2362·25	10 000·67	2658·32	0·636
Dairy products (g/d)	357·70	218·66	359·56	212·18	354·03	231·59	0·799
Meat (g/d)	139·01	52·32	138·63	53·25	139·75	50·60	0·830
Vegetables (g/d)	284·41	109·90	280·60	104·10	291·92	120·55	0·323
Fruits (g/d)	344·19	184·49	344·65	183·04	343·27	187·93	0·940
Alcohol (g/d)	13·00	18·03	11·98	17·14	15·02	19·55	0·105
Mg (mg)	363·08	83·82	361·20	79·40	366·79	92·10	0·523
Vitamin D (mg/d)	5·69	3·20	5·77	3·22	5·54	3·18	0·474
Vitamin E (mg/d)	9·92	3·63	9·81	3·40	10·14	4·05	0·369
Fe (mg/d)	16·13	3·77	16·02	3·55	16·35	4·17	0·403
Haem Fe (mg/d)	1·47	0·58	1·44	0·58	1·52	0·58	0·138
Biochemistry							
Total cholesterol (mmol/l)	5·80	0·97	5·84	0·91	5·73	1·08	0·251
LDL-cholesterol (mmol/l)							0·161
Median	3·57		3·64		3·47		
IQR	3·03–4·20		3·07–4·23		2·97–4·60		
HDL-cholesterol (mmol/l)	1·39*	0·03	1·41*	0·03	1·34*	0·03	0·030
TAG (mmol/l)	1·45*	0·02	1·41*	0·02	1·54*	0·02	0·031
Fasting glucose (mmol/l)							<0·001
Median	5·38		5·16		6·11		
IQR	4·94–6·83		4·83–5·49		5·55–6·80		
Fasting insulin (pmol/l)	33·45*	1·80	32·00*	1·79	36·54*	1·86	0·022
HOMA-IR	1·14*	1·88	1·03*	1·83	1·40*	1·89	<0·001
SF (μg/l)	112·27*	2·53	105·22*	2·53	127·59*	2·53	0·037
sTfR (mg/l)	1·27*	1·27	1·27*	1·26	1·26*	1·29	0·930
sTfR:ferritin ratio	11·27*	2·81	12·03*	2·80	9·9*	2·80	0·057
hs-CRP (mg/l)							0·756
Median	1·49		1·40		1·65		
IQR	0·33–4·01		0·33–4·12		0·30–3·92		
Metabolic syndrome (%)	41·50		30·80		62·70		<0·001
High waist circumference (%)†	71·40		65·20		83·70		<0·001
Low HDL-cholesterol (%)‡	19·60		17·50		23·50		0·129
HTG (%)§	34·10		30·10		41·80		0·013
HT (%)‖	96·90		97·00		96·70		0·867
High fasting glucose (%)¶	21·80		8·90		47·10		<0·001
Insulin resistance (%)	25·10		19·90		35·30		<0·001

HOMA-IR, homeostasis model assessment for insulin resistance; SF, serum ferritin; sTfR, soluble transferrin receptor; hs-CRP, high-sensitivity C-reactive protein; HTG, hypertriacylglycerolaemia; HT, hypertension.
* Geometric mean.
† High waist circumference for men ≥ 102 cm and women ≥ 88 cm.
‡ Low HDL-cholesterol for men ≤ 1·03 mmol/l and women ≤ 1·30 mmol/l.
§ HTG (TAG ≥ 1·70 mmol/l).
‖ HT (blood pressure ≥ 135–85 mmHg).
¶ High fasting glucose (≥ 5·6 mmol/l).

Table 2. Partial correlation coefficients (adjusted for sex, age and BMI) between serum ferritin (SF), soluble transferrin receptor (sTfR) and sTfR:ferritin ratio and risk factors of type 2 diabetes in the overall study sample, and segregated by cases and controls

	Whole sample (*n* 455)			Non-incident diabetics (*n* 302)			Incident diabetics (*n* 153)		
	SF	sTfR	sTfR:ferritin ratio	SF	sTfR	sTfR:ferritin ratio	SF	sTfR	sTfR:ferritin ratio
sTfR (mg/l)	−03·39*			−0·369*			−0·281*		
sTfR:ferritin ratio	−0·974*	0·542*		−0·976*	0·563*		−0·971*	0·503*	
Waist circumference (cm)	0·071	0·083	−0·043	0·030	0·055	−0·014	0·088	0·151	−0·041
Systolic blood pressure (mmHg)	−0·089	0·115*	0·107*	−0·039	0·114*	0·061	−0·202*	0·123	0·213*
Diastolic blood pressure (mmHg)	−0·056	0·114*	0·077	−0·062	0·138*	0·088	−0·040	0·070	0·053
TAG (mmol/l)	0·109*	0·117*	−0·070	0·134*	0·117*	−0·092	0·050	0·115	−0·016
HDL-cholesterol (mmol/l)	−0·046	−0·129*	0·010	−0·069	−0·143*	0·027	0·039	−0·112	−0·.063
Fasting glucose (mmol/l)	0·116*	−0·053	−0·116*	0·115*	−0·036	−0·110	0·026	−0·090	−0·.046
Fasting insulin (pmol/l)	0·147*	0·015	−0·128*	0·135*	−0·025	−0·126*	0·168*	0·081	−0·131
HOMA-IR	0·168*	−0·001	−0·150*	0·154*	−0·034	−0·145*	0·163*	0·055	−0·133
hs-CRP (mg/l)	−0·018	0·092*	0·038	−0·070	0·110	0·088	0·062	0·066	−0·039

HOMA-IR, homeostasis model assessment for insulin resistance; hs-CRP, high-sensitivity C-reactive protein.
* $P<0·05$.

In the crude model, low levels of the sTfR:ferritin ratio showed a significant trend towards increased incidence of T2DM (OR 1·73, 95% CI 0·99, 3·05; $P=0·042$). In model 1 the trend increased (OR 2·32, 95% CI 1·08, 4·98; $P=0·035$). The introduction of hs-CRP did not alter the relationship very much (OR 2·31, 95% CI 1·08, 4·97; $P=0·036$). Finally, following the inclusion of glucose as a potential confounding variable, the association increased considerably (OR 3·02, 95% CI 1·09, 8·39; $P=0·42$).

In contrast to SF and the sTfR:ferritin ratio, no significant association was observed between sTfR and T2DM after applying the same models. The corresponding multivariate OR for the lowest *v.* highest quartile of sTfR was 1·29 (95% CI 0·51, 3·23; $P=0·722$).

When we introduced SF and fasting glucose as 'conditional' mode (CLR-conditional), we observed that fasting glucose was the strongest predictor of diabetes (OR 20·07, 95% CI 8·36, 48·20; $P<0·001$) along with SF (OR 3·62, 95% CI 1·32, 9·95; $P<0·017$). In the model of sTfR, the OR of glucose was 16·58 (95% CI 7·15, 38·41; $P<0·001$) and of sTfR was 1·29 (95% CI 0·51, 3·23; $P<0·722$). Finally, in the sTfR:ferritin ratio model, the OR of glucose was 17·51 (95% CI 7·50, 40·89; $P<0·001$) and of the sTfR:ferritin ratio was 3·02 (95% CI 1·09, 8·39; $P<0·042$).

Discussion

In the present study, a direct relationship was demonstrated between high body Fe stores (measured as SF and sTfR:ferritin ratio) and the incidence of T2DM in a Mediterranean cohort with an elevated risk of CVD. This association was found after adjustment for hs-CRP, fasting glucose and other components of the MetS. These findings add data from population that is different from those previously studied, i.e. a southern European population. We did not observe any association between the levels of sTfR and the incidence of T2DM.

The prospective design of the study helps reduce temporality bias. Also, the study design enables a better control of confounding factors such as sex and ranges of age and BMI since each case in the incident T2DM group was broadly matched for these variables with two control individuals without T2DM.

SF and sTfR were measured using immunochemiluminescence, a widely used method with high sensitivity and specificity. The diagnosis of T2DM was according to the criteria of the reference organisation, i.e. the American Diabetes Association[21]. Of note is that, in the present study, not only was there an adjustment made for classical variables predictive of T2DM risk (such as age, family history of T2DM, smoking, dietary intake, waist circumference and inflammation), but also adjustment for fasting glucose and other components of the MetS since these components are strongly associated with the development of T2DM[28].

We need to highlight the limitation of extrapolating the results of the present study to the general population, given that the study was conducted in a population with various CVD risk factors. As occurs in cohort studies, there is no

Table 3. Risk of type 2 diabetes segregated by quartiles of serum ferritin (SF), soluble transferrin receptor (sTfR) and sTfR:ferritin ratio, and adjusted for sex

(Odds ratios and 95 % confidence intervals)

	Q1	Q2		Q3		Q4		P_{trend}
SF								
Men (μg/l)	<78·80	78·80–161·60		161·60–256·90		>256·90		
Women (μg/l)	<48·13	48·13–96·27		96·27–139·00		>139·00		
Cases	30	36		31		56		
Controls	75	76		76		75		
Crude*	1	1·25	0·68, 2·31	1·06	0·57, 1·96	1·99	1·12, 3·52	0·022
Model 1†	1	1·09	0·50, 2·40	0·76	0·35, 1·67	2·39	1·11, 5·16	0·030
Model 1 + hs-CRP‡	1	1·09	0·49, 2·39	0·75	0·34, 1·66	2·38	1·10, 5·14	0·031
Model 1 + hs-CRP‡ + glucose§	1	1·17	0·44, 3·07	0·65	0·24, 1·79	3·62	1·32, 9·95	0·017
	Q4	Q3		Q2		Q1		
sTfR								
Men (mg/l)	>1·45	1·22–1·45		1·07–1·22		<1·07		
Women (mg/l)	>1·46	1·27–1·46		1·09–1·27		<1·09		
Cases	36	39		36		42		
Controls	76	76		76		74		
Crude*	1	1·11	0·63, 1·97	1·02	0·59, 1·77	1·21	0·70, 2·09	0·499
Model 1†	1	1·76	0·82, 3·78	1·10	0·55, 2·21	1·39	0·68, 2·87	0·575
Model 1 + hs-CRP‡	1	1·76	0·82, 3·79	1·11	0·55, 2·22	1·39	0·68, 2·86	0·583
Model 1 + hs-CRP‡ + glucose§	1	1·59	0·61, 4·18	1·14	0·47, 2·76	1·29	0·51, 3·23	0·722
	Q4	Q3		Q2		Q1		
sTfR:ferritin ratio								
Men (μg/l)	>15·04	7·29–15·04		4·35–7·29		<4·35		
Women (μg/l)	>25·41	14·02–25·41		8·53–14·02		<8·53		
Cases	32	33		34		54		
Controls	75	76		76		75		
Crude*	1	1·04	0·56, 1·93	1·10	0·61, 1·97	1·73	0·99, 3·05	0·042
Model 1†	1	1·39	0·61, 3·14	1·04	0·48, 2·22	2·32	1·08, 4·98	0·035
Model 1 + hs-CRP‡	1	1·38	0·61, 3·13	1·03	0·48, 2·21	2·31	1·08, 4·97	0·036
Model 1 + hs-CRP‡ + glucose§	1	1·65	0·58, 4·67	1·06	0·40, 2·79	3·02	1·09, 8·39	0·042

Q, quartile; hs-CRP, high-sensitivity C-reactive protein.

* Crude: unadjusted.

† Model 1: marital status (married/not married); educational level (low/medium/high); smoking status (current/former/never); physical activity (<200 metabolic equivalents (MET)-min/d or ≥200 MET-min/d); alcohol consumption (yes/no); family history of diabetes (yes/no); waist circumference (men <102 or ≥102 cm, women <88 or ≥88 cm); hypertension (<135, 85 or ≥135/85 mmHg); hypertriacylglycerolaemia (<1·70 or ≥1·70 mmol/l); HDL-cholesterol (men ≥1·03 or <1·03 mmol/l, women ≥1·30 or <1·30 mmol/l); diet (energy, dairy products, meat, vegetables, fruits, Mg, and vitamins D and E).

‡ hs-CRP in mg/l.

§ Glucose <5·6 or ≥5·6 mmol/l.

assurance that some of the control individuals would not develop T2DM subsequent to the follow-up period. However, the median period of follow-up of 6·0 (interquartile range 3·9–6·5) years is greater than that in the majority of studies conducted to date[2,5–7,10,13].

SF, which closely reflects the estimation of body Fe levels, has been observed to be influenced by inflammation status. Hence, hs-CRP was measured in the present study. The objective was to adjust for this confounding variable when evaluating the effect of excess Fe in relation to T2DM.

Also, we assessed the relationship of another marker that measures Fe status, i.e. sTfR. sTfR in plasma is directly proportional to the cell requirements for Fe[4]. Hence, it is considered to represent a good biomarker in the evaluation of body Fe status[3]. However, some studies have suggested that sTfR levels are increased by other factors such as the degree of glucose tolerance or IR[29], hyperinsulinaemia[30], inflammation[31], general obesity and/or abdominal obesity[32]. Also, the sTfR:ferritin ratio appears to be a better marker of Fe stores[33]. It is especially useful in population studies since it is sensitive not only to elevated, but also decreased, levels of Fe stores[34].

In the present results, we observed significantly higher levels of ferritin (127·59 (SD 2·53) *v.* 105·22 (SD 2·53) μg/l) and lower levels of sTfR:ferritin ratio (9·9 (SD 2·80) *v.* 12·03 (SD 2·80)) with borderline significance in subjects with incident diabetes compared with those with non-incident diabetes. However, we did not observe any difference in sTfR levels. When analysing the relationship using adjusted CLR models, we observed that the values of SF >257 μg/l in males and >139 μg/l in females were associated with a high risk of the appearance of T2DM. These values of SF are typical of Fe overload status, according to the WHO (>200 μg/l in males and >150 μg/l in females)[35], and are similar to the values found in the studies of Norfolk[6] and Potsdam[2] of the EPIC cohort and the Atherosclerosis Risk in Communities (ARIC) study[5] conducted in the general population.

The associations encountered between SF and T2DM were maintained following the adjustment for classic risk factors including inflammation, fasting glucose and other components of the MetS. The MetS, and its components, have been strongly associated with the development of T2DM, especially with an elevated level of fasting glucose[28]. Many prospective studies have adjusted their models using some of these classic

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risk factors, including inflammation[2,5–7,9–11]. However, the associations, following the adjustment for fasting glucose and other components of the MetS, have not been observed previously. Only the ARIC study[5], conducted with US males and females between 45 and 64 years of age, observed the association, and which was lost when adjusted for the components of the MetS. Another study, conducted with cohorts from the Finish FINRISK and Health 2000[8] studies composed of males and females >25 years of age, encountered increased SF levels related to the incidence of T2DM even following the adjustment for glucose, but not for the components of the MetS. In the present study, SF and fasting glucose entered the CLR-conditional model significantly, suggesting that the two variables are risk factors for T2DM. This is in concordance with the current consensus that glucose is the principal risk factor for T2DM, which can indicate that, apart from glucose, SF plays an aetiologic role in the development of this pathology. Another study performed in the general adult population have as well observed SF and glucose as the risk factors of T2DM[8].

