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Genetic Risk Score: An Insight into MASLD and Infertility

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Abstract

Background: Metabolic dysfunction-associated steatohepatitis (MASLD) is highly influenced by metabolic, inflammatory, hormonal, and genetic pathways. As these factors may be responsible for reproductive dysfunction as well, therefore, this study was performed to investigate MASLD-related clinical, hepatic, and genetic factors among infertile patients.

Method: A case-control study in prospective single-center infertile patients and fertile control subjects was done. Parameters related to clinical, anthropometric, biochemical, and Fibroscan parameters like controlled attenuation parameter (CAP) and liver stiffness were evaluated. The genetic parameters involved PNPLA3, TM6SF2, GCKR, MBOAT7, and HSD17B13. Risk score was calculated through PRS-HFC and PRS-5 and analyzed by logistic regression models.

Result: In 253 individuals, 213 performed FibroScan and 192 performed complete genotyping. There were higher mean levels of BMI, abdomen circumference, AST, ALT, and CAP levels for infertile individuals in contrast to control group. There were no significant differences between the two groups for liver stiffness and other fibrosis parameters. PRS-HFC was not significantly related to case/control status. PRS-5 showed marginal significance with respect to infertile status with OR=2.871, 95% CI=0.988-8.338, and p-value =0.053.

Conclusion: Infertility was also correlated with elevated metabolic and hepatic steatosis-associated values, implying that subclinical alterations in relation to MASLD might be important among patients with infertility. PRS-5 may offer more data regarding the genetic predisposition of the subjects, but it is not a definite independent factor for infertility in the studied population.

1. Introduction

1.1 Metabolic dysfunction-associated steatotic liver disease (MASLD) and infertility

Metabolic dysfunction-associated steatotic liver disease (MASLD), formerly termed non-alcoholic fatty liver disease (NAFLD), is a chronic liver disease associated with excessive accumulation of fat in the liver of people with metabolic risk factors, who are not alcohol consumers or have secondary reasons for steatosis.[1,2] The condition is now regarded as one of the most common chronic liver disorders around the world, with more than a third of the global population being affected.[1,3,4] There are several presentations of MASLD, from simple steatosis of the liver to metabolic dysfunction-associated steatohepatitis (MASH), progressing to fibrosis, cirrhosis.[5-8] With the rising prevalence of MASLD and its close relationship with obesity, insulin resistance, type 2 diabetes, and dyslipidemia, it poses a significant public health problem.[3,4,8]

The etiology of MASLD is complex and involves the interplay between metabolic, environmental, hormonal, inflammatory, and genetic factors.[9,10] Insulin resistance and visceral obesity play a central role in disease development and result in increased free fatty acids flux to the liver, lipogenesis de novo, and triglycerides accumulation.[11,12] Chronic low-grade inflammation, oxidative stress, adipokine profile disturbance, altered gut microbiota composition, and increase in intestinal permeability can contribute to the liver damage and disease development.[11-15]

MASLD is particularly common in high-risk metabolic groups, including individuals with type 2 diabetes and obesity, in whom the prevalence is substantially higher than in the general population.[4] Increased MASLD prevalence has also been reported in conditions characterized by metabolic and endocrine dysregulation, including polycystic ovary syndrome (PCOS), psoriasis, and hypothyroidism.[16-19] PCOS is especially relevant because both PCOS and MASLD are closely linked to insulin resistance, central adiposity, dyslipidemia, and metabolic syndrome. Therefore, hepatic fat accumulation may occur as part of the same systemic metabolic disturbance that contributes to reproductive dysfunction.[16,17]

1.2 MASLD and reproductive dysfunction

MASLD and infertility are clinically connected through shared metabolic, endocrine, and inflammatory disturbances.[16,17,20-24] In this regard, hepatic steatosis becomes

relevant to the issue of infertility in population owing to its involvement of metabolic, endocrine, and inflammatory problems that can lead to dysfunction in reproduction in women and men.[16,17,20-24] In women, this association has been studied most extensively in PCOS.[16,17] Also, MASLD may become relevant to the issue of male reproductive problems due to the presence of metabolic disorders in addition to hypogonadism, oxidative stress, systemic inflammation, and poor semen characteristics.[20-24]

1.3 Shared pathophysiological mechanisms linking MASLD and infertility

There are many similar metabolic, inflammatory, and hormonal factors which make MASLD and infertility interlinked.[16,17,20-24] Although the manifestation of these two diseases in men and women might be somewhat different, both are characterized by metabolic dysfunction.[16,17,20-24] There are such biological factors as insulin resistance, visceral adiposity, chronic inflammation, oxidative stress, dyslipidemia, and endocrine disturbances that can lead to hepatic steatosis and reproductive dysfunction at the same time.[11,12,14,15,20-25]

Insulin resistance is one of the main mechanisms that connect MASLD and infertility.[11,12,16,17,26,27] When there is insulin resistance, fat breakdown in the adipose tissue rises, and free fatty acids appear in the bloodstream.[11,12] Then these acids move to the liver where they cause accumulation of hepatic triglycerides.[11,12] In addition, insulin resistance enhances hepatic de novo lipogenesis and deteriorates the metabolic processes of glucose and lipids.[11,12]

The role of visceral fat mass is another critical determinant in the link between liver disease and infertility.[11,12,20-24] The accumulation of visceral fat leads to increased availability of free fatty acids to the liver via the portal circulation.[11,12] Furthermore, visceral fat acts as an endocrine tissue, producing inflammatory cytokines and adipokines.[11,12,21] Changes in adipokine secretion, decreased levels of adiponectin, and increased leptin levels can affect insulin sensitivity, hepatic steatosis, and metabolic dysfunction.[11,12,21] They can also lead to reproductive hormonal dysregulation and impaired fertility.[20-24]

Low-grade inflammation contributes to both MASLD progression and fertility disorders.[11,12,14,15,17,20-25] In MASLD, liver fat accumulation initiates the inflammatory response, including production of TNF-alpha, IL-6, and IL-1beta, leading to hepatocyte injury, activation of hepatic stellate cells, and fibrosis formation.[11,12,14,15] Similarly, pro-

inflammatory cytokines can affect reproductive performance by interfering with steroidogenesis, follicular growth, endometrial receptivity, and sperm function.[20-25] Thus, inflammation is one more common mechanism that unites these two conditions.[11,12,14,15,17,20-25]

MASLD is also involved in oxidative stress in the connection between MASLD and infertility.[11,12,14,15,20,24,28] Oxidative stress results from excessive lipid accumulation, mitochondrial dysfunction, lipid peroxidation, and formation of reactive oxygen species in the liver and leads to cell damage and development of MASLD.[11,12,14,15] In reproductive organs, oxidative stress causes deterioration of the quality of oocytes, sperm motility, sperm DNA fragmentation, and embryogenesis.[20,24,28] This mechanism is especially relevant in case of patients who suffer from obesity, insulin resistance, and metabolic syndrome.[20-24,28]

The association between MASLD and infertility is reinforced by hormonal disorders. In females, hyperandrogenism is usually associated with PCOS and can cause visceral fat accumulation, insulin resistance, and liver steatosis.[29-33] In males, there is a connection between MASLD and metabolic syndrome with low testosterone levels and hypogonadism along with negative effects on the quality of semen.[20-24] The interaction between the reproductive system and the liver through the endocrine metabolism can be reciprocal.

