

# **The susceptibility to metabolic and proliferative disease**

**- from genetic predisposition to  
treatment**

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The susceptibility to metabolic and proliferative disease  
- from genetic predisposition to treatment

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# ABSTRACT

Obesity and type 2 diabetes increase the risk of cardiovascular disease. Insulin resistance is highly correlated to type 2 diabetes and both obesity and insulin resistance are risk factors for cancer. Bariatric surgery is an effective strategy to reduce cardiovascular and cancer risk.

In Paper I we tested if bariatric surgery prevents the incidence of cardiovascular events in 607 diabetic participants of the Swedish Obese Subjects (SOS) study. In a long-term follow-up, the incidence of myocardial infarction was lower in subjects who underwent bariatric surgery than in those treated with conventional therapies for obesity. No effect of the surgical treatment was observed on stroke prevention. Paper I shows that bariatric surgery is an effective strategy to prevent myocardial infarction in obese subjects with type 2 diabetes.

In Paper II we aimed to test if carriers of the Insulin receptor substrate 1 (IRS1) rs2943641 T allele, which is associated with lower insulin resistance, have lower cancer incidence. We showed that in morbidly obese subjects from the SOS study cancer incidence was lower in carriers of the *IRS1* T allele than in wild-type homozygotes. The cancer incidence was similar across the *IRS1* genotypes in a population-based cohort study, the Malmö Diet and Cancer (MDC) study. However, cancer incidence was slightly lower in carriers of the *IRS1* T allele than in *IRS1* wild-type homozygotes if only morbidly obese subjects were analysed. A meta-analysis of morbidly obese subjects from those two cohorts confirmed the association of *IRS1* T allele with lower cancer incidence.

Familiar hypercholesterolemia (FH) is a severe form of monogenic hypercholesterolemia associated with increased cardiovascular risk. Both clinical criteria and genetic tests allow performing a diagnosis of FH. Paper III aimed at performing a diagnosis of FH by combining an accurate selection of at-risk individuals through the Dutch Lipid Clinic Network criteria with next-generation sequencing (NGS). We recruited 77 individuals fulfilling clinical criteria for FH. NGS of four genes involved in FH was performed. We detected

26 mutations in 50 subjects (65% success rate). Moreover, we identified a previously unreported splicing-site mutation that seems to be causative of FH.

**Keywords:** genetics, insulin resistance, cancer, cardiovascular disease, familial hypercholesterolemia.

## SAMMANFATTNING PÅ SVENSKA

Fetma och typ 2 -diabetes ökar risken att drabbas av hjärt-kärlsjukdom. Insulinresistens är en del i utvecklingen av typ 2 -diabetes och det har visat sig att både fetma och insulinresistens är riskfaktorer för att drabbas av cancer. Magsäckskirurgi är en effektiv metod för viktnedgång men det har även visat sig att det kan minska risken för hjärtsjukdom och cancer.

I delarbete I studerades huruvida magsäckskirurgi förhindrade hjärt-kärlsjukdom i en studiepopulation bestående av 607 diabetiker från Swedish Obese Subjects (SOS) studien där deltagarna följts under en lång tid. Resultaten visade att förekomsten av hjärtinfarkt var lägre hos patienter som genomgick kirurgi än hos dem som behandlades med konventionell behandling av fetma. Däremot visade studien inte någon effekt av magsäckskirurgi på risken att drabbas av stroke. Sammantaget visar delarbete I att magsäckskirurgi är en effektiv behandling för att förebygga hjärtinfarkt hos patienter med typ 2 -diabetes. Vanligt förekommande genetiska varianter av insulin receptor substrat 1-genen (IRS1) är kopplat till lägre insulinresistens. I delarbete II testades hypotesen att bärare av Insulin receptor substrat 1 (IRS1) rs2943641 T-allel har lägre förekomst av cancer. Resultat från en utvald grupp individer med grav fetma i SOS studien visar att bärare av T-allelen i IRS1 genen har lägre risk att drabbas av cancer. När man studerade denna koppling mellan bärare av T-allelen och förekomsten av cancer i Malmö Diet and Cancer studien kunde man inte påvisa denna koppling när individer med olika BMI analyserade. Om man däremot analyserade enbart individer med grav fetma så var förekomsten av cancer lägre hos de individer som hade T-allelen i IRS1 genen. En metaanalys där individer med grav fetma från både SOS-studien och Malmö Diet and Cancer studien ingick kunde också påvisa ett samband mellan T-allelbärarskap och lägre förekomst av cancer.

Familjär hyperkolesterolemi (FH) är en ärftlig form av grav blodfettsubstansnivå som är starkt associerad med ökad risk för hjärt-kärlsjukdom. För att ställa diagnosen familjär hyperkolesterolemi kan man använda kliniska kriterier samt

genetiska test. Delarbete III syftade till att diagnosticera FH genom att identifiera ett urval av individer där misstanke om FH fanns igenom att använda Dutch Lipid Clinic Network kriterier och därefter utföra sekvensering av utvalda gener med next generation sequencing (NGS). I denna studie identifierades 77 individer som uppfyllde de kliniska kriterierna för FH. Därefter genomfördes sekvensering av fyra gener som är involverade i FH. Sekvenseringen kunde påvisa 26 st olika mutationer hos 50 patienter (65% framgång success rate). Dessutom kunde vi i denna studie identifiera en tidigare icke känd mutation som kan orsaka FH.

**Nyckelord:** genetik, insulinresistens, cancer, hjärt-och kärlsjukdomar, familjär hyperkolesterolemi .



# LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals (I-III):

- I. Romeo S, Maglio C, Burza MA, Pirazzi C, Sjöholm K, Jacobson P, Svensson PA, Peltonen M, Sjöström L, and Carlsson LM.

**Cardiovascular events after bariatric surgery in obese subjects with type 2 diabetes**

*Diabetes Care* 2012; 35(12): 2613-7.

- II. Maglio C, Ericson U, Burza MA, Mancina RM, Pirazzi C, Assarsson JA, Sjöholm K, Baroni MG, Svensson PA, Montalcini T, Pujia A, Sjöström L, Wiklund O, Carlsson LM, Borén J, Orho-Melander M, and Romeo S.

**The IRS1 rs2943641 variant and risk of future cancer among morbidly obese individuals**

*Journal of Clinical Endocrinology and Metabolism* 2013; 98(4):E785-9

- III. Maglio C, Mancina RM, Motta BM, Stef M, Pirazzi C, Palacios L, Askaryar N, Borén J, Wiklund O, and Romeo S.

**Genetic diagnosis of familial hypercholesterolemia by targeted next generation sequencing**

*In manuscript*

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# ABBREVIATIONS

|           |   |
|-----------|---|
| A         | Adenine   |
| APOB      | Apolipoprotein B  |
| BMI       | Body Mass Index   |
| C         | Cytosine  |
| CI        | Confidence Interval   |
| DNA       | Deoxyribonucleic Acid                                       |
| ERK       | Extracellular Signal-Regulated Kinase                       |
| FH        | Familial Hypercholesterolemia                               |
| G         | Guanine   |
| GWAS      | Genome-Wide Association Study                               |
| HDL-C     | High-Density Lipoprotein Cholesterol                        |
| HOMA-IR   | Homeostasis Model Assessment for Insulin Resistance         |
| HR        | Hazard Ratio  |
| IGF1      | Insulin-Like Growth Factor-1                                |
| IRS1      | Insulin Receptor-Substrate 1                                |
| LDL-C     | Low-Density Lipoprotein Cholesterol                         |
| LDLR      | LDL-Receptor  |
| LDLRAP1   | LDLR Adapter Protein 1                                      |
| MALDI-TOF | Matrix-Assisted Laser Desorption/Ionization Time Of- Flight |
| MAP       | Mitogen-Activated Protein                                   |
| MDC       | Malmö Diet and Cancer                                       |
| NGS       | Next-Generation Sequencing                                  |
| NNT       | Number Needed To Treat                                      |
| PCSK9     | Proprotein Convertase Subtilisin/Kexin Type 9               |
| PI-3K     | Phosphatidylinositol 3 Kinase                               |
| RNA       | Ribonucleic Acid  |
| SNP       | Single-Nucleotide Polymorphisms                             |
| SOS       | Swedish Obese Subjects                                      |
| T         | Thymine   |

# 1 INTRODUCTION

## 1.1 Obesity

### 1.1.1 Definition and classification

Obesity is a pathological condition characterized by an excessive accumulation of fat mass into the body which presents a risk to health<sup>1,2</sup>. Since the accumulation of fat mass is not of easy assessment, the most common way to measure obesity in adults is body mass index (BMI). BMI is calculated as weight in kilograms divided by the square of the height in meters<sup>1</sup>. Although BMI does not directly measure the fat mass nor discriminate between lean and non-lean tissue, it correlates with percentage of body fat in large population studies<sup>1,3</sup>. According to the World Health Organization, men and women with BMI  $\geq 30$  kg/m<sup>2</sup> are considered obese<sup>4</sup>. Specifically, BMI 30-35 kg/m<sup>2</sup> identifies class I obesity, BMI 35-40 kg/m<sup>2</sup> class II obesity, and BMI  $\geq 40$  kg/m<sup>2</sup> class III obesity<sup>4</sup>. These criteria for obesity classification represent only imposed cut off values that approximate a continuum between health and increased risk associated with BMI.