As we have stated above, the sTfR:ferritin ratio seems to be a good biomarker of Fe deposits given that it is sensitive to elevated as well as decreased levels of Fe[34]. However, it is possible that, since we did not find any association between sTfR and T2DM, the association encountered between the sTfR:ferritin ratio and T2DM could be due exclusively to SF concentrations. Further support for this hypothesis comes from the observation of a strong partial correlation (adjusted for sex, age and BMI) between SF and the sTfR:ferritin ratio, but not so strong a correlation between sTfR and the sTfR:ferritin ratio (Table 2).

The present results and those of previous studies[2–14] suggest that the observed association between ferritin and the sTfR:ferritin ratio *v.* T2DM was very probably due to the excess levels of Fe. Ferritin, in addition to reflecting body Fe status, increases with inflammation. We measured hs-CRP concentrations to control for this effect in the multivariate analyses of the relationship between excess Fe and the onset of T2DM. In the present results, we observed that hs-CRP levels were similar in incident and non-incident diabetic individuals, while SF was significantly higher in the incident diabetic group. This supports the hypothesis that excess Fe acts independently of the level of inflammation in the development of T2DM. Further support for this interpretation comes from previous studies that have analysed the relationship between ferritin and T2DM following adjustments for one[2,7,9,10] or more[6] inflammatory markers showing similar findings to ours.

In the DPP[7] cohort of obese subjects with impaired basal glucose, an association was found not only between SF and T2DM, but also between sTfR and T2DM. This latter relationship was contrary to expectation, i.e. increased levels of sTfR, indicative of low Fe stores, were associated with an increased risk of T2DM. More recently, the Finnish KIHD cohort[11], conducted with middle-aged men, also observed that Fe deficiency and excess Fe (using sTfR as a biomarker) increased the risk of T2DM. A recent review has concluded that extreme conditions of Fe deficiency, as well as Fe overload, were associated with increased risk of CVD[36]. As such, the hypothesis is that Fe deficiency could also cause an increase in the incidence of T2DM.

Of considerable note as well is that sTfR levels were much higher in those cohorts in whom an association was observed between sTfR and T2DM, e.g. DPP[7] (median 4th quartile 4·4 mg/l; mean of the overall sample 3·4 mg/l) and KIHD[11] (0·6–8·2 mg/l) studies compared with the present study (0·69–2·65 mg/l) and the EPIC Potsdam study[2] (mean of the overall sample 1·13 mg/l), albeit Fe stores measured by SF were similar in these four cohort studies. Also, sTfR levels have been documented to be affected by mechanisms other than those related to Fe metabolism (such as insulin sensitivity and obesity), and they could be causally linked to T2DM[4]. As such, not only lower Fe storage would lead to increased levels of sTfR, but also sTfR would be a biomarker of another factor causally related to the risk of T2DM.

The essential role of ferritin in the organism is in the storage of Fe. Fe is a catalyst for oxygen reactive species and, as such, contributes to oxidative stress[37]. A proposed underlying mechanism is that certain 'trigger' molecules associated with some pathologies could open up the structure of the ferritin molecule, thus provoking the liberation of the stored Fe[37]. This, in turn, could favour the risk associated with oxidative stress and its consequences. Experimental data and clinical studies have suggested that an oxidative environment contributes to the development of IR[38] and β-cell dysfunction, which are two key events in the clinical development of T2DM[15]. Also, the increase in oxidative stress provokes an increase in β-cell apoptosis in studies with animal models[39] and an increase in IR in human *in vivo* studies[40]. Similarly, evidence exists from cross-sectional studies[41] that elevated levels of SF are associated with increased IR. We observed this relationship in the present study ($\beta = 0{\cdot}001$; $P = 0{\cdot}020$), i.e. for each μg/l of SF, there was an increase of 0·001 in HOMA-IR. Also, ferritin was correlated with HOMA-IR in the overall study sample, as well as in incident and non-incident diabetic individuals. This suggests that this relationship with HOMA-IR could be occurring in the entire general population. The mechanisms that underlie this relationship have not been identified to date, although there has been speculation that the pro-oxidant role of Fe would activate a series of stress avenues related to the family of serine/threonine kinases and, finally, causing a disruption in the insulin signalling process[38]. It is possible then that IR is an intermediate link in the relationship between high Fe deposits and the risk of T2DM.

Conclusion

Excess body Fe (measured as SF and sTfR:ferritin ratio) is associated with an increased risk of T2DM in a Mediterranean population at a high risk of CVD, even following the adjustment for hs-CRP, fasting glucose and other components of the MetS. This association was not evident with sTfR.

The potential mechanism that mediates this relationship could be related to IR. More studies are warranted to confirm this mechanism since it is becoming increasingly evident that excess Fe is related to the incidence of T2DM.

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The authors' responsibilities are as follows: V. A. took responsibility for designing the study, directing and performing the statistical analyses, interpreting the results, and drafting of the manuscript; J. C. F.-C. contributed to the statistical analyses, interpretation of the data, and the drafting of the manuscript; J. B. conceived and participated in the design of the PREDIMED study, coordinated the fieldwork, participated in the interpretation of the results, and revised the manuscript; M. B. contributed to the interpretation of the results and revised the manuscript; N. A. coordinated the biochemical analyses, contributed to the interpretation of the results, and revised the manuscript; R. E. and M. A. M.-G. conceived and participated in the design of the PREDIMED study and revised the manuscript; J. S.-S. conceived and participated in the design of the PREDIMED study, participated in the interpretation of the results, and revised the manuscript. All authors read and approved the final manuscript.

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DISCUSSION

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DISCUSSION

The findings obtained in this thesis contribute to existing scientific evidence in the field of iron and T2DM. Specifically, we have analysed, for the first time, how excess iron in the diet and/or body affects the risk of T2DM in a large elderly Mediterranean population at high cardiovascular risk. To better understand the mechanisms behind the associations between iron and T2DM reported in the current thesis, we have assessed the relationships between iron parameters and novel T2DM-associated biomarkers such as osteocalcin (OC).

1. STUDY DESIGN AND POPULATION

The participants selected for the study were elderly men and women from three cities of Spain, Reus-Tarragona, Pamplona and Barcelona, who were free of T2DM and CVD at baseline, but were at high cardiovascular risk. These features of the sample participants - advancing age and high cardiovascular risk – are strengths of this prospective study to analyse the relationship between iron in the diet and/or body and risk of T2DM. Firstly, since the incidence of this metabolic disease in Spain is around 9 cases per 1,000 person-years (Martín Martínez et al., 2013), the sample of high cardiovascular risk subjects led to a higher incidence of T2DM than would be expected in a healthy population. We would otherwise have needed an extremely large sample to ensure a significant number of T2DM cases. And secondly, the subjects' advancing age, one of the most prominent T2DM risk factors, also helped to increase the incidence of diabetes in our sample. This is easily seen if we compare the sample size for our study on iron intake and T2DM risk with the size of American samples (see table 10), and the respective percentage of diabetic subjects. Our study enrolled 1,073 participants of whom 131 (12.2%) developed T2DM. Most American prospective cohorts (HPFS, IWHS, WHS and NHS) of healthy populations have comprised very large samples (over 35,000 and even as many as 121,000 subjects), with a high number of incident diabetics (over 1,100) in those cohorts but a significantly lower percentage of T2DM subjects (between 3 and 5.4%).

Moreover, our follow-up period of up to 8 years, was larger than those of most other studies except those of the largest prospective American cohorts with healthy populations. This length of time also helped to reduce the possibility that some control individuals would develop T2DM after the follow-up period.

2. METHODS

New-onset T2DM during follow-up was diagnosed in accordance with ADA criteria (see table 8) (American Diabetes Association, 2013). Whenever cases of new-onset T2DM were identified by medical diagnosis from the medical charts or by glucose test during routine biochemical analyses (performed at least once a year), reports were sent to the Clinical Events Committee, whose members were blinded to treatment allocation. Only when a second test had been conducted with the same criteria and repeated within the following three months was a new case of diabetes definitively confirmed by the adjudication committee. In contrast, in most previous studies that have assessed the relationship between iron intake and/or body iron stores and risk of T2DM, diabetes was identified by self-reporting (Jiang et al., 2004a) (Jiang et al., 2004b) (Lee et al., 2004) (Song et al., 2004) (Rajpathak et al., 2006) (Forouhi et al., 2007a) (Shi et al., 2010) (Montonen et al., 2012).

Trained dieticians completed a 137-item semi-quantitative FFQ in a face-to-face interview with each participant (**Appendix III**). This questionnaire had been previously validated in an elderly Spanish population at high cardiovascular risk (Fernández-Ballart et al., 2010). These widely used dietary assessment tools in epidemiological studies have several strengths (see point 1.2.2). For instance, they are a retrospective and direct method for estimating usual intake over a certain period of time. They are also highly cost-effective and, since they have a standard format, are easy to administer.

The concentrations of SF and soluble transferrin receptor (sTfR) were measured using immunochemiluminescence and OC and uncarboxylated OC using electrochemiluminescence. These are widely used methods with high sensitivity and specificity. SF, which closely reflects estimates of body iron levels, is reported to be

influenced by inflammation status. In this study, we therefore also measured hs-CRP to adjust for this confounding variable when evaluating how excess iron affects the development of T2DM. We also assessed this relationship with sTfR, another marker that measures iron status. sTfR in plasma is directly proportional to the cell requirements for iron (Speeckaert et al., 2010) and is therefore considered a good biomarker for evaluating body iron status (Désidéri-Vaillant et al., 2011). However, some studies have suggested that sTfR levels may be increased by other factors such as the degree of glucose tolerance or IR (Fernández-Real et al., 2007), hyperinsulinaemia (Davis et al., 1986), inflammation (Kasvosve et al., 2006), and general and/or abdominal obesity (Tussing-Humphreys et al., 2010). The sTfR/ferritin ratio appears to be a better marker of iron stores (Malope et al., 2001). It is particularly useful in population studies since it is sensitive to both elevated and decreased levels of iron stores (Skikne, 2008).

Table 10. Characteristics of prospective studies about haem iron intake and risk of T2DM.

Author, year	Study	Location	Follow-up (years)	Ethnicity	Gender	Age (years)	Sample size	Incident diabetics	T2DM (%)	Ascertainment of T2DM	Dietary assessment method	Haem iron intake (mg/day)	Effect size estimate (95% CI), P-trend
Jiang, 2004	HPFS	U.S.	12	American	Male	40–75	38,394	1,168	3.0	Self-report	VFFQ	Median Q5: 1.9	1.28 (1.02-1.61), P = 0.045
Lee, 2004	IWHS	U.S.	11	American	Female	55-69	35,698	1,921	5.4	Self-report	VFFQ	Median Q5: 2.2	1.28 (1.04-1.58), P = 0.020
Song, 2004	WHS	U.S.	8.8	American	Female	≥45	37,309	1,558	4.2	Self-report	VFFQ	Median Q5: 1.55	1.46 (1.20-1.78), P<0.001
Rajpathak, 2006	NHS	U.S.	20	American	Female	34-59	121,700	4,599	3.8	Self-report	VFFQ	Median Q5: 1.9	1.28 (1.14-1.45), P<0.001
Shi, 2010	JNS	China	5	Chinese	Male / Female	≥20	1,056	23	2.2	Self-report	VFFQ	Median Q4: 4.4	9.84 (1.41-68.75), P = 0.033
de Oliveira Otto, 2012	MESA	U.S.	4.8	White, African American, Hispanic, and Asian	Male / Female	45-84	4,982	499	10.0	Self-report, glucose levels or new use of hypoglycemic medication	VFFQ	Q5 ≥ 1.07	1.24 (0.86-1.78), P = 0.210
Fernandez-Cao, 2013	PREDIMED	Spain	8	Caucasians	Male / Female	55-80	1,073	131	12.2	ADA criteria	VFFQ	Median Q5: 5.2 sample median: 3.65	1.30 (1.02-1.66), P = 0.037
Overall risk of high versus low haem iron intake and the risk of T2DM (Heterogeneity = 0%, P = 0.467)													1.31 (1.21-1.42)

CI (Confidence Interval), HPFS (Health Professionals' Follow-up Study), VFFQ (validated food frequency questionnaire), BMI (Body Mass Index), IWHS (Iowa Women's Health Study), WHS (Women's Health Study), NHS (Nurses' Health Study), JNS (Jiangsu Nutrition Study), MESA(Multi-Ethnic Study of Atherosclerosis), PREDIMED (PREvención con DIeta MEDiterránea).

Table 11. Characteristics of prospective studies about serum ferritin levels and risk of T2DM.

Author, year	Study	Location	Study design	Follow-up (years)	Ethnicity	Gender	Age (years)	Sample size	Incident diabetics	Ascertainment of T2DM	Laboratory measurement of ferritin	Serum ferritin (ng/mL)	Effect size estimate (95% CI), P-trend
Jiang, 2004	NHS	U.S.	Prospective nested case-control	10	American	Female	30-55	1,414	698	Self-report	ITA	Q5 ≥ 107.2	2.61 (1.68-4.07), P<0.001
Jehn, 2007	ARIC	U.S.	Prospective nested case-cohort	7.9	White, African American	Male / Female	45-64	1,289	599	DDC or self-reported	ITA	Median Q5: 354.5	0.79 (0.48-1.32), P= 0.078
Forouhi, 2007	EPIC	U.K.	Prospective nested case-control	Mean: 5.1±1.7	White	Male / Female	40-74	1118	360	Self-report	TR-FIA	Median Q5: 300 (male), 150 (females)	3.2 (1.3-7.6), P=0.007
Le, 2008	ACLS	U.S.	Prospective cohort	up to 12	White (93.2 %)	Male / Female	20-83	5,512	220	ADA criteria	-	Q4 >188 (male), >60 (pre-female), >90 (post-female)	Male: 1.67 (1.05-2.66), P = 0.027; female: 0.59 (0.20-1.76), P >0.050
Rajpathak, 2009	DPP	U.S.	Prospective nested case-control	Mean: 2.8	Multiethnic	Male / Female	≥ 25	560	280	ADA criteria	ITA	Median Q4: 203	1.61 (0.85-3.02) , P= 0.030
Montonen, 2012	EPIC	Germany	Prospective nested case-cohort	7	White	Male / Female	35-65	1,969	607	Self-report	ADVIA Centaur XP	Q5 ≥ 280 (male), ≥110 (female)	1.73 (1.15-2.61), P= 0.002
Aregbesola, 2013	KIHD	Finland	Prospective cohort	Mean: 16.8	White	Male	42-60	1,613	331	DDC or self-reported	RIA	Q5 ≥ 228	1.5 (1.0-2.2), P=0.050
Sun, 2013	NHAPC	China	Prospective cohort	6	Chinese	Male / Female	50-70	2,198	538	DDC or self-reported	ITA	Geometric median Q5: 325 (male), 235 (females)	1.59 (1.12-2.25), P= 0.002
Arija, 2014	PREDIMED	Spain	Prospective nested case-control	Median: 6	Caucasian	Male / Female	55-80	459	153	ADA criteria	ICL	Q4 >257 (male), >139 (female)	3.62 (1.32-9.95), P= 0.022
Overall risk of high versus low serum ferritin levels and the risk of T2DM (Heterogeneity = 56.1%, P = 0.015)													1.64 (1.27-2.12)

CI (Confidence Interval), NHS (Nurses' Health Study), ITA (ImmunoTurbidimetric Assay), ARIC (The Atherosclerosis Risk in Communities study), DDC (Diabetes Diagnostic Criteria: fasting glucose ≥7.0 mmol/L; or nonfasting glucose ≥11.1 mmol/L), EPIC (European Prospective Investigation of Cancer); TR-FIA (Time-Resolved FluoroImmunoAssay), ACLS (Aerobics Center Longitudinal Study), HPFS (Health Professionals' Follow-up Study), ADA (American Diabetes Association), Q4 (Quartile 4), pre-females (premenopausal females), post-females (postmenopausal females), DPP (Diabetes Prevention Program), KIHD (Kuopio Ischemic Heart Disease Risk Factor), RIA (RadioImmunoAssay), NHAPC (The Nutrition and Health of Aging Population in China study), PREDIMED (PREvención con DIeta MEDiterránea), ICL (ImmunoChemiLuminiscence).