Alterations of the gut microbiome can play a role in the common pathophysiology of MASLD and infertility, through promoting the intestinal permeability and translocation of bacterial products, which stimulate inflammation on the systemic level and hepatic inflammatory processes.[13,17] Alteration in the composition of gut microbiota can affect the metabolism of bile acids, insulin sensitivity, lipid metabolism, and sex hormones regulation.[13,17] While further research is needed to clarify this hypothesis, it may be viewed as one more connection between metabolic liver disease and infertility.[13,17]

1.4 MASLD in women with PCOS

PCOS is one of the most widespread endocrine diseases in women of reproductive age and one of the most common causes of anovulatory infertility.[34] All these factors predispose women with PCOS to the development of MASLD.[16,17,35-46]

The association between PCOS and MASLD is explained by overlapping metabolic and hormonal factors. One of the key factors in PCOS is insulin resistance, which is

responsible for ovarian hyperandrogenism and fatty infiltration of the liver.[11,12,26,27,47,48] Hyperinsulinemia induces androgen production in the ovaries and inhibits the production of sex hormone-binding globulin in the liver, thus leading to the increase in free androgens in circulation.[49-52]This mechanisms also promote hepatic triglyceride accumulation, increasing the risk of MASLD in women with PCOS.[11,12] Thus, insulin resistance influences negatively reproductive and metabolic processes at the same time.

Hyperandrogenemia is another significant feature contributing to the association between PCOS and MASLD. Elevated androgen concentration has been related to visceral fat storage, dyslipidemia, inflammation, and further deterioration of insulin resistance.[29-33] This can lead to higher fatty acid infiltration into liver cells, making metabolic impairment more pronounced. Thus, hyperandrogenic PCOS subtypes are regarded as having increased metabolic burden and a high risk of MASLD compared with non-hyperandrogenic types.[29,33]

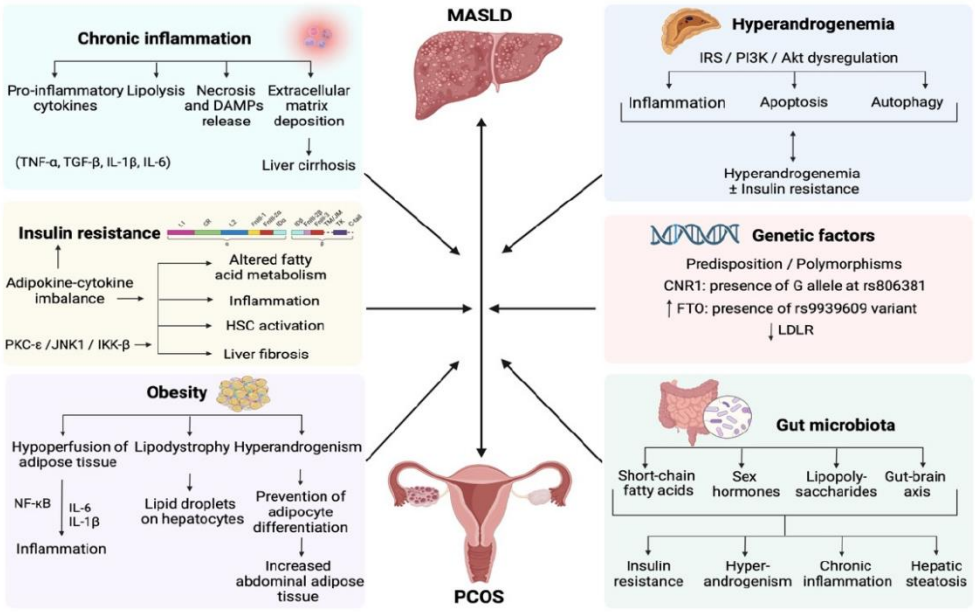

Figure 1. Schematic representation of the major mechanisms connecting PCOS with MASLD, including insulin resistance, hyperandrogenemia, visceral adiposity, chronic inflammation, genetic susceptibility, and gut microbiota dysbiosis. These pathways may promote hepatic steatosis, metabolic dysfunction, and reproductive impairment.[11,17]

It has been shown in many studies that the prevalence of hepatic steatosis in women with PCOS is higher than that in healthy individuals.[16,35-46] The prevalence rate of MASLD among PCOS patients can differ a lot from one study to another due to differences in diagnostic criteria, demographic features, and techniques used for

detecting liver steatosis.[17,35-46] Notably, despite obesity being one of the factors increasing the risks of PCOS and MASLD development, it has been shown that liver steatosis may occur in lean women with PCOS, particularly those who suffer from insulin resistance.[44,45,53] This means that the occurrence of MASLD may be connected with PCOS-related endocrine and metabolic disorders independently of being associated with overweight/obesity.[16,29,33]

It is clinically significant to note that there might be an occurrence of liver involvement in patients with PCOS even when liver enzyme levels are normal.[36,54-59] While conventional liver function markers like ALT and AST may indicate liver injury, normal levels of transaminases do not exclude liver steatosis.[55-59] That is why non-invasive methods of MASLD diagnosis like ultrasound, transient elastography, liver stiffness measurement, controlled attenuation parameter (CAP), and serum-based indices might prove to be helpful.[60-66]

In summary, PCOS is a significant example of a woman suffering from a combination of infertility, metabolic disorders, and MASLD.[16,17] Male reproductive disorders are discussed independently in the following section.

1.5 MASLD and male reproductive dysfunction

Although research on the relationship between MASLD and infertility has been conducted more widely in female patients, particularly in cases of PCOS, there is growing evidence showing the correlation between MASLD and male reproductive disorders as well.[20-24] Patients suffering from MASLD usually show some metabolic abnormalities, including obesity, insulin resistance, dyslipidemia, chronic low-level inflammation, and metabolic syndrome.[20,21] It is worth noting that all the listed conditions can also negatively affect men's reproductive system and lead to their infertility.[20-24]

One of the main processes connecting MASLD and male infertility is related to hormonal imbalance. Metabolic disorders and liver fat accumulation are connected to decreased testosterone concentrations and hypogonadism.[20,21] Changes in the function of the hypothalamus-pituitary-gonads axis can decrease the level of androgens and cause problems with normal spermatogenesis. As testosterone is required for proper spermatogenesis, its deficiency can negatively affect men's semen quality and fertility.[20-22]

One more mechanism linking MASLD and reproductive abnormalities includes oxidative stress.[11,12,14,15,20,24,28] In men, MASLD-related oxidative stress may impair sperm motility, morphology, and DNA integrity.[20,24] Since spermatozoa are vulnerable to oxidative stress, general metabolic inflammation may negatively influence male fertility.[20,24]

Inflammation can impair reproductive system functioning as well due to pro-inflammatory cytokine effects on endocrine regulation, testicular physiology, and spermatogenesis.[20,21,24] Moreover, obesity and abdominal fat accumulation exacerbate the inflammatory process and raise scrotal temperature, what influences sperm production and quality.[20,22,24]