### 1.1.2 Epidemiology

Obesity is a burden whose prevalence is dramatically increasing worldwide. Since 1980 mean BMI increased of 0.4 kg/m<sup>2</sup> per decade for men and 0.5 kg/m<sup>2</sup> for women<sup>5</sup>. Nowadays approximately one third of the population of the United States of America is obese<sup>6</sup> and the consequences of obesity are responsible for or contribute to about 300,000 deaths per year<sup>7</sup>.

### 1.1.3 Obesity comorbidities

Obesity is responsible for higher risk of developing several conditions that impair quality of life, leading to increased morbidity and mortality<sup>8</sup>. According to the International Diabetes Federation, obesity *per se*, or an increased abdominal obesity as measured by waist circumference, is one of the criteria for the diagnosis of the metabolic syndrome<sup>9</sup>. The other characteristics of the metabolic syndrome are insulin resistance, impaired glucose intolerance/type 2

diabetes, hypertension, and dyslipidemia with hypertriglyceridemia and low serum high-density lipoprotein cholesterol (HDL-C). The increase in the prevalence of obesity explains the higher prevalence of type 2 diabetes that occurred worldwide in the last years as the risk of diabetes increases linearly with the increase of BMI<sup>10</sup>. Obesity is also associated with increased risk of developing dyslipidemia, including hypertriglyceridemia, reduced serum HDL-C levels, and increased low-density lipoprotein cholesterol (LDL-C) levels<sup>11</sup>. Moreover, a linear association relates obesity to hypertension<sup>11</sup>. Obesity is therefore a risk factor for several conditions associated with cardiovascular disease. However also after adjusting for several risk factors, obesity associates with increased cardiovascular disease incidence and it is considered as an independent cardiovascular risk factor<sup>12,13</sup>. Obesity increases also the risk of developing cancer<sup>8</sup>. It has been reported that overweight and obesity are responsible for about 14% of cancer deaths in men and 20% in women<sup>14</sup>.

#### **1.1.4 Treatment options**

In obese subjects intentional weight loss is associated with an improvement in obesity-related conditions, including type 2 diabetes, dyslipidemia and hypertension<sup>15,16</sup>. Short-term intentional weight loss can be easily achieved through changes in lifestyle; however a successful long-term weight loss is more complicated to be achieved<sup>17,18</sup>.

Lifestyle modifications consist of several approaches to achieve weight-loss including dietary interventions and increased physical activity<sup>18</sup>. Weight-loss diets usually involve modifications not only in the energy content (hypocaloric diets) but also in macro and micro nutrients composition. Physical activity is a milestone in the treatment of obesity. Physical activity aims at increasing the energy expenditure thus making negative the energy balance<sup>19</sup>. Unfortunately, conventional obesity treatment through diet and physical activity is associated with a high rate of recidivism<sup>18</sup>.

Bariatric surgery involves several surgical procedures whose aim is to achieve weight loss in severely obese subjects<sup>20</sup>. According to National Institute of Health guidelines, bariatric surgery is recommended for individuals with BMI  $\geq$

40 kg/m<sup>2</sup> or for those with a BMI  $\geq$  35 kg/m<sup>2</sup> in presence of significant obesity comorbidities<sup>21</sup>. The American Diabetes Association recommends bariatric surgery in adults with type 2 diabetes and a BMI  $\geq$  35 kg/m<sup>2</sup>, particularly for those whose diabetes is difficult to control with lifestyle modifications and pharmacological therapy <sup>22</sup>.

Bariatric surgery procedures are classified as restrictive or predominantly malabsorptive according to their effect on the gastro-intestinal system<sup>20,23</sup>. Restrictive procedures aim to reduce the capacity of the stomach<sup>23,24</sup>; typical examples of restrictive procedures are gastric banding, vertical gastropasty and sleeve gastrectomy. Predominantly malabsorptive procedures are effective mainly by inducing malabsorption<sup>23,24</sup>. Many malabsorptive procedures are now abandoned because of serious side effects; a technique which is still used is biliopancreatic diversion. An example of mixed procedure, applying both techniques simultaneously (restriction and malabsorption), is the gastric bypass.

Bariatric surgery is associated with a decrease in morbidity in obese subjects. It has been shown that bariatric procedures improve obesity complications and prevent type 2 diabetes, cardiovascular disease and cancer in subjects with severe obesity<sup>25-30</sup>. In a long-term follow up they are also associated with a decrease in mortality<sup>31</sup>.

## 1.2 Insulin resistance

### 1.2.1 Insulin and signalling

Insulin is a peptide hormone secreted by the  $\beta$ -cells in the Islets of Langerhans of the endocrine pancreas<sup>32,33</sup>. Insulin is essential to maintain glucose and lipid homeostasis in the body and is released by the pancreatic cells when glucose enters the  $\beta$ -cells by a process of facilitated diffusion<sup>34-36</sup>. Once secreted by the  $\beta$ -cells insulin mediates glucose uptake in the tissues by binding the insulin receptor on the cell surface<sup>37-39</sup>. The binding and activation of the insulin receptor initiates a cascade of phosphorylation that results in the activation of different metabolic and mitogenic pathways in several tissues (Figure 1)<sup>40</sup>. The first key event in the insulin signalling is the activation by phosphorylation of the insulin receptor-substrate 1 (IRS1)<sup>41-43</sup>. Among the several pathways activated, insulin stimulates the translocation of the glucose transporter type 4 onto the cell membrane and the increase of the glucose uptake in different tissues. Insulin increases also glycogen, protein, and lipid synthesis while inhibiting gluconeogenesis, proteolysis, and lipolysis. It stimulates cell growth and proliferation<sup>44</sup>.

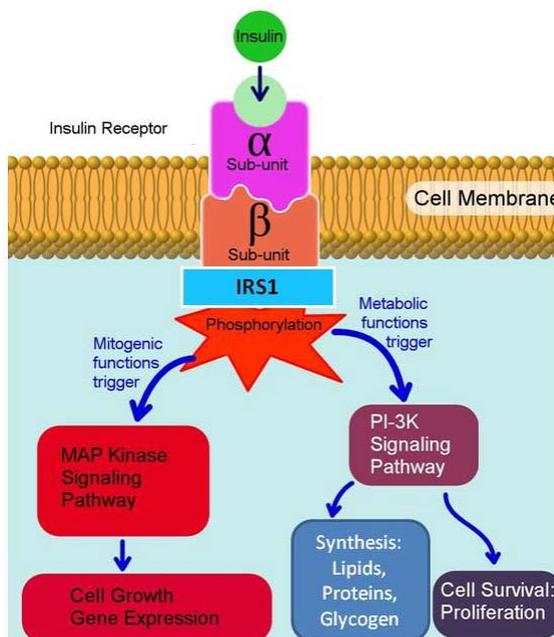


Figure 1. Insulin signalling. Adapted by James Foreman's figure licensed under CC BY-SA 3.0 (original:

[http://en.wikipedia.org/wiki/File:Bioe\\_Article\\_Pic.svg#filelinks](http://en.wikipedia.org/wiki/File:Bioe_Article_Pic.svg#filelinks)).

Abbreviations: MAP, mitogen-activated protein; PI-3K; phosphatidylinositol 3-kinase

## 1.2.2 Insulin resistance

Insulin resistance is a pathological condition characterized by an impaired sensitivity of the body tissues to the action of insulin<sup>45-47</sup>. Subjects with insulin resistance have a decreased transport of glucose into the cells and show lower ability of insulin to inhibit lipolysis in the adipocytes<sup>48</sup>. In the liver, impaired insulin action is responsible for a lower suppression of gluconeogenesis and for stimulation of free fatty acid production<sup>49,50</sup>. This results in hyperglycaemia and hypertriglyceridemia<sup>48</sup>.