3. IRON INTAKE AND TYPE 2 DIABETES MELLITUS

In our prospective cohort study, we assessed, for the first time in an European population, the relationship between high haem iron intake and T2DM. Our results showed that a high haem iron intake increases the risk of T2DM by 30%, in an elderly Mediterranean population at high cardiovascular risk. However, total iron and non-haem iron intake are not significantly related to T2DM.

The relationship between haem iron intake and the risk of developing T2DM has been previously analysed in five long-term prospective epidemiological studies in US populations (Jiang et al., 2004a) (Lee et al., 2004) (Song et al., 2004) (Rajpathak et al., 2006) (de Oliveira Otto et al., 2012), and in cross-sectional (Shi et al., 2006) (Luan de et al., 2008) and prospective (Shi et al., 2010) epidemiological studies in Chinese populations. Data from US cohorts - such as the HPFS (Jiang et al., 2004a) of men aged 40 to 75, the IWHS (Lee et al., 2004) of postmenopausal women aged 55 to 69, the Women's Health Study (WHS) (Song et al., 2004) of women aged ≥ 45, and the Nurse's Health Study (NHS) (Rajpathak et al., 2006) of women aged 34 to 59, showed that subjects in the highest quantile of haem iron intake increased the risk of T2DM by between 28% (Jiang et al., 2004a) (Lee et al., 2004) (Rajpathak et al., 2006) and 46% (Song et al., 2004) with respect to the lowest. In addition, no associations were observed between total iron, non-haem iron intake and/or iron supplementation and T2DM. Our results are consistent with those from the above American studies of men and pre- and postmenopausal women, which showed a similar T2DM risk (30%) in subjects aged 50-80 with an elevated intake of haem iron but not with total iron or no-haem iron intake. However, other prospective study of U.S. subjects aged 45 to 84, in the MESA cohort, found no association between haem iron intake and T2DM (de Oliveira Otto et al., 2012). The ethnic diversity of the MESA cohort could be the reason for the lack of association observed, since we know that certain ethnicities are more susceptible to T2DM than others. For example, T2DM is up to six times more common in people of South Asian descent and up to three times more common in people of African and African-Caribbean origin than in white populations (Ntuk et al., 2014) (Tillin et al., 2013) (Bennet et al., 2014). South Asians followed by Chinese and black populations seem to have the highest

prevalence of diabetes, while with white populations have the lowest prevalence (Ntuk et al., 2014).

Results from studies of Chinese populations are inconsistent (Shi et al., 2006) (Luan de et al., 2008). While Luan et al. also found an association between haem iron intake and T2DM (Luan de et al., 2008) as in our cohort and in four American cohorts (Jiang et al., 2004a) (Lee et al., 2004) (Song et al., 2004) (Rajpathak et al., 2006), Shi et al. in the JIN study, surprisingly observed a positive relationship between high total iron (Shi et al., 2006) and non-haem iron (Shi and Pan, 2008) intake and T2DM risk in women, but not in men. However, in accordance with previous studies, a subsequent prospective analysis of 1,056 participants from this cohort with FPG <5.6mmol/L and without diabetes at baseline showed a higher risk of hyperglycaemia and T2DM among subjects in the highest quartile of haem iron intake than among subjects in the lowest (Shi et al., 2010).

It has been suggested that the association between haem iron intake and the risk of T2DM is confounded by food source (Jiang et al., 2004a) since the most important source of haem iron in the diet is red meat. It is known - as Loma Linda University's Adventist Health Study first reported in 1985 (Snowdon and Phillips, 1985) and has since been consistently observed in several other studies (Pan et al., 2011) - that a high meat intake is associated with the risk of developing T2DM. However, later prospective studies of American (Lee et al., 2004) (Rajpathak et al., 2006) and Chinese (Shi et al., 2010) cohorts - and now of our European cohort - confirm earlier findings of associations between haem intake from all sources and T2DM.

Although haem iron absorption is less stringently regulated in response to dietary inhibitors and enhancers, recent studies have shown that certain dietary components, such as animal and vegetal proteins (Hallberg et al., 1993) (Villarroel et al., 2011), calcium (Hallberg et al., 1991) (Hallberg et al., 1993) and polyphenols (Ma et al., 2010) (Ma et al., 2011), may inhibit its absorption, while other components such as ascorbic acid (Ma et al., 2011), may enhance it. It has also been found that intestinal iron absorption increases two-fold in chronic alcoholics, possibly due to changes in intestinal permeability (Duane et al., 1992). In addition to sociodemographic, anthropometric, and other variables, dietary components have been associated with T2DM risk (Salas-

Salvadó et al., 2011). This is why the following dietary variables were included in the proportional hazard models for assessing the relationship between haem iron and T2DM: meat, fish and eggs (animal protein); cereals, legumes and nuts (vegetable protein); fruit; alcoholic beverages; tea; coffee; and calcium.

Of our initial sample (1,073 subjects), 12.2% (131 subjects) developed T2DM with a median follow-up of 4.8 ± 1.3 years. At baseline, we described the general characteristics, and food, energy, and nutrient consumption in incident diabetics in comparison with non-incident diabetics among a Mediterranean population at high cardiovascular risk. Known risk factors, such as BMI (Abdullah et al., 2010), waist circumference (Adegbija et al., 2015) and smoking (Cho et al., 2009) were significantly higher in incident diabetics than in non-incident diabetics. Moreover, incident diabetics consumed significantly less fruit and coffee - which have a protective effect against the development of T2DM (Wu et al., 2015) (Ding et al., 2014) - but more haem iron.

After adjusting the proportional hazard model for energy intake, we observed a significant association between haem iron intake and the risk of developing T2DM. In the final model applied, in addition to haem iron, we also observed a positive association between the consumption of alcohol beverages and an increased risk of T2DM. On the other hand, as has also been observed in other studies (Ding et al., 2014), coffee consumption was associated with a lower risk. The negative relationship between coffee consumption and risk of T2DM could also be explained by the positive relationships between caffeine and insulin sensitivity, and between decaffeinated coffee and β-cell function (Loopstra-Masters et al., 2011). Polyphenols, including those found in coffee and tea, seem to inhibit the uptake of intestinal haem iron (Ma et al., 2010) (Ma et al., 2011), thus reducing its adverse effects. Alcohol seems to have a detrimental effect on the metabolism of iron. In the IWHS, an association was observed between higher haem iron intake and a greater risk T2DM, especially in those who also consumed alcohol (Lee et al., 2004). Consuming two or more alcoholic drinks per day was associated with a higher risk of iron overload (Ioannou et al., 2004). It seems that acute or chronic exposure to alcohol suppresses the expression of hepcidin in the liver, thus leading to greater iron absorption (Bridle et al., 2006) (Harrison-Findik et al., 2006) (Ohtake et al., 2007) (Flanagan et al., 2007) (Harrison-Findik, 2007) (Harrison-Findik et al., 2007) (Harrison-

Findik, 2009). Alcohol intake at low levels increases serum iron and ferritin and, therefore, body iron stores (Whitfield et al., 2001), and this in turn is associated with the risk of developing T2DM (Kim et al., 2011a).

Although it is not fully understood why the risk of T2DM has been specifically linked with haem iron intake, Haem iron is known to be more bioavailable than non-haem iron (roughly 25% compared to 5–15%, respectively) (Rajpathak et al., 2009a). There is also strong evidence that non-haem iron absorption in the proximal intestine is accurately regulated by hepcidin to ensure that overall body iron levels are maintained at adequate levels (Gulec et al., 2014). In conditions of elevated iron stores, hepcidin therefore causes FPN internalization and proteolysis into lysosomes (De Domenico et al., 2007) and, probably, the proteasomal-mediated degradation of DMT1 (Brasselagnel et al., 2011). Non-haem iron absorption therefore becomes blocked, thus preventing enterocytes from allowing iron into the hepatic portal system and preventing an increase in body iron stores. Cao et al. recently observed that liver hepcidin explained 59% and 63% of the variation in haem and non-haem iron absorption, respectively, but had a greater relative impact on non-haem iron absorption (Cao et al., 2014). It is known that inside enterocytes, haem iron is mostly catabolised by HO-1 in biliverdin, CO and free iron. Free iron probably enters in the same pathways as non-haem iron and so some haem iron is probably regulated by hepcidin. Accordingly, a fraction of the haem iron absorbed seems to respond to changes in body iron status. However, it has been suggested that an intact haem iron fraction passes directly into the bloodstream (Mendiburo et al., 2011) (Cao et al., 2014). This fraction of haem iron that traverses enterocytes may avoid hepcidin control. Under these assumptions, people with an elevated intake of animal products could keep on absorbing iron - at least a fraction of haem iron - despite having high iron stores. Body iron levels would therefore continue to increase even when the hepcidin levels were elevated. In this context, elevated haem iron intake has been associated with high levels of serum ferritin (SF) (Vander et al., 2006).

It is speculated that the increase in iron stores may play a role in the production of ROS, such as hydroxyl radicals, thus inducing tissue damage (Lipinski, 2011) identified by increased peroxidation of lipids, protein modification, and DNA damage (Förstermann, 2008). If continued for a longer duration, iron-induced oxidative stress may lead to T2DM

(Swaminathan et al., 2007). This mechanism could explain why high dietary haem iron intake has been related to the risk of T2DM, and diseases, such as gestational diabetes (Qiu et al., 2011) (Bowers et al., 2011), MetS (de Oliveira Otto et al., 2012), cardiovascular disease (Fang et al., 2014) (stroke (Kaluza et al., 2013), acute myocardial infarction (Kaluza et al., 2014) and coronary heart disease (Hunnicutt et al., 2014)) and oesophagus (Ward et al., 2012) gastric (Jakszyn et al., 2012), colorectal (Qiao and Feng, 2013), breast (Kabat et al., 2010), lung (Tasevska et al., 2009), and endometrial cancer (Genkinger et al., 2012).

4. BODY IRON STATUS AND SERUM OSTEOCALCIN LEVELS

We have observed, for the first time, that body iron status, measured as SF and sTfR levels, is inversely associated with total and uncarboxylated OC in an elderly Mediterranean population at high cardiovascular risk. This relationship was independent of potentially confounding variables such as lifestyle and biomarkers of glucose metabolism, inflammation and oxidative stress. Since OC has been related not only to bone metabolism but also to IR and sensitivity, our results would help to explain one of the possible mechanisms relating iron metabolism with glucose homeostasis.

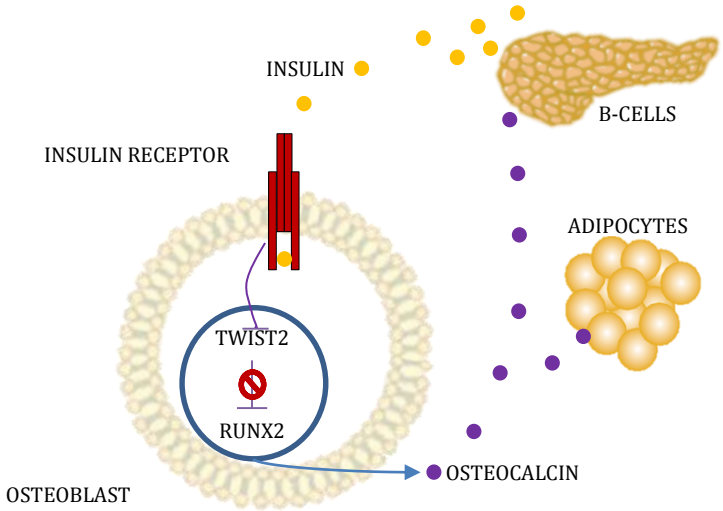
We have also reported the general, lifestyle, energy intake, and biochemical characteristics and risk factors for T2DM (MetS and its components, IR, β-cell function, obesity, inflammation) in IFG versus normal glucose metabolism (NGM) subjects in a Mediterranean population at high cardiovascular risk. As expected, IFG subjects had a significantly larger waist circumference, fasting glucose, fasting insulin, and IR, and lower β-cell function, HDL-cholesterol, and adiponectin levels, which indicates a greater risk of developing T2DM. Also, total and uncarboxylated OC were lower in IFG subjects than in NGM subjects, and both these biomarkers have recently been related to the risk of T2DM (Ngarmukos et al., 2012) (Díaz-López et al., 2013). Our findings therefore support the extra-skeletal roles of OC involved in the regulation of energy, glucose, and fat metabolism (Lee et al., 2007) (Neve et al., 2013). It has been observed that mice lacking OC show lower β-cell proliferation, glucose intolerance and IR, and that UOC mediates the metabolic functions of this hormone (Lee et al., 2007). Also, OC knockout mice have

lower levels of serum adiponectin, which suggests a potential role for OC in insulin sensitivity and secretion. Studies in humans seem to be consistent with previous findings in animal models. In an elderly Mediterranean population at high cardiovascular risk, high total or uncarboxylated OC serum concentrations were directly associated with HOMA-β and inversely associated with IR determined by HOMA-IR (Bulló et al., 2012), thus suggesting a possible role of bone in the development of T2DM.

Iron overload can damage important organs such as liver, pancreas and heart (Whittaker et al., 1996). Many lines of research also indicate that it affects bone tissue, causing both osteopenia and osteoporosis (Weinberg, 2006) and a high prevalence of fractures (Vogiatzi et al., 2009a). For example, in a sample of 38 untreated HFE-related haemochromatotic patients, 79% were osteopenic and 34% were osteoporotic. Interestingly, recent in vitro studies have shown that ferritin itself can directly inhibit osteogenesis in a dose-responsive manner through its ferroxidase activity (Zarjou et al., 2010). Iron has also been shown to have a detrimental effect on the differentiation, proliferation and activity of osteoblasts (Yamasaki and Hagiwara, 2009). Moreover, iron exposure on human osteoblast-like cells reduced the expression of genes involved in bone matrix formation or osteoblast differentiation such as COL1AI, Runx2 or OC (Yang et al., 2011) (Doyard et al., 2012). It has been suggested that this deleterious effect is probably due to iron overload-associated oxidative stress (Zhao et al., 2012). High SF levels, reflecting high iron stores, may therefore inhibit OC synthesis through down-regulation of osteoblasts. This would be consistent with our findings, in which we observed an inverse association between SF and total OC. However, we found no relationship between SF and UOC following adjustment for potentially confounding variables such as lifestyle and biomarkers of glucose metabolism, inflammation and oxidative stress. OC synthesis, but not the subsequent carboxylation, probably depends on ferritin levels. Moreover, we found these results both in the whole sample and in NGM and IFG subjects. Surprisingly, however, the association found in IFG subjects disappeared in the full adjustment model, after the introduction of fasting plasma glucose and insulin and other variables. This suggests that the relationship between SF and OC depends on glucose metabolism in IFG subjects. The elevated glucose levels and, consequently, the characteristic high insulin concentration found in IFG subjects probably influence the regulation of OC homeostasis. In earlier study, Bulló et al.,

reported that fasting insulin increased OC values (β = 0.97, P = 0.028) (Bulló et al., 2012). Curiously, Fulzele et al. suggested the existence of a novel bone-pancreas feedback loop through which insulin signalling in the osteoblast promotes osteoblast differentiation, and simultaneously stimulates OC production by suppressing the Runx2 inhibitor Twist2, which in turn regulates pancreatic insulin secretion and insulin sensitivity (see figure 15) (Fulzele et al., 2010). High insulin levels may therefore reduce the deleterious effect of iron excess on the synthesis of OC.