A number of studies have shown that there is an association between metabolic syndrome, obesity, diabetes and poor sperm quality and quantity (lower sperm concentrations, motility, semen volumes, normal morphology).[20,22,23] Despite the fact that there is no enough scientific evidence comparing with PCOS and female infertility, it can be assumed that MASLD is one aspect of the metabolic disorders, influencing male reproductive ability.[20-24]

Thus, the risk of MASLD-related reproductive abnormalities is not only about women suffering from PCOS but also concerns male infertility.[20-24]

1.6 Genetic susceptibility to MASLD

The development and progression of MASLD are determined by both external and internal causes.[9,10] While metabolic abnormalities, insulin resistance, obesity, diet, and lifestyle are the primary factors, genetic predisposition may influence the vulnerability of individuals to hepatic steatosis, inflammation, and fibrosis, as well as the disease progression.[9,10,67]

Some genetic variant have been identified for their relationship to MASLD traits, especially the ones that regulate the processes of hepatic lipids storage and secretion, phospholipids remodeling, glucose metabolism, and liver damage.[9,67-76] The most extensively studied MASLD-associated genes are Patatin-like phospholipase domain-containing 3 (PNPLA3), Transmembrane 6 superfamily member 2 (TM6SF2), Glucokinase regulatory protein (GCKR), Membrane-bound O-acyltransferase domain-containing 7 (MBOAT7), and Hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13).[9,67-79]

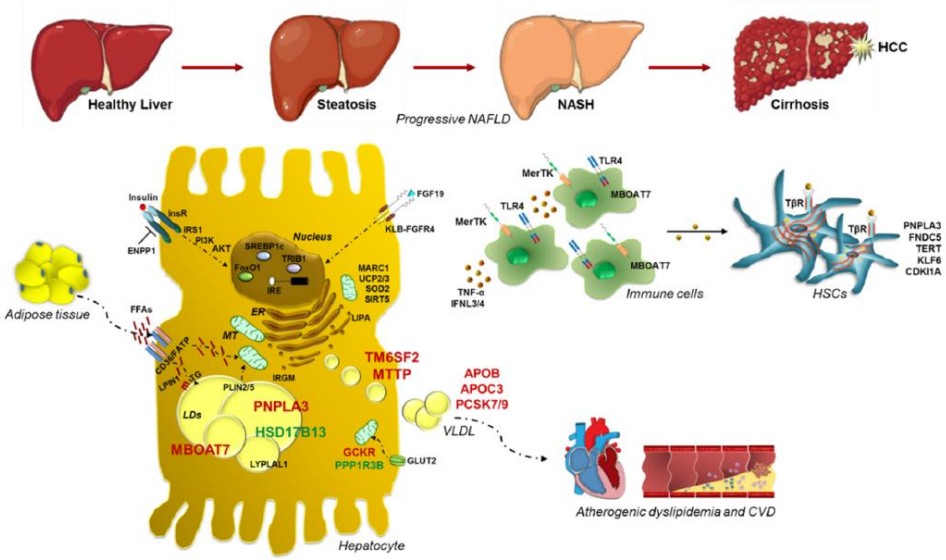

***Figure-2**. Schematic representation of genetic factors involved in hepatic lipid accumulation, lipid droplet remodeling, VLDL secretion, inflammation, fibrosis, and progression toward advanced liver disease. Variants in PNPLA3, TM6SF2, MBOAT7, GCKR, and HSD17B13 may influence individual susceptibility to MASLD and related hepatic outcomes[69].*

1.6.1 PNPLA3

The PNPLA3 also called adiponutrin, involved in the metabolism of lipid droplets in the hepatocytes.[71,72] The rs738409 C>G variant results in the isoleucine-to-methionine change at position 148 called p.I148M.[71] This polymorphism is one of the most powerful and consistently replicated risk factors for MASLD.[67,71] The risk allele is linked with the elevated hepatic accumulation of triglycerides, increased vulnerability to steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma.[67,71,72] The adverse effects of the PNPLA3 I148M variant may also depend on the environment and metabolism factors such as obesity, insulin resistance, and diet.[10,67]

1.6.2 TM6SF2

The TM6SF2 participating in the metabolism of hepatic lipids and VLDL secretion.[70] The rs58542926 C>T variant leads to the glutamate-to-lysine replacement at position 167 referred to as p.E167K.[70] This is the loss-of-function polymorphism that impairs the ability of hepatocytes to deliver triglycerides via VLDL.[70] Therefore, individuals carrying the risk allele have an increased risk of hepatic steatosis and MASLD development.[67,70] Interestingly, this genetic variant can be related to decreased levels of lipoproteins in the circulation, which means that some genetic variants may have opposite effects on hepatic and cardiovascular complications.[70]

1.6.3 GCKR

The GCKR is responsible for the regulation of glucose uptake and metabolism via glycolysis.[76] The rs1260326 C>T polymorphism causes a proline-to-leucine change at position 446 of the protein, which is termed p.P446L.[76] The rs1260326 C>T variant leads to alterations in glucokinase regulation and favors the increase in glucose uptake and glycolysis in the liver.[76] In turn, this can lead to an increased influx of glucose-derived carbons to the formation of acetyl-CoA and the formation of triglycerides and lipogenesis, which contributes to triglyceride accumulation and steatosis.[76] Thus, the GCKR gene variants relate glucose metabolism to lipid deposition in the liver and MASLD pathogenesis.[76]

1.6.4 MBOAT7

The MBOAT7 is involved in the process of remodeling of phospholipids, including the Lands' cycle, thus regulating the composition of membranes and lipid mediators of inflammation.[68,69,80] The rs641738 C>T variant results in lower levels of MBOAT7 and changes in phosphatidylinositol remodeling.[69] These effects are likely to cause increased hepatic triglyceride accumulation and inflammation and fibrosis.[68,69] Although the effect of the variant is typically smaller than in PNPLA3, it can potentially contribute to MASLD susceptibility, especially when combined with other genetic, metabolic, and environmental risk factors.[9,69]

1.6.5 HSD17B13

The HSD17B13 is a protein bound to lipid droplets and primarily expressed in the liver.[73-75,78] In contrast to the other variants mentioned previously, the loss-of-function variants in the HSD17B13 gene, such as the rs72613567 variant, have been linked to increased resistance to liver damage, steatohepatitis, fibrosis, and chronic liver disease progression.[73,75] Importantly, it means that this protective effect may prevent patients from developing an advanced form of liver disease despite being carriers of other MASLD risk variants.[67,73] Hence, proper coding of alleles is critical when considering HSD17B13 in genetic risk scores.[67,73,75]

1.7 Polygenic risk scores (PRS) and non-invasive liver assessment

Although each MASLD-linked genetic variant may have an impact on hepatic steatosis and progression of liver diseases, its effect remains relatively low.[9,81-83] MASLD represents a multifactorial disorder whose development is linked to interplay of several

factors such as genetics, metabolism, environment, and hormones.[9,10] Therefore, combined assessment of several genetic variants can provide a more reliable prediction of inherited predisposition to MASLD compared to assessment of single genetic variant.[81-84]