The aetiology of insulin resistance is not completely clear and involves many factors including down-regulation of insulin receptor and of post-receptor pathways<sup>51-53</sup> and compensative insulin hypersecretion by pancreatic  $\beta$ -cells<sup>54</sup> which worsens the signalling desensitization. Among the environmental factors influencing insulin resistance, obesity is the most well-known<sup>45</sup>. Genetics plays an important role in the development of insulin resistance as shown by the fact that first-degree relatives of subjects with type 2 diabetes show signs of insulin resistance even when they are not diabetic or obese<sup>55</sup>.

The hyperinsulinemic euglycaemic clamp is considered the gold-standard technique to measure insulin resistance in adults<sup>56</sup>. However this technique is demanding, time consuming and not feasible in large study cohorts. The homeostasis model assessment for insulin resistance (HOMA-IR) index is widely used to quantify insulin resistance in large cohorts<sup>57</sup>. HOMA-IR is an index calculated from fasting glucose and insulin according to the following equation:  $[\text{glucose (mmol/L)} * \text{insulin (mIU/L)} / 22.5]$ . HOMA-IR is highly correlated with the hyperinsulinemic euglycaemic clamp and it has also been validated in obese individuals<sup>58-61</sup>. Despite not directly measuring insulin resistance, HOMA-IR is a well-accepted surrogate index to assess this trait in large epidemiological genetic studies.

## 1.2.3 Comorbidities: type 2 diabetes and cancer

Insulin resistance plays a pivotal role in the development of type 2 diabetes<sup>46,52</sup>. Subjects with type 2 diabetes consistently show signs of insulin resistance,

although manifest diabetes is rarely seen in insulin-resistant individuals without pancreatic  $\beta$ -cells dysfunction. Type 2 diabetes develops in genetically predisposed individuals having environmental risk factors<sup>62</sup>. Among risk factors for type 2 diabetes there is obesity and insulin resistance *per se*, but also age, gender and the ethnic background<sup>63-65</sup>. In the last decades type 2 diabetes has become one of the most important public health problems whose incidence is increasing worldwide<sup>66</sup>.

The criteria for the diagnosis of type 2 diabetes are fasting plasma glucose levels  $\geq 7$  mmol/L or plasma glucose levels two hours after oral glucose tolerance test  $\geq 11.1$  mmol/L. Glycated haemoglobin  $\geq 6.5\%$  is also sufficient to perform type 2 diabetes diagnosis<sup>22</sup>.

Insulin resistance is known to increase the risk of malignancy development<sup>67,68</sup>. Insulin binds and activates the insulin receptor and the Insulin-like Growth Factor-1 (IGF1) receptor thus mediating both metabolic and mitogenic effects that stimulates cancer initiation and development<sup>38,39,41,69-75</sup>. Insulin stimulation of the insulin receptor induces transformation in normal breast cells<sup>76</sup> while hyperinsulinemia has been associated with increased risk for breast cancer in women without type 2 diabetes<sup>77,78</sup>. It has been shown that interventions that aim to reduce body weight and insulin resistance are associated with a decrease in the risk of cancer development<sup>28,79,80</sup>.

## 1.3 Human Genetics

Genetics is a branch of biology that studies heredity and variation in living organisms<sup>81</sup>. Inheritance is due to the transmission from generation to generation of heritable units called gene. Genes are constituted of deoxyribonucleic acid (DNA) and carry the information required to create a protein. According to the central dogma of molecular biology, the flow of information in cells goes from DNA, via ribonucleic acid (RNA) to protein (also known as “DNA makes RNA makes protein”)<sup>82</sup>.

From a molecular point of view, DNA is a chain of four nucleotides: adenine (A), cytosine (C), guanine (G), and thymine (T). In the DNA’s double strand helix each nucleotide pairs with its partner nucleotide in the opposite strand: A with T and C with G<sup>83</sup>. In human cells DNA is packed into 22 autosomal and two sexual chromosomes<sup>81</sup>.

In 2003 the Human Genome Project was declared complete and the sequence of the human DNA was published in 2004<sup>84</sup>. The project concluded that human genome contains approximately 3 billion base pairs organized into 20,500 protein coding genes. Between unrelated healthy individuals DNA differs by only about 0.2% or 1 in 500 bases<sup>85</sup>. Genetic variations contribute to normal phenotypic diversity in humans and have been implicated in the development of several diseases<sup>86</sup>. A mutation is any change in the DNA sequence that deviates from normality with a frequency of less than 1% in the population. Conversely, polymorphisms are common sequence variations with a frequency higher than 1%. Single-nucleotide genetic polymorphisms (SNPs) occur after a single nucleotide change and represent a major source of genetic variation. SNPs are widespread in the human genome and are known to modulate the susceptibility to many common and rare diseases<sup>87-89</sup>. A nonsynonymous SNP is a polymorphism that results into an aminoacidic change in the protein.

### 1.3.1 Single- gene association studies

Genetic association studies are performed to assess the association of a specific genotype with a phenotype of interest<sup>90</sup>. The phenotype may be a disease (e.g. diabetes) or a quantitative trait (e.g. HOMA-IR or serum LDL-C levels). Using a

candidate gene approach, the SNPs to be tested are chosen based on a *a priori* knowledge of the gene or of the SNP, such as previous reports on the gene function or previous genetic associations<sup>86</sup>. The easiest way to perform a genetic association study is genotyping the variant of interest in the population of choice. Genotyping techniques aim to determine which genetic variant one individual has. Genotyping may be performed using different biological assays, including matrix-assisted laser desorption/ionization time of- flight (MALDI-TOF) analysis<sup>91</sup>.

### **1.3.2 Genome- wide association studies**

A genome-wide association study (GWAS) is a hypothesis-free approach where genetic variations are tested against a trait (e.g., HOMA-IR) or a condition (e.g., diabetes vs. healthy control)<sup>86,92</sup>. In contrast to genetic association studies, GWAS investigates several variations at the same time. From the DNA of each study participants hundreds of thousands of SNPs are simultaneously read using SNP arrays. If a specific SNP is more frequent in the group with the disease (or in subjects in whom the quantitative trait is higher; e.g. high HOMA-IR), the SNP is considered to be associated with the specific disease/trait. Once the association is described further genetic association studies in different populations are needed to confirm the finding, as well as *in vitro* study to assess the molecular mechanism behind the association.

### **1.3.3 DNA resequencing studies**

DNA sequencing consists of the determination of the nucleotide order in a DNA sequence<sup>93</sup>. The first sequencing method was developed by Frederick Sanger in 1977 and it is based on the selective incorporation of chain-terminating dideoxynucleotides by a polymerase during a process of DNA replication<sup>93</sup>. This procedure creates DNA fragments of different lengths labeled with a fluorescent molecule that is specific for each dideoxynucleotide terminating the chain. The fragments are then separated by size through capillary electrophoresis. The average read length for Sanger sequencing is around 500-800 bases.

Next-generation sequencing (NGS) uses micro- and nano- technologies that diminish the size of samples components thus reducing reagents costs and allowing sequencing reactions to run in parallel<sup>94</sup>. Pyrosequencing is a NGS technique based on the detection of a pyrophosphate released when a deoxyribonucleotide triphosphate is added to the end of a new DNA strand<sup>95,96</sup>. The light emitted during the incorporation allows determining the DNA sequence since only one out four nucleotides (A, C, G, or T) is added at a time<sup>96</sup>. For pyrosequencing the average read length is between 100 to 400 bases<sup>97</sup>.

Exome sequencing consists of the sequencing of all protein-coding sequences of a genome (i.e., the exome) and it is now possible thanks to NGS techniques<sup>98,99</sup>. Exome sequencing is a very efficient technology to extensively analyze the common and rare genetic defects in the coding regions of the genome. However, the amount of information provided by exome sequencing is huge and difficult to handle. When genes involved in a disease or a phenotype are known, targeted sequencing of such genes may be an efficient solution to look for variations in an efficient, time-saving and economic way<sup>100</sup>.

## 1.4 The *IRS1* rs2943641 variant

IRS1 is a cytosolic protein which is the main substrate of the insulin and IGF1 receptors<sup>41,101</sup>. Once activated IRS1 is phosphorylated and triggers several intracellular pathways. IRS1 plays a key role in mediating both metabolic and mitogenic pathways activated by insulin and IGF1<sup>70</sup>.