Figure 15. Bone-pancreas feedback loop model.



Adapted from (Fulzele et al., 2010)

We also found that higher levels of sTfR, which are indicative of low iron status, were inversely associated with serum total and uncarboxylated OC. Recent experimental studies have shown that osteogenesis is optimal in a certain range of iron concentration, i.e. while mild low iron promoted osteoblast activity, both, severely low iron and iron overload may inhibit osteoblast activity (Zhao et al., 2012), and therefore, OC synthesis. Studies in animal models have consistently found that serum OC concentration was significantly lower in rats fed an iron-deficient diet than in rats fed a control diet, and given free access to the experimental diets (Katsumata et al., 2006) (Katsumata et al.,

2009). ID in humans as severe as in these studies conducted on rats is probably rare. However, even in humans a consistently low iron intake and/or severe ID may alter osteoblast activity and therefore also serum OC concentrations. Our data support the hypothesis regarding the detrimental effect of ID and iron overload on serum total and uncarboxylated OC concentration. On other hand, we observed no relationship between sTfR/ferritin ratio and serum total or uncarboxylated OC concentration. However, as expected, we did observe a negative tendency in the association between this ratio and HOMA-IR.

In summary, we have described an inverse association between SF and sTfR with total and/or uncarboxylated OC in elderly Mediterranean subjects at high cardiovascular risk. These findings suggest that both high body iron stores and ID may contribute to the alteration of glucose metabolism through the inhibition of OC. Further research is required in order to understand the precise mechanisms by which iron status induces T2DM.

5. BODY IRON STATUS AND TYPE 2 DIABETES MELLITUS

In this prospective nested case-control study conducted on an elderly Mediterranean population at high cardiovascular risk, we confirmed a direct association between high body iron stores, measured as SF and sTfR/ferritin ratio, and the incidence of T2DM. This association was found after adjusting for established risk factors, such as hs-CRP, fasting glucose and other MetS components. These findings provide data from a different population – from southern Europe – to add to those previously studied. In addition, we found no association between sTfR levels and the risk of developing T2DM.

Our results showed significantly higher levels of ferritin (127.59 ± 2.53 vs. 105.22 ± 2.53mg/L, P = 0.037) and lower sTfR/ferritin ratios (9.9 ± 2.80 vs. 12.03 ± 2.80) with borderline significance (P = 0.057) in subjects with incident T2DM than in those with non-incident T2DM. However, we found no difference in sTfR levels. When we analysed the relationship using adjusted conditional logistic regression models, we found that the SF values >257mg/L in males and >139mg/L in females were associated with a high risk

of T2DM. According to the WHO (>200mg/L in males and >150mg/L in females) (World Health Organization, 2011a), these SF values, which are typical of iron overload status, are similar to those found in the studies of Norfolk (Forouhi et al., 2007a) and Potsdam (Montonen et al., 2012) of the EPIC cohort and the ARIC study (Jehn et al., 2007) conducted in the general population.

Serum ferritin

This association between SF and T2DM observed in our prospective study in a southern Europe population is consistent with previous prospective studies conducted with U.S. (Jiang et al., 2004b) (Le et al., 2008) (Rajpathak et al., 2009b), Chinese (Sun et al., 2013) and northern European (Forouhi et al., 2007b) (Salomaa et al., 2010) (Montonen et al., 2012) (Aregbesola et al., 2013) populations. As in our study, these studies showed that subjects in the highest quantile of ferritin had between a 50 % (Aregbesola et al., 2013) and more than three times (Forouhi et al., 2007b) higher risk of developing T2DM than the lowest quantile. Our results were maintained after we adjusted for classic risk factors such as inflammation, fasting glucose and, for the first time, MetS components since MetS, and its components, especially an elevated level of fasting glucose (Zeng et al., 2011), have been strongly associated with the development of T2DM. Previous prospective studies adjusted their models using some classic risk factors, including inflammation (Jiang et al., 2004b) (Forouhi et al., 2007b) (Le et al., 2008) (Rajpathak et al., 2009b) (Salomaa et al., 2010) (Montonen et al., 2012) (Aregbesola et al., 2013) (Sun et al., 2013). However, association, after adjustment for fasting glucose and other MetS components, has not previously been observed. Only the ARIC study (Jehn et al., 2007), conducted with U.S. subjects aged between 45 and 64 observed this association until the MetS components were introduced into the model. Another study, conducted with cohorts from the Finnish FINRISK and Health 2000 (Salomaa et al., 2010) studies comprising men and women aged >25, encountered increased SF levels related to the incidence of T2DM even after adjustment for glucose but not after adjustment for other MetS components.

Moreover, in our study, through the different proportional hazard models, SF and fasting glucose were significantly associated with the risk of T2DM, which suggests that both variables are risk factors for this disease. This is in agreement with the current consensus

that glucose is the main risk factor for T2DM, and that SF may have an aetiological role in the development of this pathology.

sTfR

We found no association between sTfR levels and the risk of T2DM. However, in the DPP (Rajpathak et al., 2009b) cohort of obese subjects with impaired basal glucose, an association was found not only between SF and T2DM, but also between sTfR and T2DM. This latter relationship was contrary to our expectations, i.e. higher levels of sTfR, which are indicative of low iron stores, were associated with a greater risk of T2DM. More recently, the Finnish KIHD cohort (Aregbesola et al., 2013), conducted with middle-aged men, also observed that ID and excess iron (using sTfR as a biomarker) increased the risk of T2DM. A recent review concluded that extreme conditions of ID, as well as iron overload, are associated with a higher risk of CVD (Lapice et al., 2013). The hypothesis is therefore that ID may also increase the incidence of T2DM. Note also that sTfR levels were much higher in cohorts in which an association was observed between sTfR and T2DM, e.g. the DPP study (Rajpathak et al., 2009b) (median 4th quartile 4.4mg/L; mean of the overall sample 3.4mg/L) and the KIHD study (Aregbesola et al., 2013) (0.6–8.2mg/L) compared with our study (0.69–2.65mg/L) and the EPIC Potsdam study (Montonen et al., 2012) (mean of the overall sample 1.13mg/L). However, iron stores measured by SF were similar in these four cohort studies. sTfR levels are also reported to be affected by mechanisms other than those related to iron metabolism (such as insulin sensitivity and obesity) and could be causally linked to T2DM (Speeckaert et al., 2010). Not only would lower iron storage therefore lead to higher sTfR levels but, as suggested by Rajpathak et al. (Rajpathak et al., 2009b) sTfR would be a biomarker of another factor causally related to the risk of T2DM.

sTfR/ferritin ratio

As in previous studies (Salonen et al., 1998) (Jiang et al., 2004b) (Montonen et al., 2012) (Aregbesola et al., 2013), we observed a significant association between higher sTfR/ferritin ratios and a greater risk of T2DM. As stated earlier, the sTfR/ferritin ratio seems to be a good biomarker of iron deposits since it is sensitive both to elevated and

decreased levels of iron (Skikne, 2008). However, since we found no association between sTfR and T2DM, the association observed between the sTfR/ferritin ratio and T2DM could be due exclusively to SF concentrations. Further support for this hypothesis comes from the observation of a strong partial correlation, adjusted for gender, age and BMI, between SF and the sTfR/ferritin ratio (r = -0.974), and by the not-so-strong correlation between sTfR and the sTfR/ferritin ratio (r = 0.542).

At baseline, lifestyle, biochemical, and risk factors for T2DM (MetS and its components, IR, obesity, and inflammation) have been described in incident diabetics and non-incident diabetics in a Mediterranean population at high cardiovascular risk. Of the incident diabetics, 83.7% had abdominal obesity, 62.7% had MetS, 96.7% had hypertension and 35.3% had IR. In addition, cases had greater waist circumference and higher levels of fasting glucose, fasting insulin, triglycerides and IR than non-incident diabetics. Despite these differences at baseline, and after adjusting for these and other risk factors in the proportional hazard models, we found an association between ferritin and the sTfR/ferritin ratio with T2DM.

Our results and those of previous studies (Salonen et al., 1998) (Jiang et al., 2004b) (Forouhi et al., 2007b) (Jehn et al., 2007) (Le et al., 2008) (Rajpathak et al., 2009b) (Salomaa et al., 2010) (Montonen et al., 2012) (Aregbesola et al., 2013) (Sun et al., 2013) suggest that the association between ferritin and T2DM is probably due to excess levels of iron, even though ferritin, as well as to reflecting body iron status, increases with inflammation. This is support in our study because hs-CRP levels were similar in incident and non-incident diabetic subjects, while SF was significantly higher in incident diabetic subjects. We also measured hs-CRP concentrations to control for this effect in multivariate analyses of the relationship between excess iron and the onset of T2DM. Moreover, previous studies that analysed the relationship between ferritin and T2DM after adjustments for one (Jiang et al., 2004b) (Rajpathak et al., 2009b) (Montonen et al., 2012) or more inflammatory markers (Forouhi et al., 2007b) reported similar findings to ours. All these data support the hypothesis that elevated iron status acts independently of the level of inflammation in the development of T2DM.

The essential role of ferritin in the organism lies in the storage of iron, since iron is a strong pro-oxidant agent that catalyses the generation of powerful ROS such as hydroxyl radicals, thus increasing oxidative stress (Schulze and Hu, 2005) (Drews et al., 2010). A proposed underlying mechanism is that certain 'trigger' molecules associated with certain pathologies may open up the structure of the ferritin molecule, thus provoking the liberation of the stored iron (Watt, 2011). This, in turn, could favour the risk associated with oxidative stress and its consequences. Oxidative stress could herefore be the mechanism by which iron excess is associated with a higher incidence of T2DM. Specifically, through its oxidative properties (Schulze and Hu, 2005) (Drews et al., 2010), body iron is suggested to play a role in IR (Syrovatka et al., 2009) and β-cell dysfunction (Kolberg et al., 2009) two key events in the clinical development of T2DM (Swaminathan et al., 2007). In mouse models of haemochromatosis, excess iron in the islets induces oxidative stress that leads to islet cell apoptosis, thus leading to a decrease in insulin secretory capacity (Cooksey et al., 2004). In addition, the peroxidation of lipids, especially free fatty acids, induced by increased iron stores in muscle reduces insulin sensitivity (Felber et al., 1987) (DeFronzo, 1988). Another mechanism that could lead to the development of T2DM is iron deposition in the liver, which could induce hyperinsulinaemia by impeding the liver's capacity for insulin extraction, thus resulting in impaired suppression of hepatic glucose production (Niederau et al., 1984) (Ferrannini, 2000).

Evidence from cross-sectional studies suggests that higher levels of SF are associated with increased IR (Pham et al., 2013). We have also observed this relationship in the present study ($\beta = 0.001$; $P = 0.020$), i.e. for each mg/L of SF, there was an increase of 0.001 in HOMA-IR. Moreover, ferritin was correlated with HOMA-IR in the overall study sample, as well as in incident and non-incident diabetic subjects. This suggests that the relationship with HOMA-IR may occur in the entire general population. The mechanisms underlying this relationship have not yet been identified, though there is speculation that the pro-oxidant role of iron may activate a series of stress avenues related to the family of serine/threonine kinases, thus finally disrupting the insulin signalling process (Rains and Jain, 2011). IR may therefore be an intermediate link in the relationship between high iron deposits and the risk of developing T2DM.

6. STRENGTHS AND LIMITATIONS

Given that this study was conducted in a population with various CVD risk factors, we should highlight the danger of extrapolating these results to the general population. However, this feature of the sample led to a higher incidence of T2DM than would be expected in a healthy population. We would otherwise have needed an extremely large sample to ensure a significant number of T2DM cases. As with cohort studies, there is no assurance that some control subjects would not develop T2DM after the follow-up period. Also, given the observational nature of our studies, we cannot completely establish a cause-effect relationship between independent variables and the risk of developing T2DM.

One of the strengths of this study was that the diagnosis of T2DM was not self-reported but verified by a second analytical test, in accordance with ADA criteria (American Diabetes Association, 2013), enabling new incident cases to be identified more reliably and accurately.

Also, the dietetic variables used in the analysis were obtained from a semiquantitative FFQ (**Appendix III**) that had been validated in the same population in order to reduce the possibility of error (Fernández-Ballart et al., 2010). Finally, the prospective nested case-control study design used to assess the relationship between body iron stores and the risk of T2DM enabled better control of confounding factors such as gender, age ranges and BMI because each case in the incident T2DM group was broadly matched for these variables with two control individuals without T2DM.

In summary, our results support the deleterious effect of iron excess on the risk of T2DM independently of known risk factors. Previous prospective studies had found association between elevated haem iron intake and a greater risk of developing T2DM in U.S., and Chinese populations. We confirm these results, and extend the evidence to another population – a European population from the Mediterranean region. Moreover, as in studies of U.S., Chinese and northern European populations, we also observed, for the first time in a southern European population from the Mediterranean area, a greater risk of T2DM in those subjects with elevated body iron status.

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CONCLUSIONS

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CONCLUSIONS

In a prospective eight-year follow-up study of an adult Mediterranean population at high cardiovascular risk, we observed that:

1. At baseline, incident diabetics consume more haem iron and less fruit and coffee than non-incident diabetics. BMI, waist circumference and the consumption of tobacco are also higher in incident subjects. No significant differences in obesity, hypertension, hypercholesterolaemia, and hypertiglyceridaemia are found between the groups.

2. The incidence of T2DM is higher in the upper tertile of haem iron intake than in the lower tertile.

3. High dietary haem iron intake increases the risk of developing T2DM by 30%, when other factors associated with this relationship such as socio-demographic, anthropometric, lifestyle, and dietary factors, are taken into account. In addition, alcohol consumption increases the risk of T2DM by 2%, while coffee consumption decreases the risk by 7%.

4. A controversial association between iron status and serum osteocalcin (OC) is observed. While low iron status, measured as soluble transferrin receptor (sTfR), is inversely associated with serum concentrations of total and uncarboxylated OC, in adjusted models high iron status, measured as serum ferritin (SF), is inversely associated with total OC in normal glucose metabolism subjects but not in impaired fasting glucose subjects.

5. At baseline, incident diabetics have a worse lipid profile and higher levels of iron, inflammation, fasting glucose, insulin, insulin resistance and metabolic syndrome (MetS) than non-incident diabetics.

Conclusions

6. High body iron status, measured as SF and the sTfR/ferritin ratio, increases the risk of T2DM more than threefold, when other factors associated with this relationship, such as socio-demographic, anthropometric, lifestyle, and dietary variables, inflammation and MetS components (including high glucose levels) are taken into account. However, the relationship between sTfR and the risk of T2DM is not evident in this population.