The calculation of PRS involves consideration of contribution of several genetic variants linked to a specific disease or trait.[81,83] In the case of MASLD, PRS is used to evaluate the genetic predisposition to MASLD-related liver steatosis and damage.[82,84] The weighted PRS that puts more weight on SNPs with stronger effect may help to achieve better estimation of genetic risk.[81-83] It can be especially helpful for population with metabolic or reproductive disorders due to interaction of the inherited genetic predisposition with metabolic factors.[10,16,17,20-24,82,84]

Two PRS were considered: PRS_HFC and PRS_5. PRS_HFC is based on the hepatic fat content score developed from established hepatic-fat genetic variants and estimates inherited predisposition to liver fat accumulation by combining risk alleles in PNPLA3, TM6SF2, GCKR, and MBOAT7, weighted according to their reported effects on hepatic fat content.[10,85] PRS_5 extends this model by including the HSD17B13 rs72613567 variant, which has been associated with protection against liver injury and fibrosis; therefore, its contribution is considered protective rather than risk-increasing.[73,75,84] These scores provide the genetic framework for evaluating whether inherited MASLD susceptibility is associated with metabolic risk among patients within the infertility population.[17,20-24,81-84]

Nevertheless, the interpretation of the risk scores should take into account the clinical and hepatic factors. Indeed, the MASLD diagnostics and risk stratification is strongly dependent on the data obtained using anthropometry, metabolic analysis, biomarkers, and non-invasive liver assessments.[55-63] BMI and waist circumference indicate general and abdominal adiposity levels, respectively, whereas liver enzymes and serum-based liver injury, fibrosis, and steatosis markers can provide information about the possible liver injury and fibrosis risk.[55-63]

The use of non-invasive imaging tools is particularly valuable for assessing liver involvement.[55,62-66] Transient elastography helps to obtain liver stiffness, which is used as an indirect indicator of liver fibrosis, and controlled attenuation parameter (CAP) that estimates the extent of hepatic steatosis.[65,66] CAP becomes especially important in

MASLD due to the possibility of non-invasive evaluation of liver fat content and identification of patients with an elevated metabolic load on their liver irrespective of conventional changes in liver enzymes.[65,66]

In this regard, the current research aimed to integrate genetic factors with clinical, anthropometric, and non-invasive hepatic parameters to assess MASLD-related risks in patients with infertility. [17,20-24,55-63,65,66,81-84]

1.8 Rationale of the present study

Despite the established connection between MASLD and metabolic abnormalities, its significance in infertility populations is less well-established. The clinical significance of such populations lies in the frequent occurrence of reproductive dysfunction alongside metabolic and hormonal abnormalities.[16,17,20-24]

Therefore, PRS may be useful for evaluating inherited susceptibility to MASLD in infertility populations.[17,20-24,81-84] It is unknown whether PRS is associated with poor metabolic health in these individuals. [17,20-24,82,84]

2. Objectives of the study

To evaluate the association between infertility and MASLD-related metabolic, hepatic, and genetic risk factors. The study aimed to compare infertile subjects and fertile controls using clinical, anthropometric, biochemical, FibroScan-derived, and genetic parameters. It also assessed whether MASLD-related PRS could support the identification of individuals with increased metabolic-hepatic risk in an infertility population.

3. Material and Methods

3.1 Patients and Ethical Committee

A prospective, single-center, case-control study was conducted on a cohort of couples (male/female) attending the Assisted Reproduction Center at the Galliate (Novara) branch of the Division of Obstetrics and Gynecology. The cases were recruited consecutively through face-to-face interviews starting in January 2021; couples who had their first visit in 2020 were also contacted by telephone.

Cases are represented by patients recruited at the hospital outpatient clinic, adults of both sexes.

Inclusion criteria for the study were: infertility and age >18 years. Exclusion criteria included pregnancy or suspected pregnancy. Because transient elastography has not been validated for use during pregnancy, women who had recently undergone embryo transfer were excluded, as an early pregnancy could not be reliably ruled out. Moreover, other exclusion criteria are pre-existing diagnosis of chronic liver disease with fibrotic evolution (chronic hepatitis C to B, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis), presence of a focal liver lesion of suspected malignant pathogenesis, high alcohol consumption, and inability to obtain valid measurements.

Controls were healthy volunteers (male-female couples, not necessarily having children), matched for age (+1- 2.5 years) to cases.

All patients underwent hepatic elastometry to measure liver stiffness (LS) and CAP at the Hepatology Clinic of SCDU Internal Medicine 1, performed by personnel experts in this method using FibroScan (Echosens).

The study received approval from the Comitato Etico Interaziendale di Novara (N. CE288/20) and was conducted in accordance with the ICH-GCP (International Conference on Harmonization Good Clinical Practice) guidelines, national laws, and the Declaration of Helsinki.

3.2 DNA extraction

The peqGOLD Blood and Tissue DNA Mini Kit was used to extract and purify the DNA from whole blood following the manufacturer's instructions. The extracted DNA was stored at -20°C.

3.3 Restriction Fragment Length Polymorphism-PCR (RFLP-PCR)

RFLP-PCR is a molecular method that analyzes changes in DNA fragment lengths to find genetic variants. First, a particular DNA region is amplified using polymerase chain reaction (PCR). Next, restriction enzymes are used to digest the amplified DNA sequence of interest. These enzymes cut the DNA at specified recognition sites. The pattern of fragment sizes is visualized when the resultant fragments are separated by gel electrophoresis.

3.3.1 RFLP-PCR for PNPLA3 and HSD17B13

The PCR was used to amplify PNPLA3 and HSD17B13 genes. For PNPLA3 amplification, the following primer sequences were utilized:

Forward Primer: 5'-CCTGCAGGCAGGAGATGTGT-3'

Reverse Primer: 5'-GCCCTGCTCACTTGGAGAAA-3'

While for HSD17B13 amplification, the following PCR primer sequences were used:

Forward: 5’-GGCCTGTATTGGAGACAGATG-3’

Reverse: 5’-GTCTGAGGCATGAGAATTGCT-3’

After obtaining gene amplification, enzyme digestion was performed. PNPLA3 PCR product was digested with NLA-III enzyme. Three possible results were obtained: wild type homozygous sample where the restriction site for the NLA III enzyme is absent on both alleles, resulting in a single band of 213 bp, mutated homozygous sample, two fragments, one measuring 93 bp and the other 120 bp and the heterozygous sample, three bands of 213 bp, 93 and 120 bp, respectively. While, HSD17B13 was cut using Tru1I enzyme. In the presence of a wild-type homozygous sample, the Tru1I enzyme's restriction site is absent on both alleles, resulting in a single band of 294 bp. Conversely, in a mutated homozygous sample, the enzyme cleaves both alleles, producing two fragments of 116 bp and 178 bp. In a heterozygous sample, three bands are observed: one of 294 bp, and two of 116 bp and 178 bp, respectively. All samples underwent amplification twice, and any inconsistent findings were subjected to a third amplification.