Genetic variants near or in the *IRS1* gene have been previously related to insulin resistance and type 2 diabetes<sup>102,103</sup>. A nonsynonymous SNP in the *IRS1* gene (G972R, rs1801278) has been associated not only with insulin resistance<sup>102-106</sup> but also with cardiovascular disease<sup>107-109</sup> and cancer<sup>74,110,111</sup>.

A GWAS has identified a SNP (rs2943641) near the *IRS1* gene that is associated with insulin resistance and type 2 diabetes<sup>112</sup>. The *IRS1* rs2943641 T allele

carriers show a reduced insulin resistance<sup>112-114</sup> and lower type 2 diabetes prevalence compared to C allele carriers<sup>112,114-116</sup>. Moreover, the *IRS1* T allele is also associated with higher serum HDL-C levels and lower serum triglyceride levels in subjects with type 2 diabetes<sup>117</sup>.

## 1.5 LDL- C and cardiovascular disease

Lipoproteins transport lipid in the bloodstream. LDL particles are the main cholesterol-carrier lipoproteins in the plasma; they are composed of lipids (75%) and proteins (25%). The main lipid component is cholesterol and APOB-100 is the principal protein in the LDL<sup>118-120</sup>. LDL particles bind the LDL-receptor (LDLR) on the cell surface and are taken up by hepatocytes (75%) and other peripheral tissues<sup>121</sup>.

An increase in serum LDL-C is a major risk factor for atherosclerosis<sup>122</sup>. Atherosclerosis is a process characterized by a thickening of the arterial wall due to lipid accumulation<sup>123</sup>. Specifically, atherosclerosis starts developing when LDL particles pass by and are retained behind the cellular monolayer of the endothelium<sup>123,124</sup>. Once inside the arterial wall, the LDL particles binds the proteoglycans<sup>125</sup> and are prone to be oxidized<sup>126</sup>. Oxidized LDL particles recruit monocytes from the bloodstream into the sub endothelium space. Monocytes penetrate the intima and differentiate into macrophages that absorb the oxidized LDL forming foam cells. Foam cells tend to accumulate inside the arterial wall thus leading to an atheromatous plaque<sup>127,128</sup>. The rupture of an atheromatous plaque may cause an acute cardiovascular event.

Cardiovascular disease, including both myocardial infarction and stroke, is the leading cause of mortality worldwide<sup>129,130</sup>. Cardiovascular risk factors that are immutable include age, male gender, and family history. Among the modifiable risk factors for myocardial infarction and stroke there are smoking, high blood pressure and hypercholesterolemia. Increased serum LDL-C levels are associated with higher risk of developing cardiovascular disease independently of other main risk factors. The third report of the National Cholesterol

Education Program expert panel recommends having serum LDL-C levels lower than 2.6 mmol/L in adults (lower than 1.8 in those with advanced cardiovascular disease)<sup>131</sup>.

## 1.6 Familial hypercholesterolemia

Familial Hypercholesterolemia (FH) is a genetic disorder of LDL metabolism<sup>132,133</sup>. It is characterized by high serum levels of LDL-C and early atherosclerosis development. Subjects with FH represent a population of individuals at risk for premature coronary artery disease as a consequence of the long time exposure to high serum levels of LDL-C<sup>134</sup>. About half of men and one third of women with untreated FH develop coronary disease by the age of 60 years<sup>135</sup>. In heterozygous FH subjects serum LDL-C levels are usually two-three-fold higher while serum triglycerides are within the normal range<sup>136</sup>. Typical physical signs of FH are tendon xanthomas<sup>137</sup> and premature arcus corneae<sup>138</sup>. Homozygous FH is much rarer than the heterozygous form and it is characterized by a more serious phenotype with incidence of cardiovascular disease during childhood/adolescence<sup>139</sup>.

The commonly reported prevalence is 1/500 for heterozygous FH<sup>136</sup>; however, a recent study on a Danish population showed a prevalence of approximately 1/137 for FH diagnosed as definitive or probable according to the Dutch Lipid Clinic Network criteria<sup>134</sup>. The most common causes of FH are mutations in the *LDLR* gene, which is responsible for the cellular uptake of the LDL particle<sup>140</sup>. In Sweden, more than 30 mutations in the *LDLR* gene have been identified, including nonsense, missense and splice site mutations, along with 4 gene rearrangements<sup>141</sup>. Mutations in the apolipoprotein B (APOB)<sup>142</sup> gene, which encodes for the ligand of the LDLR<sup>143</sup>, and proprotein convertase subtilisin/kexin type 9 (PCSK9)<sup>144</sup>, which is involved in the LDLR degradation, have been also described. Mutations in the LDLR adaptor protein 1 (*LDLRAP1*) gene, which encodes for the low density lipoprotein receptor adaptor protein 1, cause a recessive form of FH<sup>145</sup>.

It has been estimated that in most countries only about 1% of the individuals suffering from FH receive a proper diagnosis<sup>146</sup>. Subjects with FH can be identified using clinical criteria<sup>134,146</sup> or genetic testing<sup>147,148</sup>. There are several diagnostic algorithms for a clinical diagnosis of FH and one of the most used is the Dutch Lipid Clinic Network score (Table 1)<sup>134</sup>.

**Table 1 Dutch Lipid Clinic Network criteria for FH diagnosis**

|                      | Criteria  | Score     |
|----------------------|---|-----------|
| Family history       | First degree relative known with premature CAD* and/or<br>First degree relative with LDL-C >95 <sup>th</sup> percentile | 1         |
|                      | First degree relative with Tx and/or<br>Children <18 with LDL-C >95 <sup>th</sup> percentile                            | 2         |
| Clinical history     | Patient has premature CAD*  | 2         |
|                      | Patient has premature cerebral/peripheral vascular disease  | 1         |
| Physical examination | Tendon xanthomas  | 6         |
|                      | Arcus corneae below the age of 45   | 4         |
| LDL-cholesterol      | >8.5 mmol/l (>330mg/dl)   | 8         |
|                      | 6.5-8.4 mmol/l (250-329 mg/dl)  | 5         |
|                      | 5.0-6.4 mmol/l (190-249 mg/dl)  | 3         |
|                      | 4.0-4.9 mmol/l (155-189 mg/dl)  | 1         |
|                      | Definite FH   | Score > 8 |
|                      | Probable FH   | Score 6-8 |
|                      | Possible FH   | Score 3-5 |
|                      | No diagnosis  | Score < 3 |

\* Premature CAD or CVD: men before 55, women before 60 years of age

Abbreviations: FH, familial hypercholesterolemia; CAD, coronary artery disease; LDL-C, low density lipoprotein cholesterol; Tx, tendon xanthomata; CVD, cardiovascular disease.

Dutch Lipid Clinic Network score include information about family history, as premature cardiovascular disease or hyperlipidemia, personal clinical history, and data on lipid levels and presence of tendon xanthomas or arcus corneae (Table 1). Genetic testing and identification of the pathogenic mutation involved in FH can also be performed. FH genetic techniques usually involve assay systems designed to detect specific high-frequency mutations. Another strategy is a combined genetic approach that consists of targeted Sanger sequencing, then detection of deletions/duplications by multiplex ligation-dependent probe amplification of the *LDLR* gene and finally targeted testing of specific

mutations in the *APOB* and *PCSK9* genes<sup>147</sup>. Recently, the use of targeted NGS techniques has been validated to perform FH diagnosis<sup>99,149-151</sup>.

Once identified, subjects with heterozygous FH should undergo intensive education about lifestyle management and treatment with lipid-lowering medications such as statins. In subjects with FH an adequate drug treatment with lipid-lowering medications prevents the onset of cardiovascular events as long as it is started ahead of time<sup>152</sup>. Indeed if early and adequately treated individuals affected by FH will have a life expectancy comparable to that of the overall population<sup>153</sup>. Unfortunately, most of the individuals suffering from FH starts a lipid-lowering therapy only after a proper diagnosis is performed<sup>154</sup>. Therefore the identification of individuals with FH is crucial to exert an effective prevention strategy for coronary artery disease.

## 2 AIMS

My PhD project initially focused on the effect of weight loss due to bariatric surgery on obesity-related comorbidities. I then become interested in human genetics and studied how common genetic variants influence the susceptibility to obesity-related comorbidities. Finally to explore other facets of human genetics I examined rare forms of monogenic diseases. Therefore the specific aims of this PhD project are:

- **Paper I.** To examine the impact of weight loss due to bariatric surgery on cardiovascular prevention in subjects with type 2 diabetes.
- **Paper II.** To investigate whether the insulin *IRS1* rs2943641 variant reduces cancer risk in obese individuals.
- **Paper III.** To combine clinical criteria and next generation sequencing to achieve genetic diagnosis of familiar hypercholesterolemia.