The relationship we observed between excess iron and the risk of T2DM in the Mediterranean population supports the previous findings in U.S., Chinese and northern European populations. Identifying risk factors such as excess iron could help to prevent and control this disease, which has increased worldwide.

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José Cándido Fernández Cao

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APPENDICES

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José Cándido Fernández Cao

1- PREDIMED QUESTIONNAIRES

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APPENDIX I

Inclusion questionnaire

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José Cándido Fernández Cao

Inclusion questionnaire

ESTUDIO PREDIMED

Inclusión / exclusión

Identificador del participante: Nodo | C.Salud | Médico | Paciente | Visita

Nodo: anotar el número de nodo correspondiente.
01. Andalucía - Málaga / 02. Andalucía - Sevilla - S.Pablo / 03. Andalucía - Sevilla - V.Rocío / 04. Baleares / 05. Cataluña - Barcelona norte / 06. Cataluña - Barcelona Sur / 07. Cataluña - Reus - Tarragona / 08. Madrid Norte / 09. Madrid Sur / 10. Navarra / 11. País Vasco / 12. Valencia

C.Salud: anotar el número del centro de salud correspondiente.

Médico: anotar el número del médico correspondiente.

Paciente: anotar el número del paciente correspondiente.

Visita: anotar el número de visita correspondiente.
00. Inclusión - exclusión / 01. Visita Inicial / 02. Visita 3 meses / 03. Visita 1 año / 04. Visita 2 años / 05. Visita 3años

Fecha del examen Día / Mes / Año

Primer apellido | Segundo apellido | Nombre

Dirección (Calle, Plaza, Paseo, Avenida) | Número | Piso | Puerta

Población | Código postal

Teléfono | Teléfono

Fecha de nacimiento Día / Mes / Año

Sexo: ◯ Hombre ◯ Mujer

¿Evita usted habitualmente comer con mucha grasa de origen animal (mantequilla, manteca, bolleria industrial...)? En caso de no ser así, ¿estaría usted dispuesto a intentarlo?

- ◯ Sí, lo hago desde hace MÁS de 6 meses
- ◯ Sí, lo hago desde hace MENOS de 6 meses
- ◯ No lo hago, pero lo intentaré en los próximos 30 días
- ◯ No lo hago, pero lo intentaré en los próximos 6 meses
- ◯ No lo hago, y no lo intentaré en los próximos 6 meses
- ◯ datos insuficientes

¿Sigue usted una alimentación rica en fibra, es decir con abundante fruta, verdura y legumbres? En caso de no ser así, ¿estaría usted dispuesto a intentarlo?

- ◯ Sí, lo hago desde hace MÁS de 6 meses
- ◯ Sí, lo hago desde hace MENOS de 6 meses
- ◯ No lo hago, pero lo intentaré en los próximos 30 días
- ◯ No lo hago, pero lo intentaré en los próximos 6 meses
- ◯ No lo hago, y no lo intentaré en los próximos 6 meses
- ◯ datos insuficientes

Procedencia: ◯ Europea ◯ Latinoamericana ◯ Norteafricana ◯ Subsahariana ◯ Asiática ◯ Otras

¿Piensa mudarse a otro municipio en los próximos años o tiene alguna limitación que le impida o dificulte poder acudir a los controles y reuniones programados?

◯ sí ◯ no ◯ datos insuficientes

Inclusion questionnaire

¿Ha sido usted informado por personal sanitario, que padezca una enfermedad que le impida seguir alguna dieta determinada que incluya aceite de oliva y/o frutos secos ?

○ sí ○ no ○ datos insuficientes

¿Ha sido usted informado por personal sanitario, que haya tenido alguna vez un infarto de miocardio?

○ sí ○ no ○ datos insuficientes

¿Ha sido usted informado por personal sanitario, que haya tenido alguna vez una angina de pecho?

○ sí ○ no ○ datos insuficientes

¿Ha sido usted informado por personal sanitario, que haya tenido alguna vez una embolia o un accidente vascular cerebral?

○ sí ○ no ○ datos insuficientes

¿Ha sido usted informado por personal sanitario, que haya tenido alguna vez una claudicación intermitente?

○ sí ○ no ○ datos insuficientes

¿Ha sido usted informado por personal sanitario, que haya tenido una diabetes?

○ sí ○ no ○ datos insuficientes

¿Ha sido usted informado por personal sanitario, que tenga el colesterol alto?

○ sí ○ no ○ datos insuficientes

¿Sigue usted algún tratamiento hipolipemiante?

○ sí ○ no

En caso afirmativo, anotar:

Col. total ☐☐☐ **Col. HDL** ☐☐ **Col. LDL** ☐☐☐ **Triglicéridos** ☐☐☐

¿Ha sido usted informado por personal sanitario, que tenga la presión alta?

○ sí ○ no ○ datos insuficientes

¿Sigue usted algún tratamiento antihipertensivo?

○ sí ○ no

En caso afirmativo, anotar:

Presión arterial sistólica: ☐☐☐ **Presión arterial diastólica:** ☐☐☐

¿Algun familiar directo (padres, hermanos, hijos, tíos) ha sufrido o fallecido un infarto de miocardio o angina a una edad inferior a 55 años (varones)/65 años (mujeres) ?

○ sí ○ no ○ datos insuficientes

¿Fuma usted cigarrillos actualmente?

○ sí, regularmente ○ ex-fumador de 0 a 1 año ○ ex-fumador de 1 a 5 años ○ ex-fumador > de 5 años
○ nunca fumador ○ datos insuficientes

En caso afirmativo, ¿cuantos años hace que fuma? ☐☐ 88 = no procede 99 = datos insuficientes

¿Aproximadamente, ¿cuántos cigarrillos, puros o pipas fuma al día?

☐☐ cigarrillos/día ☐☐ puros/día ☐☐ pipas/día 88 = no procede 99 = datos insuficientes

¿Es usted capaz de cambiar/seguir la dieta que le aconsejen los médicos del estudio?

○ sí ○ no ○ datos insuficientes

INCLUSIÓN ○ sí ○ no

MOTIVO de exclusión:

○ No cumplir criterios de inclusión
○ Dificultad de seguimiento del estudio o cambio de hábitos alimenticios
○ Falta de interés de participación en el estudio
○ Datos insuficientes
○ Otros .....
○ Enfermedad Cardiovascular previa
○ Enfermedad médica grave
○ Imposibilidad para cambiar de hábitos

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IRON EXCESS AND RISK OF TYPE 2 DIABETES MELLITUS IN A PROSPECTIVE COHORT OF MEDITERRANEAN POPULATION
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IRON EXCESS AND RISK OF TYPE 2 DIABETES MELLITUS IN A PROSPECTIVE COHORT OF MEDITERRANEAN POPULATION
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APPENDIX II

General questionnaire

UNIVERSITAT ROVIRA I VIRGILI
IRON EXCESS AND RISK OF TYPE 2 DIABETES MELLITUS IN A PROSPECTIVE COHORT OF MEDITERRANEAN POPULATION
José Cándido Fernández Cao

General questionnaire

ESTUDIO PREDIMED

Cuestionario general

Identificador del participante: Nodo | C.Salud | Médico | Paciente | Visita

Nodo: anotar el número de nodo correspondiente.
01. Andalucía - Málaga / 02. Andalucía - Sevilla - S.Pablo / 03. Andalucía - Sevilla - V.Rocío / 04. Baleares / 05. Cataluña - Barcelona norte / 06. Cataluña - Barcelona Sur / 07. Cataluña - Reus - Tarragona / 08. Madrid Norte / 09. Madrid Sur / 10. Navarra / 11. País Vasco / 12. Valencia

C.Salud: anotar el número del centro de salud correspondiente.

Médico: anotar el número del médico correspondiente.

Paciente: anotar el número del paciente correspondiente.

Visita: anotar el número de visita correspondiente.
00. Inclusión - exclusión / 01. Visita Inicial / 02. Visita 3 meses / 03. Visita 1 año / 04. Visita 2 años / 05. Visita 3años

Información de contacto (Pariente o amigo):

Primer apellido | Segundo apellido | Nombre

Teléfono | Teléfono

GRUPO asignado: ◯ Aceite de oliva virgen ◯ Frutos secos ◯ Control

VARIABLES SOCIO DEMOGRÁFICAS

Lugar de nacimiento:

◯ Galicia ◯ La Rioja ◯ Murcia ◯ Castilla la Mancha
◯ Asturias ◯ Aragón ◯ Madrid ◯ Andalucía
◯ Cantabria ◯ Cataluña ◯ Castilla-León ◯ Canarias
◯ País Vasco ◯ Comunidad Valenciana ◯ Extremadura ◯ Baleares
◯ Navarra

País (solo rellenar en caso de extranjeros):

Estado Civil: ◯ Soltero/a ◯ Casado/a ◯ Viudo/a ◯ Divorciado/a ◯ Separado/a ◯ Religioso

¿Cúal es el nivel más alto de escolarización que ha completado?

◯ Titulado Superior o similares ◯ Técnico Escuela Universitaria ◯ Escuela secundaria o Bachiller ◯ Escuela primaria
◯ No sabe leer ni escribir ◯ Datos insuficientes

Número de personas con las que comparte el hogar:

¿Cúal es su situación laboral actual?

◯ Está trabajando ◯ Incapacidad permanente ◯ Ama de casa ◯ Estudiante ◯ Jubilado
◯ Trabaja pero tiene una baja laboral de más de tres meses ◯ Paro con subsidio ◯ Paro sin subsidio ◯ Datos insuficientes

¿Se considera una persona tensa y/o agresiva? Puntuase de 0 (más relajado) a 10 (más competitivo)

Qué trabajo concreto hace o hacía

Qué trabajo concreto hace o hacía el/la cabeza de familia

General questionnaire

Durante el último mes, ¿Ha tomado algún medicamento de los siguientes?

Aspirina, Adiro o similar	○ sí	○ no	○ no sabe / no contest
Otras medicinas para aliviar el dolor o la fiebre	○ sí	○ no	○ no sabe / no contest
Tranquilizantes, sedantes, pastillas para la ansiedad, pastillas para dormir.	○ sí	○ no	○ no sabe / no contest
Vitaminas o minerales	○ sí	○ no	○ no sabe / no contest
Medicamentos para el corazón	○ sí	○ no	○ no sabe / no contest
Medicamentos para la presión arterial	○ sí	○ no	○ no sabe / no contest
Medicamentos para el colesterol	○ sí	○ no	○ no sabe / no contest
Insulina	○ sí	○ no	○ no sabe / no contest
Medicamentos parala diabetes (diferentes de la insulina)	○ sí	○ no	○ no sabe / no contest
Solo mujeres: Tratamiento hormonal	○ sí	○ no	○ no sabe / no contest
Otros	○ sí	○ no	○ no sabe / no contest

En caso afirmativo, nombre del medicamento/s _____

indicar el nombre del fármaco, la dosis y el tiempo del tratamiento en años

LOS TRATAMIENTOS ANOTADOS POR EL PACIENTE DEBEN SER CONFIRMADOS POR LA ENFERMERA A PARTIR DE LA HISTORIA DEL CENTRO DE SALUD

General questionnaire

¿Algún familiar directo (padres, hermanos, hijos, etc...) ha fallecido por causas cardíacas, o ha tenido algún problema cardíaco?

◯ sí, antes de los 55 años (varones) / 65 años (mujeres) ◯ sí, después de los 55 años (varones) / 65 años (mujeres)
◯ no ◯ Datos insuficientes

¿Ha sido usted informado por personal sanitario, que haya tenido alguna vez arritmias o alguna enfermedad cardíaca?

◯ sí ◯ no ◯ datos insuficientes

Diagnóstico

¿Algún familiar directo (padres, hermanos, hijos...) ha tenido algún accidente vascular cerebral?

◯ sí, antes de los 55 años ◯ no ◯ sí, después de los 55 años ◯ datos insuficientes

¿Algún familiar directo (padres, hermanos, hijos...) tiene el colesterol elevado?

◯ sí, antes de los 55 años ◯ sí, después de los 55 años ◯ no ◯ datos insuficientes

¿Algún familiar directo (padres, hermanos, hijos...) tiene la tensión arterial alta?

◯ sí, antes de los 55 años ◯ sí, después de los 55 años ◯ no ◯ datos insuficientes

¿Algún familiar directo (padres, hermanos, hijos...) tiene o ha tenido cáncer?

◯ sí, antes de los 55 años ◯ sí, después de los 55 años ◯ no ◯ datos insuficientes

¿Se cansa excesivamente o le falta el aire al realizar alaún ejercicio (subir escaleras, caminar, etc.)?

◯ No disnea
◯ Disnea a grandes esfuerzos (bailar, caminar durante media hora, trabajos de jardinería, etc.)
◯ Disnea a moderados esfuerzos (ducharse, vestirse,etc.)
◯ Disnea a mínimos esfuerzos (cualquier actividad, levantarse de la cama)
◯ Disnea sin especificar grado
◯ Datos insuficientes

¿Algún médico le ha diagnosticado de alguna de estas enfermedades? Puede haber más de una respuesta.

◯ Embolia pulmonar ◯ Trombosis venosa profunda ◯ Cataratas
◯ Aneurisma de aorta ◯ Bronquitis crónica - Enfisema ◯ Apneas del sueño
◯ Insuficiencia cardíaca izquierda ◯ Depresión ◯ Cáncer o Tumores

Edad del diagnóstico: ☐☐ años

Solo mujeres: **¿Que edad tenía cuando inició la menopausia?** ☐☐ años

¿Le ha molestado a ud. alguna vez la gente criticándole su forma de beber?

◯ sí ◯ no ◯ datos insuficientes

¿Ha tenido ud. la impresión de que debería beber menos?

◯ sí ◯ no ◯ datos insuficientes

¿Se ha sentido alguna vez mal o culpable por su costumbre de beber?

◯ sí ◯ no ◯ datos insuficientes

¿Alguna vez lo primero que ha hecho por la mañana ha sido beber para calmar los nervios o para librarse de una resaca?

◯ sí ◯ no ◯ datos insuficientes

General questionnaire

EXPLORACIÓN FÍSICA

Altura ☐☐☐ cm **Cintura** ☐☐☐ cm **Cadera** ☐☐☐ cm

Peso ☐☐☐ kg **Índice tobillo-brazo** ☐☐☐ cm

		PAS	PAD	FC
Brazo no dominante	1	☐☐☐	☐☐☐	☐☐☐
(paciente sentado)	2	☐☐☐	☐☐☐	☐☐☐
Brazo izquierdo	1	☐☐☐	☐☐☐	☐☐☐
(paciente decubito supino	2	☐☐☐	☐☐☐	☐☐☐
Brazo derecho	1	☐☐☐	☐☐☐	☐☐☐
(paciente decubito supino)	2	☐☐☐	☐☐☐	☐☐☐
Tobillo izquierdo	1	☐☐☐	☐☐☐	☐☐☐
(paciente decubito supino)	2	☐☐☐	☐☐☐	☐☐☐
Tobillo derecho	1	☐☐☐	☐☐☐	☐☐☐
(paciente decubito supino)	2	☐☐☐	☐☐☐	☐☐☐

ITB Izquierdo (PAS mayor del tobillo izquierdo / PAS mayor de los brazos) ☐,☐☐

ITB Derecho (PAS mayor del tobillo derecho / PAS mayor de los brazos) ☐,☐☐

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APPENDIX III

Food frequency questionnaire

UNIVERSITAT ROVIRA I VIRGILI
IRON EXCESS AND RISK OF TYPE 2 DIABETES MELLITUS IN A PROSPECTIVE COHORT OF MEDITERRANEAN POPULATION
José Cándido Fernández Cao

Food frequency questionnaire

IDENTIFICACIÓN DEL PARTICIPANTE

NODO
01. Andalucía-Málaga
02. Andalucía-Sevilla-San Pablo
03. Andalucía-Sevilla-V. Rocío
04. Baleares
05. Catalunya-Barna Norte
06. Catalunya-Barna Sur
07. Catalunya-Reus-Tarragona
08. Madrid Norte
09. Madrid Sur
10. Navarra
11. País Vasco
12. Valencia

NODO | CENTRO | MÉDICO | PACIENTE | VISITA

PÁGINA 1

Por favor, marque una única opción para cada alimento.