3.4 TaqMan Assay

The TaqMan assay was employed to genotype specific SNPs in the TM6SF2 (rs58542926), MBOAT7 (rs641738), and GCKR (rs1260326) genes. The protocol adhered to the manufacturer’s instructions for the ProAmp TaqMan Assay. We used the Applied Biosystems QuantStudio™ 1 Real-Time PCR technology for genotyping. This technology detects fluorescent signals and simultaneously amplifies DNA, allowing us to discriminate between alleles. For each SNP, the assays distinguished between three potential genotypes using allele-specific probes labelled with two different fluorophores: reference allele homozygotes, heterozygotes, and variant allele homozygotes. An accurate assignment of genotypes to each sample was achieved by analysing the fluorescence intensity data generated during PCR amplification using the QuantStudio™ Design & Analysis Software, version 1.5.1. The findings were checked for any contamination using no-template controls (NTCs) in each run, and the assay's performance was validated by testing positive control samples with known genotypes.

3.5 Polygenic Risk Score (PRS)

The PRS was determined by combining the effects of several genetic variants associated with MASLD. Five SNPs were selected based on prior genome-wide association studies and established associations with hepatic fat content: PNPLA3 (rs738409, β = 0.594), TM6SF2 (rs58542926, β = 0.166), MBOAT7 (rs641738, β = 0.073), GCKR (rs1260326, β = 0.271), and HSD17B13 (rs72613567, β = 0.216). Beta coefficients (β) quantify the estimated effect size and direction of each SNPs influence on MASLD-related traits and were used as weights in the calculation of the weighted PRS.

Using the following formula, we determined each person's PRS by adding up their risk alleles from the varieties we chose, weighted by their relative frequency:

$$PRS = \sum_{i=1}^{n} \beta_i \times Genotype_i$$

where β_i signifies the beta coefficient of the *i-th* SNP, and $Genotype_i$ indicates the number of risk alleles (0, 1, or 2) associated with that SNP. This weighted scoring method enables an improved evaluation of genetic risk by considering both the existence of risk alleles and the magnitude of their correlation with MASLD.

3.6 Statistical Analysis

All statistical analyses were conducted using Stata (version 18.0, StataCorp LLC, College Station, TX, USA). Descriptive statistics were used to delineate the study cohort. Continuous variables were summarized as mean ± SD or median (IQR) based on data distribution, then compared using t-tests or Mann–Whitney U tests. Categorical variables were represented as counts and percentages (%) and analyzed using chi-square or Fisher's exact tests, as applicable. The PRS was computed as the weighted aggregate of effect alleles according to reported beta coefficients. Relationships between PRS and the main outcome (adverse metabolic status) were assessed by univariate and multivariate logistic regression, producing odds ratios (ORs) with 95% confidence intervals (CIs). Multivariate models were adjusted by essential factors, including age, sex, BMI, waist circumference, and CAP values. Stepwise regression was used to ascertain independent factors. A two-sided p-value < 0.05 was considered statistically significant. A two-sided (or two-tailed) test evaluates the possibility of an

effect in both directions—for example, whether the PRS is associated with an increase or a decrease in adverse metabolic status.

4. Results

4.1 Participant flow and study population

A total of 263 individuals were recruited at the onset of the study. However, ten of them did not participate in filling out the questionnaire; of these, eight were excluded due to high consumption of alcohol, and two were excluded due to tubal infertility. In other words, a total of 253 individuals answered the questionnaire.

From these 253 individuals, 213 individuals had undergone liver transient elastography through Fibroscan. Out of 213 individuals, 124 had infertile conditions, whereas 89 had fertile conditions. During the same visit, there were plans to sample their blood for biochemical analysis and genetic testing. Amongst all these 253 individuals, a total of 192 had their complete genetic analysis done, which were subjected to PRS analysis.

Table 1. Participant flow and final sample sizes

Step	Number of participants	Details
Initially considered for recruitment	263	Starting population
Excluded before questionnaire	10	8 excessive alcohol consumption; 2 tubal infertility
Completed questionnaire	253	145 cases and 108 controls
Underwent FibroScan	213	124 cases and 89 controls
Underwent complete genetic analysis	192	109 cases and 83 controls

4.2 Clinical-demographic characteristics

The total number of participants comprised 253 individuals; from these, 145 cases were infertile and 108 individuals were considered fertile controls. The studied group consisted of 108 men and 145 women. Male cases accounted for 72 participants, and 36 were male controls, while among females, 73 were cases and 72 were controls.

Median age was comparable in both groups, where the median age in cases was 37.0 years [33-40], while in controls - 38 years [34-42]. Most of the participants were Italian

in both groups. Smokers and alcohol drinkers less than the exclusion criteria were not significantly different in both groups. But the differences were observed in ethnicity and educational status, which was higher in controls.

BMI was significantly higher in cases of infertility when compared to controls who were fertile. Total sample showed significantly different BMI between cases and controls. Abdominal circumference was found to be significantly higher among cases. But no difference in hip circumference was found between groups. PCOS was found in 20 women in the total sample and did not show a significant association with the other variables analyzed.

Table 2. Main clinical and anthropometric characteristics of the total and genotype samples

Variable	Sample	Cases	Controls	p-value
Ethnicity: Italian / other origin	Genotyped sample, n=192	90 / 19	80 / 3	0.003
Smoking, n	Genotyped sample, n=192	24	13	0.252
Alcohol consumption, n	Genotyped sample, n=192	2	1	0.721
BMI: <25 / 25-29.9 / >=30 kg/m2	Genotyped sample, n=192	56 / 43 / 10	51 / 22 / 10	0.023
Abdominal circumference, mean ± SD	Genotyped sample, n=192	85.4 ± 14.9	83.2 ± 12.5	0.267
Hip circumference, mean ± SD	Genotyped sample, n=192	99.0 ± 11.7	98.3 ± 9.0	0.668
Ethnicity: Italian / other origin	Total sample, n=253	121 / 24	103 / 5	0.003
BMI: <25 / 25-29.9 / >=30 kg/m2	Total sample, n=253	68 / 57 / 20	68 / 26 / 14	0.009
Abdominal circumference, mean ± SD	Total sample, n=253	85.1 ± 23.8	69.6 ± 34.5	<0.001

Hip circumference, mean ± SD	Total sample, n=253	97.9 ± 13.6	94.9 ± 12.8	0.084

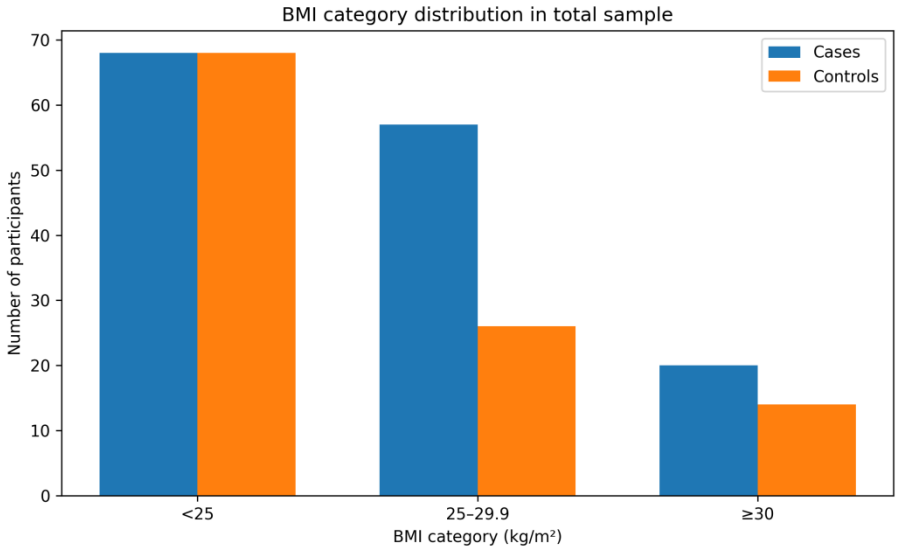

Figure 3. *BMI category distribution in the total sample. BMI distribution differed significantly between infertile cases and fertile controls.*

As regards the blood parameters, AST and ALT levels were found to be significantly higher in cases than in controls. The median AST level was 23 [18-28] in cases and 20 [17-25] in controls. The median ALT level was 22 [15-36] in cases and 17 [12-27] in controls. There were no differences observed for glycemia and platelets.