## 3 SUBJECTS AND METHODS

### 3.1 Study subjects

#### 3.1.1 The Swedish Obese Subjects (SOS) study

The Swedish Obese Subjects (SOS) study is a controlled, matched, longitudinal, interventional trial on the effects of bariatric surgery vs. conventional care in obese individuals<sup>31,155</sup>. Briefly, 4,047 subjects from September 1<sup>st</sup> 1987 to January 31<sup>st</sup> 2001 were recruited in Sweden. Inclusion criteria were age between 37 and 60 years and BMI  $\geq 34$  kg/m<sup>2</sup> in men and  $\geq 38$  kg/m<sup>2</sup> in women. Exclusion criteria include among others earlier bariatric surgery, on-going malignancy or active malignancy during the past five years, and myocardial infarction during the past six months<sup>155</sup>.

A total of 2,010 subjects who electively chose bariatric surgery constituted the surgery group, while 2,037 individuals were included in the control group. The study was not randomized due to ethical issues but the two groups were matched based on 18 variables. In the surgery group, 376 subjects underwent nonadjustable or adjustable banding, 1,369 vertical banded gastroplasty, and 265 gastric bypass. Individuals from the control group received the conventional non-surgical obesity treatment at their centers of registration, ranging from intensive lifestyle modifications to virtually no treatment whatsoever.

Participants of the SOS study were examined at matching, at baseline and during follow-up and biochemical and anthropometric parameters were measured. Type 2 diabetes was defined as fasting blood glucose  $\geq 6.1$  mmol/L (corresponding to 7.0 mmol/L or 126 mg/dL) and/or self-reported therapy with glucose-lowering medications at baseline.

During follow-up, participants in the SOS surgery group underwent a massive and sustained weight loss while no changes in BMI were detected in the SOS control group<sup>27,31</sup>. Moreover, the SOS study showed that bariatric surgery associates with lower mortality and morbidity during follow-up including lower incidence of cardiovascular disease, cancer and type 2 diabetes<sup>26-29,31,156</sup>.

Paper I included all the participants of the SOS study with type 2 diabetes at baseline (N=607). Paper II included nondiabetic participants of the SOS study with DNA available in whom the *IRS1* rs2943641 variant was successfully genotyped (N=2,988).

### **3.1.2 The Malmö Diet and Cancer cohort**

The Malmö Diet and Cancer (MDC) study is a population-based prospective cohort study in the city of Malmö, Sweden. Baseline examinations were conducted between 1991 and 1996 and all women born 1923-1950 and all men born 1923-1945, living in the city of Malmö, were invited to participate<sup>157,158</sup>. During the screening period, 28,098 participants completed all baseline examinations. The participants filled out questionnaires covering socio-economic, lifestyle and dietary factors, registered meals, and underwent a diet history interview. Clinical and anthropometric parameters (e.g., blood pressure, waist circumference) were assessed; data on glucose and insulin levels were available only in ~20% of the overall population. Prevalent diabetes diagnosis was based on self-reported diabetes diagnosis, self-reported diabetes medications or register information indicating a date of diagnosis preceding baseline examination date.

Paper II includes 23,306 non diabetic participants from the MDC cohort in whom the rs2943641 *IRS1* variant was successfully genotyped. Individuals with cancer diagnosis at baseline were not included.

### **3.1.3 The FH cohort**

Paper III included 77 adults from the Lipid Clinic at the Sahlgrenska Hospital, Västra Götaland region, Gothenburg, Sweden who were recruited over the period 2012-2013. Subjects were included if they had a Dutch Lipid Clinic Network score  $\geq 3$ , defining possible FH, probable FH or definite FH. The data available included information about family and personal history, drug therapy and habits. BMI and blood pressure were measured. Dutch Lipid Clinic Network score has been calculated as previously described (Table 1)<sup>146</sup>.

### 3.1.4 Ethical considerations

The SOS study was approved by seven Swedish Regional Institutional Review Boards (Paper I and II)<sup>31</sup>. All subjects gave their consent to participate the study. The trial has been registered in the ClinicalTrials.gov registry (NCT01479452). The ethical committee of Lund University approved the MDC study (LU 51-90) and all participants gave their informed consent (Paper II)<sup>159</sup>. The study on subjects with FH (Paper III) was approved by the regional Ethics Committee of Gothenburg (Number 145-12). All participants gave their informed consent to participate.

## 3.2 Methods

### 3.2.1 Statistical analyses

Statistical analyses in this thesis were carried out using the IBM Statistical Package for Social Sciences (IBM SPSS, version 18.0.0. or 19.0.0, Inc. Chicago, IL, USA). Two-sided P values  $<0.05$  were considered statistically significant. Intention-to-treat principle was applied in the analyses included in Paper I and II. Continuous variables across genotypes or between groups were compared by linear regression analysis or general linear model after adjustment for confounders. Categorical variables were compared by Chi-square or Fisher Exact test. Time of progression to end-points was evaluated by Kaplan-Meier estimates of cumulative incidence rates and survival distributions were compared using log-rank test. Cox proportional hazards models adjusted for baseline confounders were used to evaluate time to the outcome.

### 3.2.2 Genotyping

In Paper II, genotyping of the rs2943641 variant in *IRS1* was performed in the SOS and in the MDC cohorts. In the SOS study the *IRS1* variant was genotyped using MALDI-TOF analysis, performed on the MassARRAY Platform from Sequenom (Sequenom Inc., San Diego, California) at the Mutation Analysis core Facility of the Karolinska Institute. In the MDC cohort the *IRS1* variant was genotyped by TaqMan® (Applied Biosystems, Foster City, CA, USA).

### 3.2.3 Pyrosequencing

In the FH cohort DNA from the participants was sequenced by SEQPRO LIPO RS (Progenika Biopharma, Spain). SEQPRO LIPO RS is NGS kit conceived to detect mutations in the *LDLR*, *APOB*, *PCSK9* and *LDLRAP1* genes. It also analyses copy number variations in the *LDLR* gene. All the exons and exons-introns boundaries of the *LDLR*, *PCSK9* and *LDLRAP1* genes as well as exons 26 and 29 in the *APOB* gene (nucleotides 10416-10779 for exon 26 and nucleotides 12987-13221 for exon 29) were pyrosequenced (454 Life Science, Roche®). Targeted Sanger sequencing was used to detect mutations in family members of the probands.

### 3.2.4 Mutation effect prediction and species alignment

We performed *in silico* analyses to predict missense mutation effect. The following bioinformatic tools were used: Polymorphism Phenotyping version 2 (PolyPhen-2; <http://genetics.bwh.harvard.edu/pph2/>), Sorting Intolerant From Tolerant (SIFT; [http://sift.jcvi.org/www/SIFT\\_enst\\_submit.html](http://sift.jcvi.org/www/SIFT_enst_submit.html)), Mutation Taster and Consensus deleteriousness score of missense single-nucleotide variations (Condel; <http://bg.upf.edu/condel/home>). Multiple sequence alignment was performed using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

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## 4 RESULTS AND DISCUSSION

### 4.1 Bariatric surgery and myocardial infarction in diabetic subjects

My PhD project initially focused on the effect of weight loss after bariatric surgery on cardiovascular disease. In obese subjects bariatric surgery causes an improvement of cardiovascular risk factors, such as BMI, dyslipidaemia, hypertension and type 2 diabetes<sup>25-27,31,160</sup>. Recently, the SOS study showed that bariatric surgery is also associated with a reduction in cardiovascular events compared to conventional obesity treatment<sup>29</sup>. In a median 15 years follow-up, bariatric surgery reduced the number of fatal and nonfatal cardiovascular events; the surgical treatment was associated with a lower number of myocardial infarction and stroke events separately only after adjustment for confounders.

Obese subjects with type 2 diabetes are a population at risk for several conditions, including cardiovascular disease<sup>46,161-163</sup>. The American Diabetes Association recommends bariatric surgery for diabetic patients with BMI lower than 35 Kg/m<sup>2</sup><sup>22</sup>. However, despite such recommendations, few data are available on the effect of the surgical treatment on long-term type 2 diabetes comorbidities in obese subjects. To our knowledge no previous study investigated the role of bariatric surgery in terms of myocardial infarction and stroke prevention in obese diabetic subjects during a long-term follow-up.