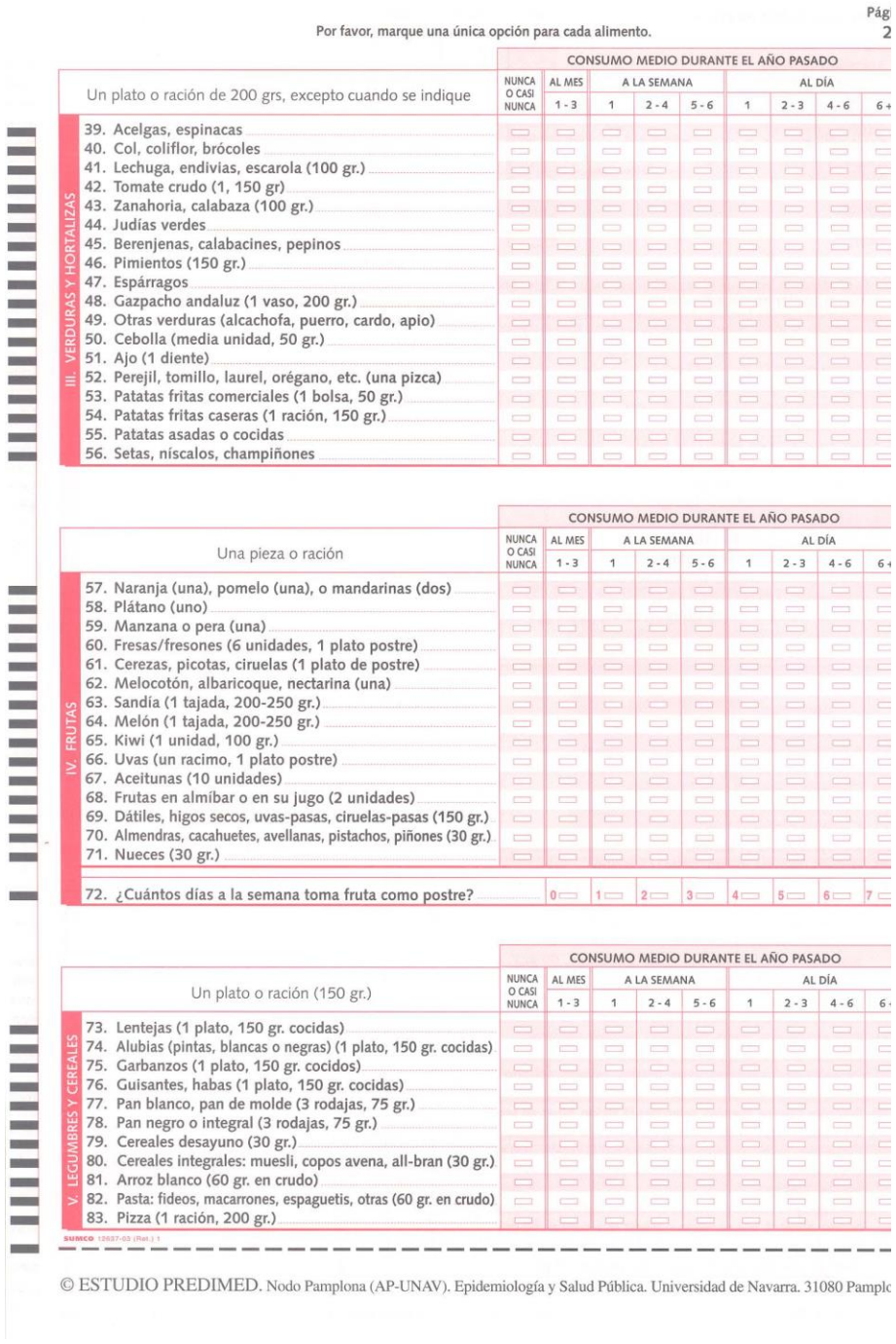
Para cada alimento, marque el recuadro que indica la frecuencia de consumo **por término medio** durante el **año pasado**. Se trata de tener en cuenta también la variación verano/invierno. Por ejemplo, si toma helados 4 veces/semana sólo durante los 3 meses de verano, el uso promedio al año es 1/semana

CONSUMO MEDIO DURANTE EL AÑO PASADO

	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6+
I. LÁCTEOS									
1. Leche entera (1 taza, 200 cc)									
2. Leche semidesnatada (1 taza, 200 cc)									
3. Leche descremada (1 taza, 200 cc)									
4. Leche condensada (1 cucharada)									
5. Nata o crema de leche (1/2 taza)									
6. Batidos de leche (1 vaso, 200 cc)									
7. Yogurt entero (1, 125 gr.)									
8. Yogurt descremado (1, 125 gr.)									
9. Petit suisse (1, 55 gr.)									
10. Requesón o cuajada (1/2 taza)									
11. Queso en porciones o cremoso (1, porción 25 gr.)									
12. Otros quesos: curados, semicurados (Manchego, Bola, Emmental...) (50 gr.)									
13. Queso blanco o fresco (Burgos, cabra...) (50 gr.)									
14. Natillas, flan, puding (1, 130 cc)									
15. Helados (1 cucurucho)									

Un plato o ración de 100-150 gr, excepto cuando se indique otra cosa	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6+
II. HUEVOS, CARNES, PESCADOS									
16. Huevos de gallina (uno)									
17. Pollo o pavo CON piel (1 ración o pieza)									
18. Pollo o pavo SIN piel (1 ración o pieza)									
19. Carne de ternera o vaca (1 ración)									
20. Carne de cerdo (1 ración)									
21. Carne de cordero (1 ración)									
22. Conejo o liebre (1 ración)									
23. Hígado (ternera, cerdo, pollo) (1 ración)									
24. Otras vísceras (sesos, corazón, mollejas) (1 ración)									
25. Jamón serrano o paletilla (1 loncha, 30 gr.)									
26. Jamón York, jamón cocido (1 loncha, 30 gr.)									
27. Carnes procesadas (salchichón, chorizo, morcilla, mortadela, salchichas, butifarra, sobrasada, 50 gr.)									
28. Patés, foie-gras (25 gr.)									
29. Hamburguesa (una, 50 gr.), albóndigas (3 unidades)									
30. Tocino, bacon, panceta (50 gr.)									
31. Pescado blanco: mero, lenguado, besugo, merluza, pescadilla,... (1 plato, pieza o ración)									
32. Pescado azul: sardinas, atún, bonito, caballa, salmón (1 plato, pieza o ración 130 gr.)									
33. Pescados salados: bacalao, salazones (1 ración, 60 gr. en seco)									
34. Ostras, almejas, mejillones y similares (6 unidades)									
35. Calamares, pulpo, chipirones, jibia (sepia) (1 ración, 200 gr.)									
36. Crustáceos: gambas, langostinos, cigalas, etc. (4-5 piezas, 200 gr.)									
37. Pescados y mariscos enlatados al natural (sardinas, anchoas, bonito, atún) (1 lata pequeña o media lata normal, 50 gr.)									
38. Pescados y mariscos en aceite (sardinas, anchoas, bonito, atún) (1 lata pequeña o media lata normal, 50 gr.)									

Food frequency questionnaire



Pág 2

Por favor, marque una única opción para cada alimento.

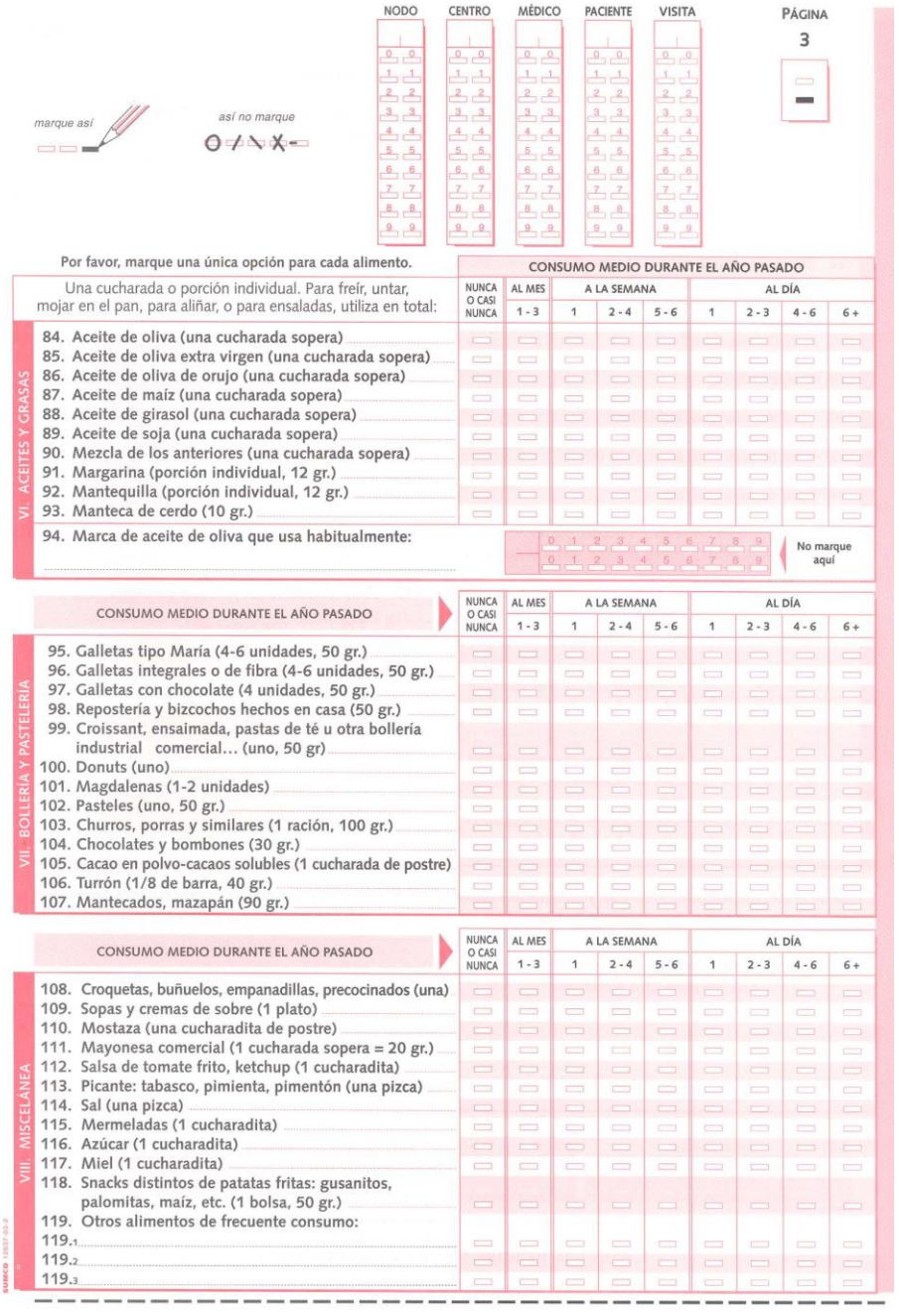
	Un plato o ración de 200 grs, excepto cuando se indique	CONSUMO MEDIO DURANTE EL AÑO PASADO									
		NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA				
			1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6+
III. VERDURAS Y HORTALIZAS	39. Acelgas, espinacas									
	40. Col, coliflor, brócoles									
	41. Lechuga, endivias, escarola (100 gr.)									
	42. Tomate crudo (1, 150 gr)									
	43. Zanahoria, calabaza (100 gr.)									
	44. Judías verdes									
	45. Berenjenas, calabacines, pepinos									
	46. Pimientos (150 gr.)									
	47. Espárragos									
	48. Gazpacho andaluz (1 vaso, 200 gr.)									
	49. Otras verduras (alcachofa, puerro, cardo, apio)									
	50. Cebolla (media unidad, 50 gr.)									
	51. Ajo (1 diente)									
	52. Perejil, tomillo, laurel, orégano, etc. (una pizca)									
	53. Patatas fritas comerciales (1 bolsa, 50 gr.)									
	54. Patatas fritas caseras (1 ración, 150 gr.)									
	55. Patatas asadas o cocidas									
	56. Setas, níscalos, champiñones									

	Una pieza o ración	CONSUMO MEDIO DURANTE EL AÑO PASADO									
		NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA				
			1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6+
IV. FRUTAS	57. Naranja (una), pomelo (una), o mandarinas (dos)									
	58. Plátano (uno)									
	59. Manzana o pera (una)									
	60. Fresas/fresones (6 unidades, 1 plato postre)									
	61. Cerezas, picotas, ciruelas (1 plato de postre)									
	62. Melocotón, albaricoque, nectarina (una)									
	63. Sandía (1 tajada, 200-250 gr.)									
	64. Melón (1 tajada, 200-250 gr.)									
	65. Kiwi (1 unidad, 100 gr.)									
	66. Uvas (un racimo, 1 plato postre)									
	67. Aceitunas (10 unidades)									
	68. Frutas en almíbar o en su jugo (2 unidades)									
	69. Dátiles, higos secos, uvas-pasas, ciruelas-pasas (150 gr.)									
	70. Almendras, cacahuetes, avellanas, pistachos, piñones (30 gr.)									
	71. Nueces (30 gr.)									
	72. ¿Cuántos días a la semana toma fruta como postre?	0	1	2	3	4	5	6	7		

	Un plato o ración (150 gr.)	CONSUMO MEDIO DURANTE EL AÑO PASADO									
		NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA				
			1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6+
V. LEGUMBRES Y CEREALES	73. Lentejas (1 plato, 150 gr. cocidas)									
	74. Alubias (pintas, blancas o negras) (1 plato, 150 gr. cocidas)									
	75. Garbanzos (1 plato, 150 gr. cocidos)									
	76. Guisantes, habas (1 plato, 150 gr. cocidas)									
	77. Pan blanco, pan de molde (3 rodajas, 75 gr.)									
	78. Pan negro o integral (3 rodajas, 75 gr.)									
	79. Cereales desayuno (30 gr.)									
	80. Cereales integrales: muesli, copos avena, all-bran (30 gr.)									
	81. Arroz blanco (60 gr. en crudo)									
	82. Pasta: fideos, macarrones, espaguetis, otras (60 gr. en crudo)									
	83. Pizza (1 ración, 200 gr.)									

SUMCO 12637-03 (Rel.) 1

Food frequency questionnaire



NODO | CENTRO | MÉDICO | PACIENTE | VISITA | PÁGINA 3

marque así | así no marque

Por favor, marque una única opción para cada alimento.