Table 3. Main liver enzyme findings in cases and controls

Variable	Cases	Controls	p-value
AST, median	23 [18-28]	20 [17-25]	0.02
ALT, median	22 [15-36]	17 [12-27]	0.01

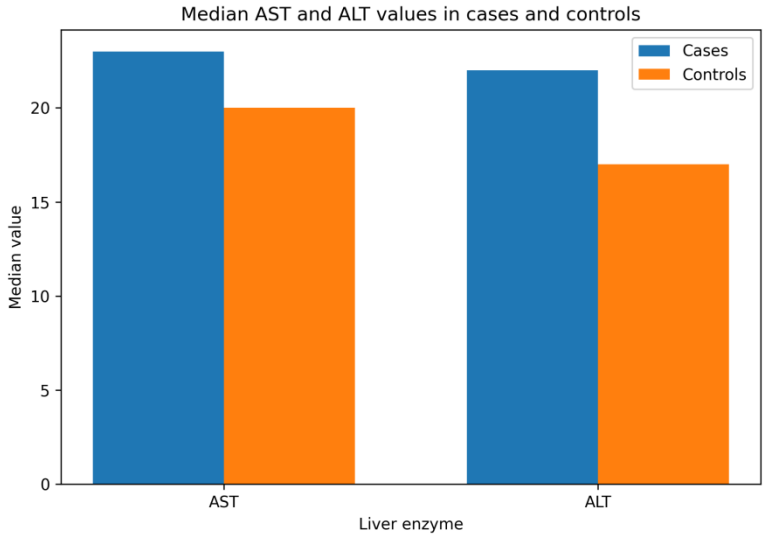

Figure 4. *Median AST and ALT values in infertile cases and fertile controls. Both AST and ALT were higher in cases.*

4.3 Liver elastometry analysis

Transient elastography was used for the assessment of hepatic steatosis using the CAP and of liver fibrosis using liver stiffness measurement. The CAP levels were found to be elevated in the group of infertile cases in comparison with controls. There were 35 cases and 16 controls in which moderate steatosis, defined as CAP >=255 dB/m, was found. Severe steatosis, defined as CAP >=280 dB/m, was found in 20 cases and 10 controls.

The median value of CAP was significantly increased in cases in comparison with controls; the CAP median in cases and controls was 223 dB/m [211-298] and 209.5 dB/m [204-302], respectively. In the stratification by sex, the difference was noted only in women. Women cases had the median CAP level of 206.0 dB/m [188.5-234], while that of controls was 198.5 dB/m [170.5-217]. The CAP levels in males were equal in both groups.

There was no statistically significant difference between cases and controls regarding the assessment of liver stiffness. The median values of liver stiffness were 4.2 kPa [3.5-5.1] for cases and 4.1 kPa [3.5-4.6] for controls. It can be seen that the measurements were within the normal or low-risk range and therefore advanced fibrosis was infrequent among these patients.

Table 4. FibroScan parameters according to sex and case-control status

Parameter	Female cases (n=64)	Female controls (n=60)	p-value	Male cases (n=60)	Male controls (n=29)	p-value
CAP >=255 dB/m, n (%)	8 (12.5)	4 (6.7)	0.27	27 (47.4)	12 (42.9)	0.69
CAP >=280 dB/m, n (%)	5 (7.8)	1 (1.7)	0.21	15 (26.3)	9 (32.1)	0.57
CAP, median	206.0 [188.5-234]	198.5 [170.5-217]	0.05	247.0 [220-288]	245.5 [210-295.5]	0.94
LS >=5 kPa, n (%)	12 (18.8)	8 (13.3)	0.41	24 (42.1)	9 (32.1)	0.38
LS >=7.1 kPa, n (%)	0 (0.0)	0 (0.0)	1.0	4 (7.0)	1 (3.6)	1.0
LS, median	3.8 [3.3-4.7]	4.0 [3.4-4.6]	0.99	4.8 [4.0-5.5]	4.4 [3.9-5.3]	0.47

4.4 Liver damage indices

HSI values did not differ statistically between infertile patients and healthy subjects. There were 43 and 29 values of HSI over the threshold for cases and controls, respectively. The median HSI level was 34.4 [31.8-37.9] in cases and 34.2 [30.6-37.5] in controls.

There was significant difference in AST/ALT ratio between infertile cases and healthy controls. The median AST/ALT ratio was 1.0 [0.8-1.3] in cases and 1.1 [0.9-1.5] in controls. Values of APRI and FIB-4 above the threshold for fibrosis were found only in a few patients. In summary, these non-invasive fibrosis indices showed no significant difference between infertile patients and healthy subjects.

Table 5. Non-invasive liver damage indices in cases and controls

Index	Cases	Controls	Interpretation (p-value)
HSI >=36, n (%)	43 (33.9)	29 (34.9)	0.30
HSI, median	34.4 [31.8-37.9]	34.2 [30.6-37.5]	0.42
AST/ALT ratio, median	1.0 [0.8-1.3]	1.1 [0.9-1.5]	0.04
APRI above threshold, n (%)	2 (1.6)	2 (2.4)	0.67
FIB-4 above threshold, n (%)	5 (3.9)	5 (6.0)	0.52

4.5 Genetic analysis

Genetic analysis was carried out on 192 individuals, including 109 cases and 83 controls. In the case group, 52 subjects were males while 57 were females. In the control group, 27 were males while 56 were females. Genetic analysis revealed five MASLD variants associated with the disease such as TM6SF2 rs58542926, GCKR rs1260326, MBOAT7 rs641738, PNPLA3 rs738409, and HSD17B13 rs72613567.

The most common genotype for TM6SF2 was the wild type accounting for 89.6% of the population under study. In addition, there was only one homozygote carrying the risk allele, meaning that this mutation was rather rare in the studied cohort. PNPLA3 was characterized by an important proportion of variant genotypes, where 35.9% of patients were heterozygotes and 12.5% were homozygotes for the risk genotype. HSD17B13 was wild type in 61.98% of subjects, 31.25% were heterozygotes and 6.77% of subjects were homozygotes for the risk allele. Genotype distribution of GCKR and MBOAT7 was well balanced. Overall, this data shows the existence of heterogeneous genetic background in the study population, suggesting that individual MASLD-associated variants may have modest effects when considered separately, whereas their combined contribution may be better evaluated through a PRS approach.