We compared the incidence of cardiovascular events in 345 obese diabetic subjects who underwent bariatric surgery compared to 262 subjects with the same characteristics who received nonsurgical obesity treatments. The endpoints analysed were fatal and nonfatal cardiovascular events (myocardial infarction and stroke, whichever came first), as well as myocardial infarction and stroke separately.

In a median 13 years follow-up, the bariatric surgery group showed a lower incidence of cardiovascular events compared to the control group (log-rank P value = 0.010, Figure 2 A; adjusted Hazard Ratio, HR 0.53 [95% Confidence Interval, CI 0.35–0.79]; P value = 0.002). We also analysed the incidence of myocardial infarction and stroke separately. The incidence of myocardial infarction was lower in the surgery group than in the control group (log-rank P value = 0.017, Figure 2 B; adjusted HR 0.56 [95% CI 0.34–0.93; P value = 0.025) while no effect of the surgical treatment on stroke incidence was detected (log-rank P value = 0.852).

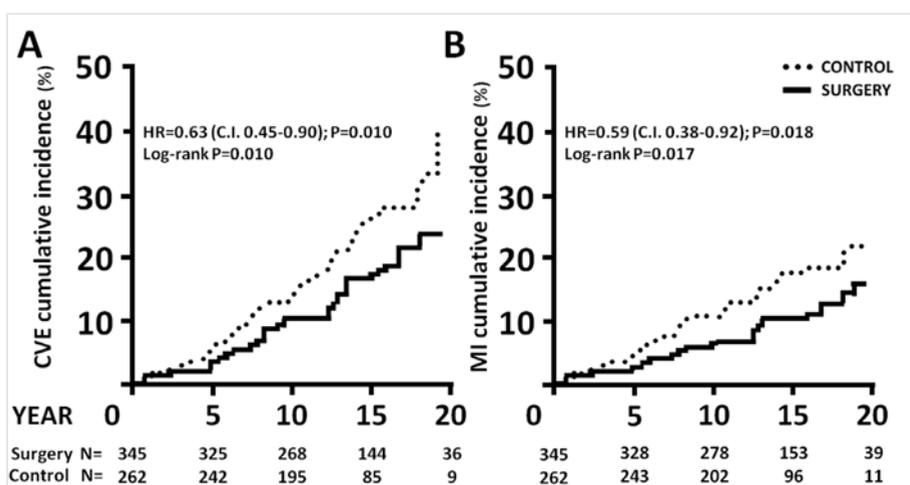


Figure 2. Cumulative incidence of cardiovascular events (A) and myocardial infarction (B) in SOS study participants with type 2 diabetes at baseline. Unadjusted hazard ratios (HR) and 95% confidence intervals (CI) are shown

Paper I shows how bariatric surgery is effective in preventive myocardial infarction in obese subjects with type 2 diabetes if compared to the conventional obesity treatment. This result is similar to what previously found in the entire SOS cohort, including both diabetic and non-diabetic subjects<sup>29</sup>. On the contrary, no effect of bariatric surgery on stroke prevention could be detected in obese diabetic subjects. Even if often referred to as “cardiovascular disease”, cerebral stroke and myocardial infarction are two distinct pathologies that share many but not all risk factors. For example, cerebral stroke recognizes as main risk factors carotid stenosis, valvular heart disease and atrial fibrillation<sup>164-166</sup>. The lack of association between bariatric surgery and stroke prevention in obese

diabetic subjects may reflect a distinct effect of bariatric surgery in modulating risk factors for myocardial infarction and cerebral stroke. The absence of an effect of bariatric surgery on stroke incidence may be also due to low statistical power, since in the SOS cohort subjects who had a stroke are fewer than the ones who had a myocardial infarction. It should be also underlined that the effect of bariatric surgery on stroke prevention in the overall SOS population is rather mild and becomes manifest only after adjustment for confounding factors<sup>29</sup>. This may suggest that the effect size is small and larger cohorts are needed to detect it.

We also performed a subgroup analysis to identify possible baseline conditions associated with a higher treatment benefit with respect to myocardial infarction. We stratified the cohort based on gender, previous myocardial infarction, smoking, and therapy with glucose-lowering medications and by the median of baseline age, BMI, weight, waist, insulin, total cholesterol, triglycerides, HDL-C, blood pressure, and diabetes duration. Subjects with higher baseline serum total cholesterol and triglycerides showed a greater relative benefit of bariatric surgery if compared to individuals with lower lipid levels. The benefit of bariatric surgery was not related to baseline BMI or other parameters.

Nowadays, eligibility to obesity surgical treatments is mainly based on BMI. The presence of comorbidities such as type 2 diabetes or hypertension is taken into account only in individuals with BMI  $\geq 35$  but  $< 40$  Kg/m<sup>2</sup><sup>21</sup>. Paper I showed that BMI is not a predictor of the effect of bariatric surgery on myocardial infarction in obese diabetic subjects. This means that obese subjects having a higher BMI do not have a greater benefit on myocardial infarction after undergoing bariatric surgery than subjects with a lower BMI. We also showed that higher serum lipid levels (total cholesterol and triglycerides) are associated with a significant higher efficacy of bariatric surgery in preventing myocardial infarction. This is consistent with what found in the overall SOS cohort, where it has been shown that baseline serum insulin levels rather than BMI predicts the effect of bariatric surgery on cardiovascular events<sup>29</sup>. In Paper I the population was not stratified according to baseline serum insulin levels, since insulin levels are not an accurate way to assess metabolic impairment in diabetic

subjects; moreover, individuals with type 2 diabetes are per definition insulin resistant. The results from the two studies may suggest that baseline metabolic parameters, such as insulin or lipid levels, are better predictors of the outcome of bariatric surgery on cardiovascular disease rather than BMI. Those results support recent reports suggesting that BMI should not be considered the only criterion to qualify obese subjects to bariatric surgery<sup>167-171</sup>. They may also suggest that subjects metabolically impaired should be prioritized.

We calculated the number needed to treat (NNT) to prevent one myocardial infarction. The study showed that 16 obese diabetic subjects need to be surgically treated to prevent one myocardial infarction. This NNT is extremely low and together with the previous findings suggest that bariatric surgery is highly effective in preventing myocardial infarction in obese diabetic subjects. This supports the guidelines of the American Diabetes Association on bariatric surgery for subjects with type 2 diabetes<sup>22</sup>. Currently American Diabetes Association guidelines underline the importance of long-term controlled studies to assess the actual benefit of the surgical procedures on individuals with type 2 diabetes.

In conclusion we showed that bariatric surgery reduces the incidence of myocardial infarction in obese subjects with type 2 diabetes and that metabolic parameters rather than BMI predicts this outcome. We also propose a model suggesting that subjects with metabolically impairment have a higher benefit after the surgical treatment and possibly should be prioritized for bariatric surgery. Prospective study are needed to test this model and its effectiveness.

## 4.2 *IRS1* rs2943641 variant and cancer

*IRS1* is a key component in the signalling of insulin and IGF1 and a mediator of both the metabolic and the mitogenic effects of the two ligands. Consistently, genetic variants in the *IRS1* locus have been previously associated with insulin resistance and cancer<sup>74,110-113,115,172</sup>.

In 2009 a GWAS identified a sequence variation (rs2943641, C to T) near the *IRS1* gene that is associated with lower insulin resistance and lower type 2 diabetes risk<sup>112</sup>. In Paper II, we investigated if the rs2943641 variant in the *IRS1* genes associates not only with insulin resistance but also with cancer incidence in subjects from the SOS and MDC cohorts.

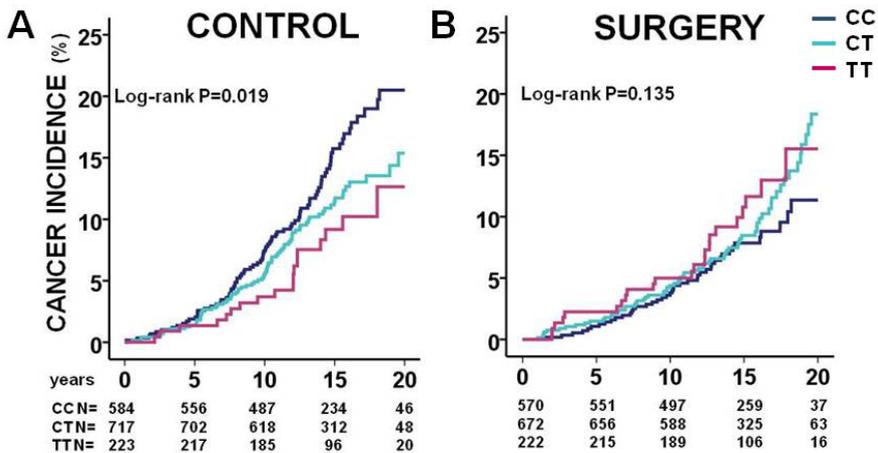


Figure 3. Cumulative incidence of cancer in the SOS study nondiabetic participants across *IRS1* genotypes in the control (A) and surgery (B) group.