Una cucharada o porción individual. Para freír, untar, mojar en el pan, para aliñar, o para ensaladas, utiliza en total:	CONSUMO MEDIO DURANTE EL AÑO PASADO: NUNCA O CASI NUNCA	AL MES 1-3	A LA SEMANA 1	A LA SEMANA 2-4	A LA SEMANA 5-6	AL DÍA 1	AL DÍA 2-3	AL DÍA 4-6	AL DÍA 6+
VI. ACEITES Y GRASAS									
84. Aceite de oliva (una cucharada sopera)									
85. Aceite de oliva extra virgen (una cucharada sopera)									
86. Aceite de oliva de orujo (una cucharada sopera)									
87. Aceite de maíz (una cucharada sopera)									
88. Aceite de girasol (una cucharada sopera)									
89. Aceite de soja (una cucharada sopera)									
90. Mezcla de los anteriores (una cucharada sopera)									
91. Margarina (porción individual, 12 gr.)									
92. Mantequilla (porción individual, 12 gr.)									
93. Manteca de cerdo (10 gr.)									
94. Marca de aceite de oliva que usa habitualmente:	No marque aquí								

CONSUMO MEDIO DURANTE EL AÑO PASADO	NUNCA O CASI NUNCA	AL MES 1-3	A LA SEMANA 1	A LA SEMANA 2-4	A LA SEMANA 5-6	AL DÍA 1	AL DÍA 2-3	AL DÍA 4-6	AL DÍA 6+
VII. BOLLERÍA Y PASTELERÍA									
95. Galletas tipo María (4-6 unidades, 50 gr.)									
96. Galletas integrales o de fibra (4-6 unidades, 50 gr.)									
97. Galletas con chocolate (4 unidades, 50 gr.)									
98. Repostería y bizcochos hechos en casa (50 gr.)									
99. Croissant, ensaimada, pastas de té u otra bollería industrial comercial... (uno, 50 gr)									
100. Donuts (uno)									
101. Magdalenas (1-2 unidades)									
102. Pasteles (uno, 50 gr.)									
103. Churros, porras y similares (1 ración, 100 gr.)									
104. Chocolates y bombones (30 gr.)									
105. Cacao en polvo-cacaos solubles (1 cucharada de postre)									
106. Turrón (1/8 de barra, 40 gr.)									
107. Mantecados, mazapán (90 gr.)									

CONSUMO MEDIO DURANTE EL AÑO PASADO	NUNCA O CASI NUNCA	AL MES 1-3	A LA SEMANA 1	A LA SEMANA 2-4	A LA SEMANA 5-6	AL DÍA 1	AL DÍA 2-3	AL DÍA 4-6	AL DÍA 6+
VIII. MISCELÁNEA									
108. Croquetas, buñuelos, empanadillas, precocinados (una)									
109. Sopas y cremas de sobre (1 plato)									
110. Mostaza (una cucharadita de postre)									
111. Mayonesa comercial (1 cucharada sopera = 20 gr.)									
112. Salsa de tomate frito, ketchup (1 cucharadita)									
113. Picante: tabasco, pimienta, pimentón (una pizca)									
114. Sal (una pizca)									
115. Mermeladas (1 cucharadita)									
116. Azúcar (1 cucharadita)									
117. Miel (1 cucharadita)									
118. Snacks distintos de patatas fritas: gusanitos, palomitas, maíz, etc. (1 bolsa, 50 gr.)									
119. Otros alimentos de frecuente consumo:									
119.1									
119.2									
119.3									

Food frequency questionnaire

Por favor, marque una única opción para cada alimento.

IX. BEBIDAS

	CONSUMO MEDIO DURANTE EL AÑO PASADO								
	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6
120. Bebidas carbonatadas con azúcar: bebidas con cola, limonadas, tónicas, etc. (1 botellín, 200 cc)									
121. Bebidas carbonatadas bajas en calorías, bebidas light (1 botellín, 200 cc)									
122. Zumo de naranja natural (1 vaso, 200 cc)									
123. Zumos naturales de otras frutas (1 vaso, 200 cc)									
124. Zumos de frutas en botella o enlatados (200 cc)									
125. Café descafeinado (1 taza, 50 cc)									
126. Café (1 taza, 50 cc)									
127. Té (1 taza, 50 cc)									
128. Mosto (100 cc)									
129. Vaso de vino rosado (100 cc)									
130. Vaso de vino moscatel (50 cc)									
131. Vaso de vino tinto joven, del año (100 cc)									
132. Vaso de vino tinto añejo (100 cc)									
133. Vaso de vino blanco (100 cc)									
134. Vaso de cava (100 cc)									
135. Cerveza (1 jarra, 330 cc)									
136. Licores, anís o anisetes... (1 copa, 50 cc)									
137. Destilados: whisky, vodka, ginebra, coñac (1 copa, 50 cc)									

138. ¿A que edad empezó a beber alcohol (vino, cerveza o licores), incluyendo el que toma con las comidas con regularidad (más de siete "bebidas" a la semana)?

Edad (años)
Decena
Unidad

139. ¿Cuántos años ha bebido alcohol con regularidad (más de siete "bebidas" a la semana)?

Años
Decena
Unidad

119. Otros alimentos de frecuente consumo

119.1 (No marque aquí)

119.2 (No marque aquí)

119.3 (No marque aquí)

Si durante el año pasado tomó vitaminas y/o minerales (incluyendo calcio) o productos dietéticos especiales (salvado, ac de onagra, leche con ácidos grasos omega-3, flavonoides, etc.), por favor indique la marca y la frecuencia con que los to

Marcas de los suplementos de vitaminas o minerales o de los productos dietéticos	CONSUMO MEDIO DURANTE EL AÑO PASADO								
	NUNCA O CASI NUNCA	AL MES	A LA SEMANA			AL DÍA			
		1 - 3	1	2 - 4	5 - 6	1	2 - 3	4 - 6	6
140.									
140.1									
140.2									

140 (No marque aquí)

140.1 (No marque aquí)

140.2 (No marque aquí)

SUMICO 12937-03-(Fhd. 2)

Muchas gracias por su colaboración

UNIVERSITAT ROVIRA I VIRGILI
IRON EXCESS AND RISK OF TYPE 2 DIABETES MELLITUS IN A PROSPECTIVE COHORT OF MEDITERRANEAN POPULATION
José Cándido Fernández Cao

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APPENDIX IV

Physical activity questionnaire

UNIVERSITAT ROVIRA I VIRGILI
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Physical activity questionnaire

ESTUDIO PREDIMED — **Cuestionario de actividad física**

Identificador del participante: Nodo | C.Salud | Médico | Paciente | Visita

Fecha del examen Dia / Mes / Año

DNI

CIP

CUESTIONARIO DE ACTIVIDAD FÍSICA EN EL TIEMPO LIBRE DE MINNESOTA

A continuación encontrara un cuadro con un listado de actividades fisicas y unas columnas con periodos de tiempo de realización de las mismas(semana,mes,trimestre y año). Cada columna esta dividida en dias y minutos.
La forma de rellenar el cuestionario es la siguiente:
1. Se lee atentamente cada actividad una a una y cuando se encuentre una que se haya realizado durante la última semana,con números claros y sin salirse del recuadro se rellenan las casillas correspondientes a los dias y minutos.
2. Seguidamente se repite la misma acción para el último mes,el último trimestre y el último año.
Ha de tener en cuenta que si ha realizado algúna actividad la última semana supone tambíen que la ha realizado el último mes,trimestre y año.
Para asegurar la uniformidad de la información recogida consideramos que:
- cada piso de escaleras = 1/2 min.
- una vuelta en esqui acuático = 5 mn.
- un set de tenis indivdual = 20 min.
- un set de tenis dobles = 15 min.
- golf 9 hoyos = 90 min.

Ejemplo:

Una persona que:

- durante la última semana ha ido a caminar media hora cada dia menos el fin de semana, ha de anotar un 5 en la columna de dias de práctica a la semana y 30 en minutos/dia de practica. Si durante el último año también ha ido a caminar pero durante 2 meses en el verano no ha hecho esta actividad , tendra que anotar 200 en la columna de dias practica al año y 30 en minutos / dia de practica .
- durante la última semana ha subido 2 veces al dia 2 pisos por la escalera a de anotar un 7 en la columna de dias de práctica a la semana y 2 a minutos/ dia de práctica. Si esta actividad la repite todo el año,tendra que anotar 365 en columna dias de práctica al año y 2 en minutos / dia de práctica.

ACTIVIDADES FÍSICAS	SEMANA		AÑO	
	DIAS DE PRACTICA	MINUTOS/DIA DE PRACTICA	DIAS DE PRACTICA	MINUTOS/DIA DE PRACTICA
ANDAR/BAILAR/SUBIR ESCALERAS				
1.Pasear	5	30	200	30
5.Subir escaleras	7	2	365	2

Physical activity questionnaire

ACTIVIDADES FÍSICAS	SEMANA		AÑO	
	DIAS DE PRACTICA	MINUTOS/DIA DE PRACTICA	DIAS DE PRACTICA	MINUTOS/DIA DE PRACTICA
ANDAR/BAILAR/SUBIR ESCALERAS				
1.Pasear				
2.Andar de casa al trabajo y del trabajo a casa o en periodos de descanso del mismo				
3.Andar (llevando carrito de la compra)				
4.Andar (llevando bolsas de la compra)				
5.Subir escaleras				
6.Andar campo a través				
7.Excursiones con mochila				
8.Escalar montañas				
9.Ir en bicicleta al trabajo				
10.Bailar				
11.Aeróbic o ballet				
12.Jugar con los niños (corriendo, saltando,..)				
EJERCICIOS DE MANTENIMIENTO GENERAL				
13.Hacer ejercicio en casa				
14.Hacer ejercicio en un gimnasio				
15.Caminar deprisa				
16.Trotar ("Jogging")				
17.Correr 8-11 km/h				
18.Correr 12-16 km/h				
19.Levantar pesas				
ACTIVIDADES ACUÁTICAS				
20.Esquí acuático				
21.Surf				
22.Navegar a vela				
23.Ir en canoa o remar (por distracción)				
24.Ir en canoa o remar (en competición)				
25.Hacer un viaje en canoa				

Physical activity questionnaire

ACTIVIDADES FÍSICAS	SEMANA		AÑO	
	DIAS DE PRACTICA	MINUTOS/DIA DE PRACTICA	DIAS DE PRACTICA	MINUTOS/DIA DE PRACTICA
26.Nadar (más de 150 metros en piscina)				
27.Nadar en el mar				
28.Bucear				
DEPORTES DE INVIERNO				
29.Esquiar				
30.Esquí de fondo				
31.Patinar (ruedas o hielo)				
OTRAS ACTIVIDADES				
32.Montar a caballo				
33.Jugar a los bolos				
34.Balonvolea				
35.Tenis de mesa				
36.Tenis individual				
37.Tenis dobles				
38.Badminton				
39.Baloncesto (sin jugar partido)				
40.Baloncesto (jugando un partido)				
41.Baloncesto (actuando de árbitro)				
42.Squash				
43.Fútbol				
44.Golf (llevando el carrito)				
45.Golf (andando y llevando los palos)				
46.Balonmano				
47.Petanca				
48.Artes marciales				
49.Motociclismo				
50.Ciclismo de carretera o montaña				

Physical activity questionnaire

ACTIVIDADES FÍSICAS	SEMANA		AÑO	
	DIAS DE PRACTICA	MINUTOS/DIA DE PRACTICA	DIAS DE PRACTICA	MINUTOS/DIA DE PRACTICA
ACTIVIDADES EN EL JARDÍN				
51.Cortar el césped con máquina				
52.Cortar el césped manualmente				
53.Limpiar y arreglar el jardín				
54.Cavar el huerto				
55.Quitar nieve con pala				
TRABAJOS Y ACTIVIDADES CASERAS				
56.Trabajos de carpintería dentro de casa				
57.Trabajos de carpinteria (exterior)				
58.Pintar dentro de casa				
59.Pintar fuera de casa				
60.Limpiar la casa				
61.Mover muebles				
CAZA Y PESCA				
62.Tiro con pistola				
63.Tiro con arco				
64.Pescar en la orilla del mar				
65.Pescar con botas altas dentro del río				
66.Caza menor				
67.Caza mayor (ciervos, osos...)				
OTROS (ESPECIFICAR)				

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2- SCIENTIFIC CONTRIBUTIONS

UNIVERSITAT ROVIRA I VIRGILI
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APPENDIX V

Articles accepted or under review

UNIVERSITAT ROVIRA I VIRGILI
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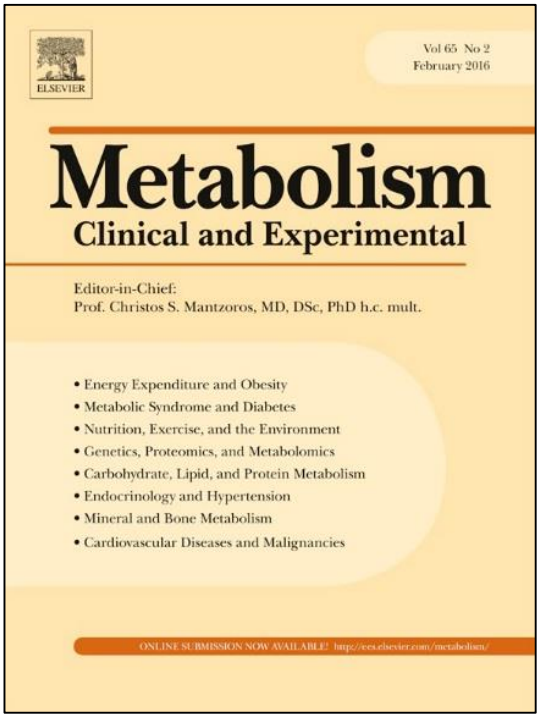
José Cándido Fernández Cao

Articles under review

Soluble transferrin receptor levels and risk of type 2 diabetes mellitus in obese and non-obese subjects: a nested case-control study



Fernández-Cao JC, Arija V, Aranda N, Basora J, Diez-Espino J, Estruch R, Fitó M, Corella D, Salas-Salvadó J

Submitted to
Metabolism Clinical and Experimental
(IF: 3.894; Q2 Endocrinology & Metabolism)

Articles under review

Metabolism Clinical and Experimental

Abstract

Introduction: High body iron stores (B-IronS), measured as ferritin levels, have been associated with an increased risk of type 2 diabetes mellitus (T2DM). However, studies evaluating the relationship between levels of soluble transferrin receptor (sTfR), biomarker inversely related with B-IronS, and risk of T2DM are scarce and inconclusive. Further, sTfR concentrations have been observed to be significantly higher in obese than in non-obese. Thereby, the aim of the present study was to assess the relationship between levels of sTfR and the incidence of T2DM in obese and non-obese, separately.

Methods: A prospective nested case-control study of 153 cases of newly diagnosed diabetic subjects, 73 obese and 80 non-obese, and 306 individually matched-controls, 138 obese and 166 non-obese, who did not develop T2DM during a median 6-year follow-up (interquartile range: 3.9-6.5) was conducted using data from the PREDIMED (PREvention with MEDiterranean Diet) cohort (http://www.controlled-trials.com/ISRCTN35739639). Subjects are Caucasian, aged 55-80 years, and at high cardiovascular risk. Cases and controls were matched for age (≤67 vs. >67 years), gender, dietary intervention group, and BMI (≤27 vs. >27kg/m2).