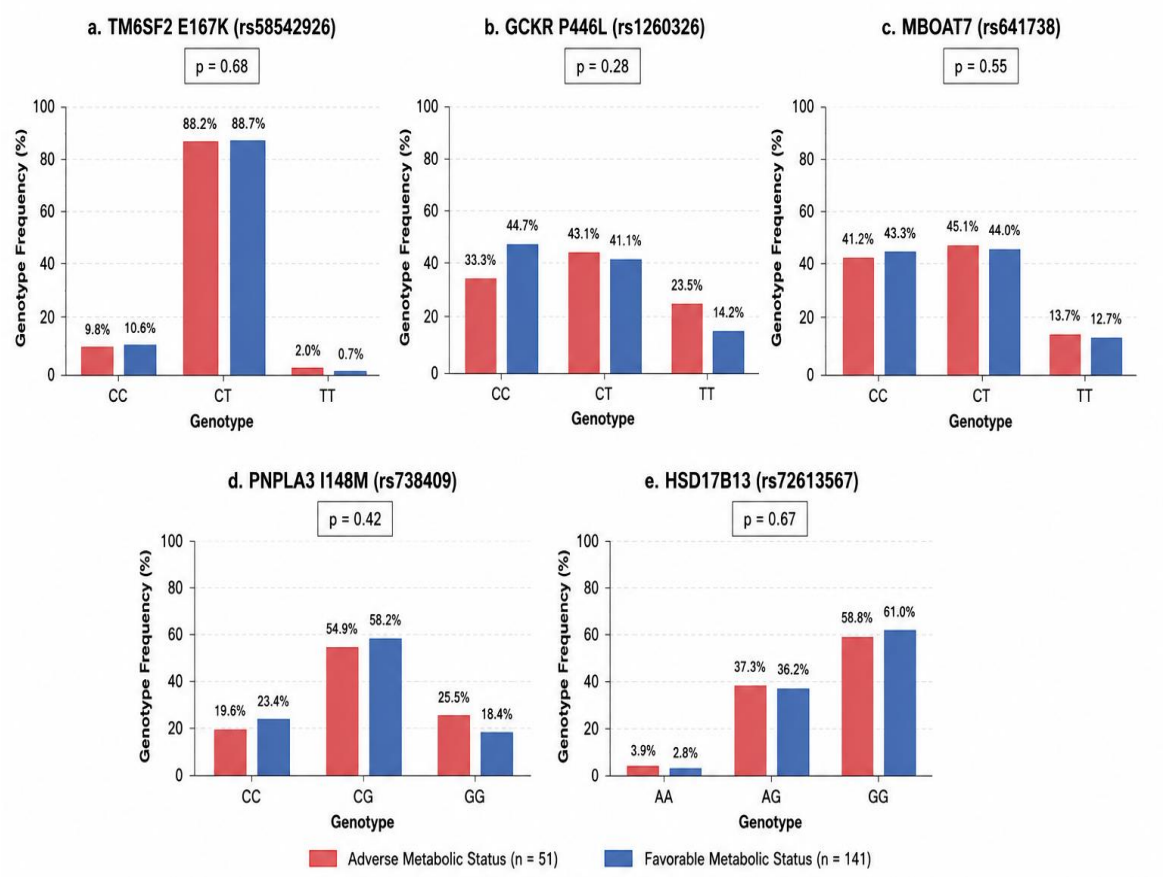

Figure 5 *shows the genotype distribution of MASLD-associated variants (TM6SF2, GCKR, MBOAT7, PNPLA3, and HSD17B13) between adverse and favorable metabolic groups. No significant differences were observed in the frequency of individual variants between the two groups (all p > 0.05).*

4.6 Polygenic risk score distribution

From the distribution of PRS, it is clear that the majority of the participants in the genotyped cohort scored below the high-risk cutoff point. In the case of PRS-HFC, the median score was 0.274 [0.128–0.456] and only 31 out of 192 participants (16.15%) scored above the high-risk point (>0.532). However, in the case of PRS-5, the median score was 0.128 [0–0.392] and only 23 out of 192 participants (11.98%) scored above the high-risk point (>0.495). This suggests that only few participants had genetic susceptibility to MASLD using PRS cut-offs.

Table 7. Distribution of polygenic risk scores in the genotyped cohort

Score	Included variants	Median	High-risk cutoff	High-risk subjects

PRS-HFC	PNPLA3, TM6SF2, GCKR, MBOAT7	0.274 [0.128-0.456]	>0.532	31/192 (16.15%)
PRS-5	PNPLA3, TM6SF2, GCKR, MBOAT7, HSD17B13	0.128 [0-0.392]	>0.495	23/192 (11.98%)

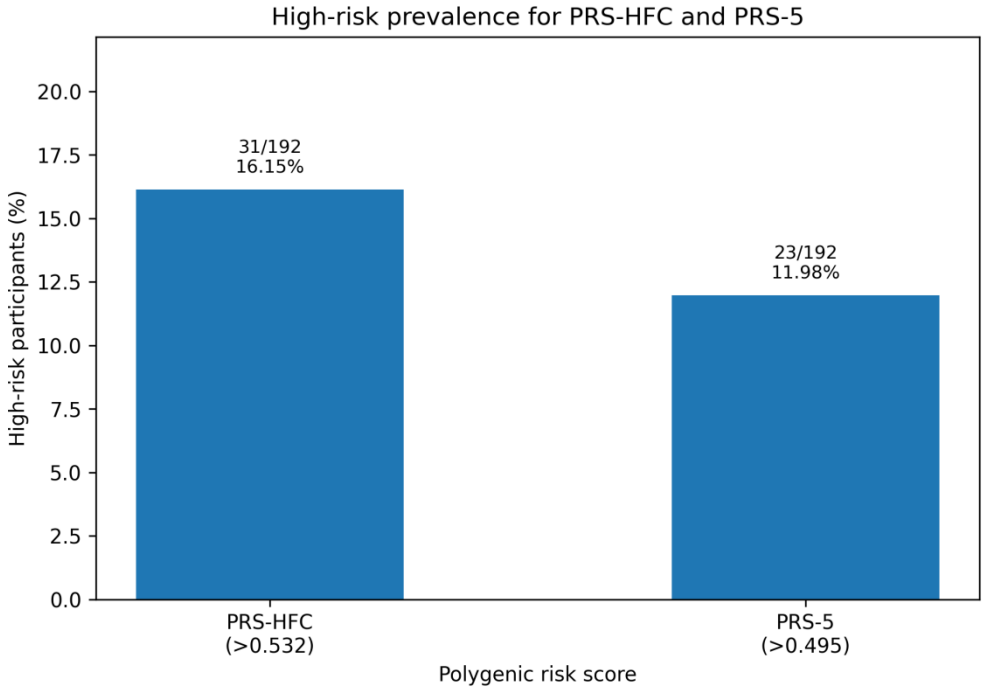

Figure 6. *High-risk prevalence for PRS-HFC and PRS-5. Most participants were below the high-risk thresholds.*

4.7 Logistic regression analysis

Logistic regression analysis was conducted to examine the relationship between the PRS and case-control status. The logistic regression model that involved PRS-HFC, age, and sex showed that PRS-HFC was not significantly related to case-control status. However, sex attained statistical significance in the model but the overall model did not show strong significance. A multiple logistic regression model was developed to examine the relationship between PRS-5, sex, age, BMI, waist circumference, and CAP. PRS-5 had a significant borderline relationship with case status with an OR of 2.871, CI 0.988-8.338, and p=0.053. These findings indicate that individuals with higher PRS-

5 had high chances of being in the infertile cases group despite the fact that the relationship was not statistically significant.