As expected, in both the cohorts carriers of the *IRS1* T allele had lower insulin resistance, as showed by lower HOMA-IR at baseline. We then analysed if the *IRS1* T allele, which associates with lower insulin resistance, is also associated with lower cancer incidence in the SOS cohort. We found that an association between the *IRS1* T allele and lower cancer incidence was present in the SOS control group, characterized by no weight changes during the 15 years follow-up (log-rank P = 0.019 Figure 3 A; adjusted HR 0.77 [95% CI 0.62–0.96]; P = 0.021; Table 2). However such an association was not detected in the SOS surgery

group that underwent a sustained and massive weight loss during follow-up (log-rank  $P = 0.135$ , Figure 3 B; Table 2).

We then decided to stratify the control group according to the median baseline BMI (40 Kg/m<sup>2</sup>, which also corresponds to the cut-off between class II and class III obesity<sup>4</sup>) to determine if the protective effect associated with the *IRS1* T allele was specifically present in morbidly obese subjects. After stratifying the control group, a significant risk reduction associated with the T allele was observed only in subjects with BMI > 40 Kg/m<sup>2</sup> (Table 2). This result, together with the lack of the association in the surgery group, suggests that the effect of the *IRS1* T allele on cancer incidence may be uncovered by morbid obesity and may be negligible in subjects with lower BMI. This is also supported by the interaction between the *IRS1* genotypes and the bariatric surgery in modulating cancer incidence ( $P=0.005$ ).

**Table 2. Multivariable Cox proportional hazards models for cancer events in participants from the SOS and the MDC studies for the *IRS1* rs2943641 T allele.**

|  | Cases/ non cases | HR (95% CI)      | P value          |       |
|--|------------------|------------------|------------------|-------|
| <b>SOS STUDY:</b>                                |                  |                  |                  |       |
| Control group                                    | 182/1,342        | 0.77 (0.62-0.96) | 0.02             |       |
| BMI ≤40  | 83/679           | 0.89 (0.65-1.22) | 0.47             |       |
| BMI >40  | 99/663           | 0.67 (0.50-0.91) | 0.01             |       |
| Surgery group                                    | 133/1,331        | 1.23 (0.97-1.57) | 0.10             |       |
| <b>MDC STUDY:</b>                                |                  |                  |                  |       |
| ALL  | 4,963/18,343     | 1.00 (0.96-1.04) | 0.92             |       |
| BMI ≤40  | 4,943/18,272     | 1.00 (0.96-1.04) | 0.98             |       |
| BMI >40  | 20/71            | 0.61 (0.29-1.29) | 0.20             |       |
| <b>SOS control and MDC cohorts (BMI&gt;40)*:</b> |                  | 99+20/663+71     | 0.66 (0.50-0.87) | 0.004 |

*Hazard ratios have been adjusted for age, gender and body-mass index.*

*\*Summary hazard ratios and corresponding 95% confidence intervals were estimated by fixed and random effect meta-analysis (Comprehensive Meta-Analysis software, Biostat, Englewood, NJ).*

*Abbreviations: SOS, Swedish obese subjects; MDC, Malmö diet and cancer; IRS1, insulin receptor substrate; HR, hazard ratio; CI, confidence interval; BMI, Body mass index.*

Although the findings in the SOS study suggest that the association between the *IRS1* variant and cancer incidence is specific for morbidly obese subjects, we could not completely exclude that the association is present at a population-based level. We tried to falsify our hypothesis by testing it in the MDC, a

population-based cohort including about 23,000 non diabetic individuals with a mean BMI of 26 Kg/m<sup>2</sup>. No association between the *IRS1* genotypes and cancer incidence was detected in the MDC cohort in a median follow-up of 15 years (Table 2). This lack of the association between the *IRS1* variant and cancer incidence in the MDC cohort suggests that the effect of such variant is negligible at a population level but does not confute our hypothesis.

To test if the association between the *IRS1* T allele and lower cancer incidence is specifically present in morbidly obese subjects, we then decided to stratify the MDC cohort according to BMI = 40 Kg/m<sup>2</sup>. Among subjects with BMI > 40 Kg/m<sup>2</sup> a non-significant trend for a lower cancer incidence was observed in T allele carriers (adjusted HR 0.61 [95% CI 0.29–1.29]; P = 0.20; Table 2). We acknowledge that no formal replication for the result found in the SOS control group was achieved in the MDC cohort. We hypothesize that this lack of association may be due to a low statistical power due to the low number of participants of the MDC cohort with BMI > 40 Kg/m<sup>2</sup> (N=91). In fact the effect of the *IRS1* T allele on cancer incidence in the SOS cohort is mild and it probably becomes not relevant in the small group of morbidly obese subjects from the MDC cohort.

Finally, we performed a meta-analysis by merging only individuals with BMI > 40 Kg/m<sup>2</sup> from the SOS control group and the MDC cohort. The meta-analysis supported the association between the *IRS1* T allele and lower cancer incidence specifically in morbidly obese subjects (HR 0.66 [95% CI 0.50–0.87]; P = 0.004; Table 2).

The rs2943641 variant lies near the *IRS1* gene and this genetic variant affects the mRNA expression of the gene in the muscle<sup>112</sup>. Signalling of both insulin and IGF1 converge on the IRS1 protein activating several pathways responsible for modulation of cell metabolism, survival and growth<sup>40,41</sup>. The importance of IRS1 in insulin and IGF1 signalling has been confirmed in several *in vitro* and *in vivo* studies<sup>42,43,71</sup>. A nonsynonymous variant inside the *IRS1* gene, Gly972Arg (rs1801278) is known to be associated with insulin resistance as well as cancer

incidence<sup>104,105</sup>. However rs1801278 and rs2943641 variants are not in linkage disequilibrium indicating that their effects are independent.

Several findings suggest a relevant role of the *IRS1* locus in tumorigenesis<sup>73,74</sup>; however the exact functional mechanism linking IRS1 and cancer development has not been completely elucidated. Regarding the *IRS1* rs2943641 variant, Rung *et al* showed that the rs2943641 T allele associates with higher IRS1 protein expression and increased IRS1-related phosphatidylinositol-3-OH kinase (PI-3K) activity in skeletal muscle biopsies<sup>112</sup>. This sensitization of the insulin signalling may account for the beneficial effect of the T allele on cancer incidence through at least two possible mechanisms: first directly through a reduced activation of the extracellular signal-regulated kinases (ERK) in the mitogenic pathways; second, indirectly through a long-term reduction of the circulating insulin levels due to lower insulin resistance that causes a lower stimulus on the insulin-activated pathways including the mitogenic ones. Both mechanisms probably contribute to the effect of the T allele on a lower cancer incidence. In this scenario, obesity may act as an enhancer pushing the system towards higher insulin resistance thus uncovering the beneficial effect of the *IRS1* rs2943641 variant on cancer incidence. We and others have previously showed that morbid obesity interacts with the genetic background in modulating metabolic traits and cancer susceptibility<sup>173-175</sup>. Moreover a recent GWAS identified genetic variants, including one in the *IRS1* locus, whose effect on insulin resistance is enhanced in obese subjects<sup>175</sup>.

In conclusion, Paper II shows that the rs2943641 genetic variant near *IRS1* gene may be associated with lower cancer incidence specifically in morbidly obese subjects.

### 4.3 Combination of NGS and clinical criteria for FH diagnosis

Familial hypercholesterolemia (FH) is a genetic disorder of lipid metabolism resulting in increased serum LDL-C levels and higher risk of developing cardiovascular disease<sup>133-136</sup>. The diagnosis of FH is performed by clinical criteria or genetic methods<sup>134,147,148</sup>. Paper III aimed at combining a well validated clinical score (Dutch Lipid Clinic Network score) with NGS (pyrosequencing) to achieve a high diagnosis rate for FH. A total of 77 subjects having a diagnosis of possible, probable or definite FH according to the Dutch Lipid Clinic Network criteria (score  $\geq 3$ ) were included in the study.