Results: Positive correlations were observed between sTfR concentrations and waist circumference (r = 0.143, P = 0.002) or BMI (r = 0.113, P = 0.016) in the whole sample, and in obese individuals (r=0.210, P = 0.002) (r = 0.169, P = 0.015), respectively. Further, sTfR concentrations were inversely and directly associated with risk of T2DM in obese (OR =0.50; 95% CI: 0.25-0.99) and non-obese (OR=2.09; 95% CI: 1.01-4.32), respectively.

Conclusions: Opposite associations were observed between sTfR levels and risk of T2DM based on the presence or absence of obesity in a population at high cardiovascular risk. While in non-obese elevated sTfR levels were associated with a decreased risk of T2DM, in obese the risk increased, suggesting that adiposity may alter the relationship between sTfR and T2DM incidence.

Clinical trial registration number: ISRCTN35739639

Keywords: Soluble transferrin receptor, body iron stores, type 2 diabetes, obesity, nested case-control, PREDIMED.

Elsevier Editorial System(tm) for Metabolism
Manuscript Draft

Manuscript Number:

Title: Soluble transferrin receptor levels and risk of type 2 diabetes mellitus in obese and non-obese subjects: a nested case-control study

Article Type: Research Paper

Corresponding Author: Prof. VICTORIA ARIJA, M.D.

Corresponding Author's Institution: UNIVERSITAT ROVIRA I VIRGILI

First Author: Jose C Fernandez-Cao

Order of Authors: Jose C Fernandez-Cao; VICTORIA ARIJA, M.D.; Nuria Aranda, PhD; Josep Basora; Javier Diez-Espino, Primary Health Care Centre of Tafalla, Servicio Na; Ramon Estruch, PhD; Montserrat Fito, PhD; Dolores Corella, PhD; Jordi Salas-Salvado, M.D,

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Departament de Biologia Fonamental i Ciències de la Salut, Espanya, Universitat de les Illes Balears
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Luis Serra Majem
Professor, Departamento de Medicina Preventiva y Salud Pública, España, Universidad Las Palmas de Gran Canaria
lserra@dcc.ulpgc.es

Opposed Reviewers:

Articles under review

Iron excess and the risk of gestational diabetes mellitus: A systematic review and meta-analysis

Fernández-Cao JC, Ribot B, Arija V

Submitted to

Obstetrics and Gynecology

(IF: 5.175; Q1 Obstetrics & Gynecology)

Abstract

Objective- The aim of this systematic review and meta-analysis of observational studies was to evaluate the association of high concentrations of hemoglobin and/or ferritin and the risk of gestational diabetes mellitus (GDM).

Data sources-The selected studies were identified through a systematic review of scientific literature published in The Cochrane Library and PubMed/MEDLINE databases from inception to October 8, 2014, in addition to citation tracking and hand-searches.

Methods of study selection- The present systematic review and meta-analysis was recorded in PROSPERO (2013:CRD42013005717). The search strategy of original articles was performed combining several terms for hemoglobin, ferritin, pregnancy and GDM. To identify associations between hemoglobin and/or ferritin levels with the risk of GDM, OR and 95% CI of the selected studies were used. Summary estimates were calculated by combining inverse-variance-weighted study-specific estimates using random-effects models.

Tabulation, integration, and results- 2032 abstracts were initially found during the search. Of these, two with both hemoglobin and ferritin data, six with hemoglobin data and one with ferritin data, were included. After conducting a meta-analysis of eight studies we observed that elevated hemoglobin levels were associated with an increased risk of GDM, 1.53 (95% CI: 1.15-2.04). A meta-analysis of three studies showed that elevated ferritin levels were associated with an increased risk of GDM, 2.08 (95% CI: 1.38-3.14). Low and undetectable heterogeneity was observed in hemoglobin (I^2= 40.7%, P = 0.107) and ferritin (I^2 = 0.0%, P = 0.692) meta-analysis, respectively. Nor publication bias was seen.

Conclusion- In this systematic review and meta-analysis of observational studies we found that high hemoglobin and/or ferritin levels were associated with an increased risk of GDM.

Keywords Hemoglobin · Ferritin · Body iron stores · Pregnancy · Gestational diabetes · Systematic review · Meta-analysis.

Obstetrics & Gynecology

Iron excess and the risk of gestational diabetes mellitus: A systematic review and meta-analysis

--Manuscript Draft--

Manuscript Number:	
Full Title:	Iron excess and the risk of gestational diabetes mellitus: A systematic review and meta-analysis
Article Type:	Review
Manuscript Classifications:	Epidemiology and public health; Medical complications of pregnancy; Pathology
Corresponding Author:	Victoria Arija, Professor Universitat Rovira i Virgili Reus, Tarragona SPAIN
Corresponding Author's Institution:	Universitat Rovira i Virgili
First Author:	José Cándido Fernández-Cao, MSc
Order of Authors:	José Cándido Fernández-Cao, MSc
	Blanca Ribot, PhD
	Victoria Arija, Professor
Manuscript Region of Origin:	SPAIN

Articles under review

Increased iron levels and lipid peroxidation in a Mediterranean population of Spain

Aranda N*, *Fernández-Cao JC*, Tous M, Arija V

Accepted in

European Journal of Clinical Investigation

(IF: 2.734; Q1 Medicine General & Internal; Q2 Medicine Research & Experimental)

Articles under review

European Journal of Clinical Investigation

Abstract

Background: Many chronic diseases are adversely affected by elevated iron levels. It has been speculated that this relationship is mediated by increased oxidative stress, due to the ability of iron to generate reactive oxygen species. The aim of this study was to assess the relationship between elevated iron levels and lipid peroxidation in Caucasian adults residing in the northeastern Mediterranean region of Spain.

Materials and methods: This cross sectional case-control study included 300 subjects: 150 adults displaying elevated iron levels (cases) selected from a representative sample of our general population and 150 age and sex matched adults exhibiting normal iron levels (controls). Dietary assessment (3-day food records), iron biomarkers (serum iron, ferritin, and transferrin saturation) and lipid profile were determined. Elevated iron levels were defined by high serum ferritin (SF >110μg/L in women and >200μg/L in men) and/or transferrin saturation (TS) >45%. Oxidized low-density lipoprotein (oxLDL) plasma levels were measured and oxLDL/LDL-cholesterol ratio was calculated to estimate lipid peroxidation. Multiple linear regression models were applied.

Results: Individuals with elevated serum iron levels showed increased oxLDL/LDL ratio, but not oxLDL levels, compared to control subjects (20.92 ± 4.89U/mmol vs. 19.72±3.573U/mmol, P = 0.028). These results were further confirmed by the regression models adjusted for demographic characteristics, diet, lipid profile and inflammation. Importantly, higher serum levels of triglycerides, LDL-cholesterol and lower intake of Vitamin E increased lipid peroxidation.

Conclusions: In our general population, we have observed that higher circulating levels of iron, measured by SF and/or TS, increased lipid peroxidation (measured by oxLDL/LDL ratio).

Keywords: Elevated iron levels; oxidative stress; lipid peroxidation; oxidized low-density lipoprotein; oxLDL/LDL-cholesterol ratio.

European Journal of Clinical Investigation

Increased iron levels and lipid peroxidation in a Mediterranean population of Spain

Journal:	*European Journal of Clinical Investigation*
Manuscript ID	Draft
Wiley - Manuscript type:	Original Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Aranda, Nuria; *Nutrition and Public Health Unit, Faculty of Medicine and Health Sciences, Research Group in Nutrition and Mental Health (NUTRISAM), Institut d'Investigació Sanitària Pere Virgili (IISPV), Universitat Rovira i Virgili, Reus, Spain Fernandez-cao, Jose Candido; *Nutrition and Public Health Unit, Faculty of Medicine and Health Sciences, Research Group in Nutrition and Mental Health (NUTRISAM), Institut d'Investigació Sanitària Pere Virgili (IISPV), Universitat Rovira i Virgili, Reus, Spain Tous, Monica; *Nutrition and Public Health Unit, Faculty of Medicine and Health Sciences, Research Group in Nutrition and Mental Health (NUTRISAM), Institut d'Investigació Sanitària Pere Virgili (IISPV), Universitat Rovira i Virgili, Reus, Spain Arija, Victoria; Nutrition and Public Health Unit, Faculty of Medicine and Health Sciences, Research Group in Nutrition and Mental Health (NUTRISAM), Institut d'Investigació Sanitària Pere Virgili (IISPV), Universitat Rovira i Virgili, Reus, Spain ; Unitat de Suport a la Recerca Tarragona-Reus, Institut Universitari d'Investigació en Atenció Primària Jordi Gol, Tarragona, Spain
Keywords:	Elevated iron levels, oxidative stress, lipid peroxidation, oxidized low-density lipoprotein, oxLDL/LDL-cholesterol ratio

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APPENDIX VI

Pre-doctoral mobility

UNIVERSITAT ROVIRA I VIRGILI
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José Cándido Fernández Cao

PROGRAMA ESTATAL DE PROMOCIÓN DEL TALENTO Y SU EMPLEABILIDAD

CERTIFICADO DEL CENTRO RECEPTOR TRAS LA ESTANCIA BREVE O TRASLADO TEMPORAL

CERTIFICATE OF STAY IN A FOREING INSTITUTION

1. Beneficiario/ *Applicant:*

Nombre y apellidos/ *Name:* José Cándido Fernández Cao

D.N.I./ *National identity Card:* 33995974L

Centro de adscripción de la beca/ *Home Institución:* Universidad Rovira i Virgili

2. Centro en el que se ha realizado la estancia/ *Host institution:*

Nombre/ *Name:* University of Central Lancashire

Dirección/ *Adress: Darwin Building, DB230*

Localidad/ *Country:* Preston

3. Investigador responsable en el centro de la estancia/ *Responsable person in the Host*

Institución/ *Institution:* University of Central Lancashire

Nombre/ *Name:* Nicola Lowe

Cargo/ *Post: Professor*

CERTIFICO:
que el becario arriba mencionado ha realizado una estancia en este centro en las siguientes fechas: desde 01 / 02 / 2015 hasta 03 / 05/ 2015

THIS IS TO CERTIFY:
that the above mentioned person has performed a stay in this Institution in the following dates: From: 01 / 02 / 2015 To: 03 / 05 / 2015

Lugar y fecha: Preston 03 / 05 / 2015
City and date: Preston 03 / 05 / 2015

Firma y Sello/ *Signature & Stamp*

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APPENDIX VII

Participation in conferences

UNIVERSITAT ROVIRA I VIRGILI
IRON EXCESS AND RISK OF TYPE 2 DIABETES MELLITUS IN A PROSPECTIVE COHORT OF MEDITERRANEAN POPULATION
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Conferences

Conference: 1st World Forum for Nutrition Research Conference: Mediterranean Food on Health and Disease. Reus, Spain.

Organizing entity: International Union of Nutrition Sciences, the World Congress on Nuts and Dried Fruits.

Date: 20-21 of May 2013.

Authors: Arija V, Ribot B, Fernández-Cao JC, Aranda N.

Title: Effect of the HFE Gene mutations on the iron status of pregnant women and their infant health.

Format: Poster.

Publication: Arija V, Ribot B, Fernández-Cao JC, Aranda N. Effect of the HFE Gene mutations on the iron status of pregnant women and their infant health. 1st World Forum for Nutrition Research Conference: Mediterranean Food on Health and Disease. Reus. Ann Nutr Metab 2013; 62 (suppl 2):71.

Conference: 1st World Forum for Nutrition Research Conference: Mediterranean Food on Health and Disease. Reus, Spain.

Organizing entity: International Union of Nutrition Sciences, the World Congress on Nuts and Dried Fruits.

Date: 20-21 of May 2013.

Authors: Pedret R, Fernández-Cao JC, Aguas D, Vinuesa A, Silva A. R, Dalmau S, Basora T, Peralta L, Arija V.

Title: Pas a Pas Program: A Community Randomized Intervention Study of Physical Activity.

Format: Poster.

Publication: Pedret R, Fernández-Cao JC, Aguas D, Vinuesa A, Silva A. R, Dalmau S, Basora T, Peralta L, Arija V. Pas a Pas Program: A Community Randomized Intervention Study of Physical Activity. 1st World Forum for Nutrition Research Conference: Mediterranean Food on Health and Disease. Ann Nutr Metab 2013; 62 (suppl 2):1-90.

Conference: IUNS 20th International Congress of Nutrition.

Organizing entity: International Union of Nutrition Sciences (IUNS).

Date: 16-20 of September 2013.

Authors: Fernandez-Cao JC, Arija V, Aranda N, Bullo M, Basora J, Martinez-gonzález MA, Espino J, Salas J.

Title: Dietary iron intake and types 2 diabetes.

Format: Poster.

Publication: Fernandez-Cao JC, Arija V, Aranda N, Bullo M, Basora J, Martinez-gonzález MA, Espino J, Salas J. Dietary iron intake and types 2 diabetes. IUNS 20th International Congress of Nutrition. Granada. Ann Nutr Metab. 2013; 63 (supp1) 448.

Conference: IUNS 20th International Congress of Nutrition.

Organizing entity: International Union of Nutrition Sciences (IUNS).

Date: 16-20 of September 2013.

Authors: Juanola-Falgarona M, Fernández-Cao JC, Salas-Salvadó J, Martínez-González MA, Estruch R, Fiol M, Arija V, Bulló M.

Title: Serum ferritin is associated to osteocalcin: a potencial mechanism of iron-induced insulin resistance.

Format: Poster.

Publication: Juanola-Falgarona M, Fernández-Cao JC, Salas-Salvadó J, Martínez-González MA, Estruch R, Fiol M, Arija V, Bulló M. Serum ferritin is associated to osteocalcin: a potencial mechanism of iron-induced insulin resistance. IUNS 20th International Congress of Nutrition. Granada. Ann Nutr Metab. 2013; 63 (supp1) 448.

Conference: III conference on public health research.

Organizing entity: L'Agència de Salut Pública de Catalunya (ASPCAT).

Date: 14 of November 2013.

Authors: Fernández-Cao JC, Pedret R, Olivé M, Basora T, Basora J, Arija V.

Title: Community physical activity intervention and impact in a hypertensive population.

Format: Poster.

Conferences

Conference: XXXIV Congreso de la semFYC.

Organizing entity: Sociedad Española de Medicina de Familia y Comunitaria (semFYC).

Date: 12-14 of June 2014.

Authors: Pedret R, Vinuesa A, Timón M, Aguas D, Basora T, Fernández-Cao JC.

Title: Community physical activity intervention, body pain and self-esteem.

Format: Oral communication.

Publication: ISSN: 2339-9333.

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3- SCHOLARSHIPS AND GRANTS

UNIVERSITAT ROVIRA I VIRGILI
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Scholarships and Grants

Institution: Ministry of Education, Culture and Sport, Government of Spain.

Scholarchip/Grant: FPU MOBILITY AID.

Date: 28 of December 2014

Period: From 1 February 2015 to 03 May 2015.

Institution: Ministry of Education, Culture and Sport, Government of Spain.

Scholarchip/Grant: Grant of the Formation of the University Staff (FPU Grants).

Date: 28 of December 2012

Period: From 1 March 2013 to 31 August 2016.

Institution: Rovira i Virgili University and Foundation Caixa Tarragona.

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