Table 8. Multivariable logistic regression model for case-control status

Predictor	Odds ratio	95% CI	p-value
PRS-5	2.871	0.988-8.338	0.053
Sex	0.529	0.250-1.119	0.096
Age	0.988	0.935-1.043	0.654
BMI	1.072	0.963-1.194	0.203
Waist circumference	0.969	0.934-1.006	0.104
CAP	1.006	0.998-1.015	0.144

5. Discussion

The purpose of this study was the assessment of the association between infertility, the presence of metabolic-hepatic disorders and the MASLD genetic susceptibility in a group of infertile subjects compared with control subjects. According to the latest data, infertile patients demonstrated unfavorable metabolic-hepatic profile in comparison with control subjects by higher BMI, abdominal circumference, AST, ALT, and CAP values.

Among other results, one of the most interesting was the high CAP found in the infertile group, in particular, in women. Taking into account the nature of CAP as a marker of hepatic steatosis, this fact means that infertile patients are prone to hepatic fat accumulation more frequently than control subjects.[65,66] At the same time, the values of liver stiffness were low and similar for both groups. This finding is very important since it means that advanced fibrosis is not a common phenomenon in infertile patients based on the cohort results.[65,66]

The non-invasive liver indexes had a more restricted discriminating value. Neither HSI, APRI, nor FIB-4 separated cases from controls, and both AST and ALT indexes were higher in cases. Thus, conventional serum-based indexes appear to be less sensitive than FibroScan to detect early hepatic changes associated with infertility in this cohort.[60-63,65,66] Consequently, CAP index might be a valid method to screen for

subclinical steatosis among patients examined for infertility, even though its interpretation should always be combined with anthropometric and metabolic data.[65,66]

Genetic assessment indicated a heterogenous distribution of MASLD-related SNPs. TM6SF2 was predominantly represented by wild-type genotype, and homozygous risk genotype was very scarce in this cohort. Heterozygous and homozygous risk genotypes were found in significant number of participants for PNPLA3 and HSD17B13 variants; thus, these two loci might contribute to the hepatic steatosis susceptibility.[9,67,71-73,75] Variant alleles of HSD17B13 could partially explain the protective part of the PRS-5.[73,75] The genotype distribution of GCKR and MBOAT7 was balanced, thus indicating a rather broad distribution of the risk variants among the population under study.[68,69,76]

As for PRS, most participants had scores below the proposed high-risk cutoff values. Nevertheless, there was a group of patients above the threshold for both PRS-HFC and PRS-5, which demonstrates that there is a certain proportion of individuals from the cohort who have an increased genetic predisposition to develop MASLD-related outcomes.[10,82,84,85] PRS-HFC was not found to be significantly associated with case-control status. On the other hand, there was a borderline association between PRS-5 and infertility-related metabolic-hepatic phenotype, particularly after adjustment for sex, age, BMI, waist circumference, and CAP. This implies that the addition of HSD17B13 and the general cumulative genetic risk can be a factor that can give additional information.[73,75,82,84]

Such results prove that genetic predisposition can supplement but not substitute clinical evaluation and imaging.[65,66,82-84] The presence of a borderline association between PRS-5 and MASLD-related outcomes proves the fact that genetic predisposition can serve as one of the factors in developing infertility-related metabolic risks.[17,20-24,82,84] Nonetheless, the sample size is too small to provide a clear conclusion on this matter.

Sex-specific variations need to be evaluated with caution as well. According to the updated data, the impact of CAP was more pronounced in women than in men, with male subjects exhibiting overall higher CAP values. However, the current data do not confirm a possible sex-specific effect on the genetic aspect. Instead, sex should be treated as the context of infertility, metabolic dysfunction, and hepatosteatosis, in which their interrelationships could be affected differently by such factors as PCOS,

hyperandrogenism, and insulin resistance in women or hypogonadism, erectile dysfunction, and altered sperm parameters in men.

The strength of the current investigation is the possibility of the assessment of hepatic steatosis and fibrosis with the help of FibroScan in the context of reproductive medicine.[65,66] It is important from a clinical point of view since liver elastometry is rapid, repeatable, non-invasive, and applicable for detection of early hepatic alterations before the development of serious liver disease.[65,66] One more novelty of the study is the evaluation of MASLD-related genetic variants and PRS, which can add insight into inherited susceptibility.[81-84]

The following are among the limitations of this study. There is a relatively small sample size that limited the analysis, especially for genetic analysis and comparison by sex. Some risk genotypes, like the homozygous risk genotype of TM6SF2, were relatively scarce and thus lowered the statistical power to detect associations with the variants. Recruiting subjects and lack of availability of data on FibroScan and genetics resulted in an even smaller final analysis sample. Self-reports of lifestyle factors, including alcohol consumption, are prone to reporting biases. Ethnicity and education are different between cases and controls in the study, which may be seen as residual confounding factors. Lastly, due to the exploratory nature of the study and case-control design, causality cannot be concluded between MASLD and infertility.

Further research with larger samples over a longer period of time would be necessary to confirm whether combining CAPs, anthropometrics, biochemical parameters, and PRS will increase risk stratification.

6. Conclusion

The results of this study suggest the possibility of a relationship between infertility and an unfavorable metabolic-hepatic phenotype. Participants from the infertile group had higher BMI, waist circumference, liver enzymes, and CAP than those of the fertile controls, which implies that liver steatosis and metabolism may occur more often among individuals with infertility problems.

The findings of genetic testing revealed that MASLD-related alleles of the PNPLA3, TM6SF2, GCKR, MBOAT7, and HSD17B13 genes were found in the population under

study at different frequencies. However, upon the creation of PRS using these alleles, the majority of participants were found to be below the suggested high-risk levels. PRS-HFC had no statistically significant relationship with case-control status, but PRS-5 had a marginal relationship after adjustment for sex, age, BMI, waist circumference, and CAP.

However, taking everything into account, one can conclude that metabolism, liver function, body dimensions, as well as genetic features might have an impact on the issue of infertility in terms of their interaction. The use of CAP might prove to be helpful in determining the presence of excess fat in the liver in this case, whereas genetic risk scores might allow evaluating an individual's genetic predisposition. Nevertheless, as the current study is exploratory and has a rather small number of participants, the findings should be considered preliminary. Future research using larger cohorts and longitudinal design should confirm whether combining genetic, anthropometric, biochemical, and imaging markers improves MASLD-related risk stratification among infertile patients.

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