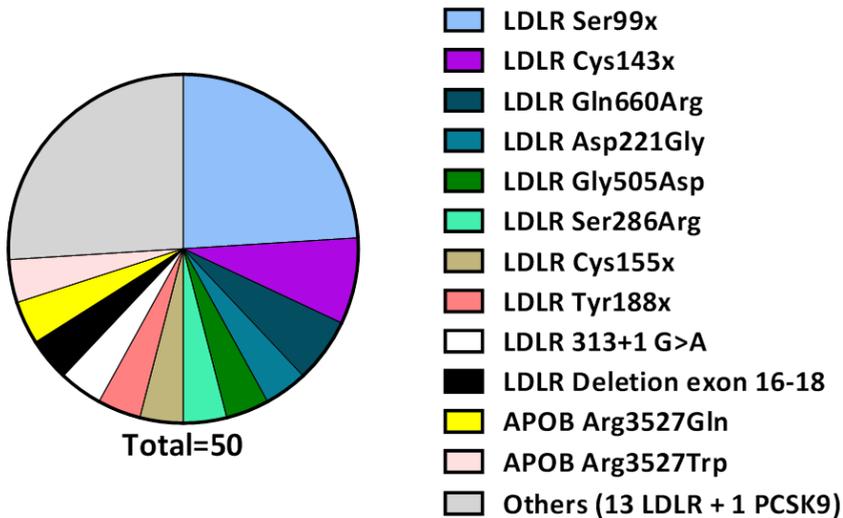


Figure 4. Spectrum of the different FH-related mutations detected

Twenty-six different mutations were detected in 50 subjects: 23 mutations in the *LDLR* gene, two in the *APOB* gene and one in the *PCSK9* gene (Figure 4). The success rate for the combined clinical and genetic approach was 65% (50 out of 77 subjects). Recently two studies performed the diagnosis of FH by using NGS in at-risk subjects and reported a success rate of about 30%<sup>95,176</sup>. To the best of our knowledge the two studies did not use a systematic way to select participants. In Paper III we accurately selected participants on the basis of the

Dutch Lipid Clinic Network score, which is a well-established, validated way to perform the clinical diagnosis of FH<sup>146</sup>. After selecting the participants we performed a pyrosequencing thus reaching a 65% success rate which is approximately 2-fold greater than the ones previously reported.

FH genetic diagnosis increases the compliance of patients to the medical treatment<sup>154,177</sup>. Most of the individuals starts a lipid-lowering therapy only after the diagnosis of FH is performed<sup>154</sup>. However, a diagnosis of FH through clinical criteria is difficult to be reached in children, since often serum LDL-C levels lie under the critical point and physical signs of lipid accumulation such as xanthomas or arcus corneae are not yet developed. Genetic diagnosis eliminates this ambiguity giving a clear-cut diagnosis. This allows performing family screening to detect subjects affected by FH at a young age and thus starting preventive procedures. Indeed when started ahead of time, the therapy with lipid-lowering medications is effective in preventing cardiovascular disease in subjects with FH<sup>178-180</sup>.

To improve the cost effectiveness of a genetic screening for FH it is of extremely importance to maximize the success rate. NGS techniques allow a deep and accurate analysis of the genes involved in FH pathogenesis<sup>95,176</sup>; however, if the population to be screened is not accurately selected the chance to detect an FH-causative mutation becomes low. The result of Paper III highlights the importance of an accurate selection of the screened population in order to maximize the chance of detecting FH-causing mutations while minimizing the costs.

Among the 26 different mutations that have been detected in Paper III, 4 mutations in the *LDLR* gene were of unknown pathogenicity, i.e. their pathogenic effect has not been previously tested or proven. Three of them (Gly505Asp, Ile585Thr and Gln660Arg) have been already reported in Europe in subjects with clinically diagnosed FH<sup>132,181</sup>. We performed *in silico* analyses through four different prediction tools that suggested that all the three mutations affect the protein function. In addition the alignment of the LDLR protein across eight different species shows that all the three aminoacids are

highly conserved. Taken all together these findings suggest that the three mutations are causative of FH.

The fourth variation with uncertain pathogenicity is a mutation affecting the proper splicing of exon 6 in the *LDLR* gene. This splicing-site mutation has never been reported before. The carrier of the mutation was a 21-year old man who developed myocardial infarction at the age of 17 years. Proband's pre-treatment serum LDL-C levels were 7.0 mmol/L and he had concomitant risk factors for cardiovascular disease, i.e. grade II obesity and smoking. To test if the mutation was causative of FH we performed a family-tree study that showed that the mutation segregated with high serum LDL-C levels within the proband's family. Both proband's father and brother, who are treated with lipid-lowering medications and reported high pre-treatment serum LDL-C levels, were carriers of the same mutation in the *LDLR* gene. Conversely, DNA from proband's mother, who has serum LDL-C levels within the normal range, did not show any mutation in the *LDLR* gene.

The detection of previously unreported mutations highlights the importance of NGS for genetic diagnosis of FH. Genetic diagnosis of FH can be also performed using different techniques such as simple chip assays or complex combined approaches including sequencing of the *LDLR* gene followed by detection of specific frequent mutations in the *APOB* and *PCSK9* genes<sup>147</sup>. However the resequencing of the genes involved in FH by NGS is a fast, reliable and affordable way to perform genetic diagnosis of FH and to detect previously unreported mutations<sup>100</sup>. This will help enlarge the range of the known mutations associated with the disease.

Paper III had important clinical impact in the health care of the Västra Götaland region. The identification of mutations causing FH in the region allowed starting a family cascade screening project to identify mutations' carriers in probands' close relatives. First-degree relatives of subjects who received a genetic diagnosis (N=50, Paper III) were offered a screening through the detection of the known FH-causing mutation. The DNA is collected through saliva samples, which makes the procedure easy and fast, especially in children. The aim is to build a

national network to improve the diagnosis of FH and in the long run to reduce the overall burden of individuals with myocardial infarction in Sweden. In the Netherlands, a systematic genetic screening is successfully ongoing since 1994 and more than 20,000 individuals with FH have been since identified<sup>182</sup>; nevertheless it has been estimated that they represent only about 50% of the population suffering from FH in the Netherlands<sup>182</sup>. The genetic cascade screening has been also proven to be cost-effective<sup>183</sup>. Similarly Norway started a genetic cascade screening that allowed identifying around 6,000 individuals with FH, out of the estimated 20,000 Norwegians affected by the disease<sup>184</sup>. The Norwegian genetic screening enabled to estimate that clinical diagnosis for FH has a sensitivity of 46% and a specificity of 88% only<sup>185</sup>. Those data confirm the importance of the genetic diagnosis for FH and suggest that family cascade screening is a cost-effective and efficient way to perform diagnosis of FH. Moreover Paper III suggests that NGS may be a first-line approach to perform genetic diagnosis in accurately selected individuals.

Paper III opens the way to future projects. Indeed 27 out of 77 subjects with Dutch Lipid Clinic Network score  $\geq 3$  do not have any mutations in the analysed FH-related genes. This may confirm that clinical criteria are not specific enough for the diagnosis of FH. However, it may also suggest that genes other than *LDLR*, *APOB*, *PCSK9* and *LDLRAP1*, are implicated in FH phenotype. Another hint for this hypothesis is that three out of 27 subjects with no FH-related mutations do have tendon xanthomas that are considered as specific for FH<sup>137</sup>. We plan to perform NGS of the entire genome in the 27 subjects with no FH-related mutations, starting with the three subjects with xanthomas. The mutations will be identified through a candidate genes approach and their pathogenicity will be tested by family-tree studies and by *in vitro* studies and confirmed in large populations<sup>99</sup>.

In conclusion, we showed that a selection based on clinical criteria together with NGS is effective to perform diagnosis of FH with a high success rate. We also described a previously unknown mutation in the *LDLR* gene.

## 5 CONCLUSION

The major findings in this thesis are:

1. Bariatric surgery associates with lower incidence of myocardial infarction in obese subjects with type 2 diabetes. Metabolic parameters rather than BMI predict the outcome of bariatric surgery in terms of myocardial infarction prevention in obese diabetic subjects.
2. The *IRS1* rs2943641 genetic variant associates not only with lower insulin resistance but also with a reduced incidence of cancer in morbidly obese individuals.
3. Next-generation sequencing in an accurately selected cohort allows performing genetic diagnosis of FH with a high success rate. This maximizes the cost-effectiveness of the technique and allows performing family cascade screening.

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