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Association Between Nonalcoholic Fatty Liver Disease and Severe Male Reproductive Organ Impairment (Germinal Epithelial Loss): Study on a Mouse Model and on Human Patients

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Abstract

Metabolic syndrome (MS) has been associated with testicular damage. Nonalcoholic fatty liver disease (NAFLD) is a multisystemic disease that affects different organs, but its effect on the testes is unknown. A study analyzing germ cell involvement on BALB/c mice was carried out. A parallel comparative study was conducted that investigated alterations in the germinal epithelium of male humans that died from an unrelated acute event. The complete medical histories and histologic samples of the thoracic aorta, liver tissue, and testicular tissue from the deceased subjects were collected. The degree of germinal epithelial loss (DGEL) was evaluated and the clinical and histologic data were compared between individuals with and without NAFLD. The only metabolic or morphologic variable that caused a significant difference in the DGEL, in both the animal model and humans, was the presence of liver steatosis. The percentage of steatosis was also correlated with the percentage of the DGEL. In humans, steatosis (greater than 20%) increased the risk 12-fold for presenting with a severe DGEL (OR: 12.5; 95% CI [1.2, 128.9]; $p = .03$). There was no association with age above 50 years or MS components. Steatosis grade was also correlated with atherosclerosis grade. NAFLD was a strongly associated factor implicated in severe DGEL, as well as the testis was identified as a probable target organ for damage caused by the disease. This finding could result in the search for new approach strategies in the management of men with fertility problems. Further studies are required to confirm these results.

Keywords

germ cells, testis, steatosis, atherosclerosis, metabolic syndrome

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Diseases related to poor alimentary habits and a sedentary lifestyle, such as metabolic syndrome (MS) and nonalcoholic fatty liver disease (NAFLD), have become a worldwide public health problem (Murillo-Zamora et al., 2016). Both conditions are risk factors for cardiovascular disease. MS consists of the presence of three or more comorbidities, including high fasting glucose (insulin resistance), high blood pressure, hypertriglyceridemia, hypercholesterolemia, or abdominal obesity. Its prevalence varies from 10% to 30% of the world population, with a greater prevalence in Western countries (Han & Lean, 2016). NAFLD is the most common cause of

chronic liver disease in the developed world and it encompasses a spectrum that ranges from simple steatosis to steatohepatitis, fibrosis, or cirrhosis. According to the United States National Health and Nutrition Examination Survey (1988–1994) study (Lazo et al., 2013), the prevalence of NAFLD in men <30 years old was about 7.4% and for men 30–40 years old was 13.3%. The overall prevalence of NAFLD varies between 20% and 50% in Western countries (Fotbolcu & Zorlu, 2016).

NAFLD has traditionally been considered the hepatic manifestation of metabolic syndrome, due to the close association between NAFLD and the various component features of MS. Many epidemiologic studies have demonstrated an association between NAFLD and metabolic syndrome (Wainwright et al., 2016). Approximately 90% of NAFLD patients present with more than one component of MS, and about 33% of the patients meet the criteria for MS (Masarone, Federico, Abenavoli, Loguercio, & Persico, 2014). MS and NAFLD are beginning to be thought of as separate entities, in which insulin resistance is the central pathophysiologic process common to both conditions. There is growing evidence that the relationship between NAFLD and metabolic syndrome is bidirectional, in that NAFLD can predispose to metabolic syndrome features, which, in turn, can exacerbate NAFLD or increase the risk for its development in those patients without a preexisting diagnosis (Masarone et al., 2014). It has recently been postulated that NAFLD should be considered not only a liver-specific disease, but also an early mediator of systemic diseases (Fotbolcu & Zorlu, 2016).

Most of the studies on the general population that search for causes of male reproduction disorders attempt to associate testicular alterations with MS or its components. There are reports that MS is associated with decreased fertility parameters in males (Leisegang et al., 2016). Obesity is associated with low levels of testosterone and with subfertility or infertility (Fan et al., 2015). Despite all these data, the association between MS or its comorbidities with spermatogenic disorders has not been fully established. A large part of the experimental or morphologic information comes from animal models that do not necessarily accurately reflect what may occur in humans. Epidemiologic association studies have produced controversial results. Ventimiglia et al. (2016) recently carried out an analysis with statistical adjustments to avoid interpretation confusion (multivariate logistic regression analysis) and concluded that semen parameters and the rate of either obstructive or nonobstructive azoospermia had no association with MS.

Perhaps the presence or not of MS or any of its components is not the determining factor for presenting with sperm production disorders. NAFLD has recently been suggested to be a multisystemic disease that affects different organs. It most likely affects the testes, becoming a determining factor for germinal epithelial damage. To analyze this, testicular damage in a mouse NAFLD model was evaluated, as well as the clinical data (MS components) and histologic aspects of the liver, arteries, and testes in deceased human patients. This was done to determine which clinical or morphologic characteristic was conclusively correlated and associated with testicular germinal epithelial loss.

Materials and Methods

Mouse Model Study

An experimental study on a mouse model to determine the relation between nonalcoholic fatty liver disease and germinal epithelial damage was carried out.

Two groups of 6- to 8-week-old male BALB/c mice (Harlan, Mexico), weighing 25–30 g, were included in the experiment. The control group of mice (n = 16) was fed a standard diet (the SD group) (2018S Tekl and Global 18% Protein Rodent Diet, Harlan®, KY) and the NAFLD group (n = 13) was fed a high-fat diet (Atherogenic Rodent Diet, TD.02028, Harlan, KY). This model has been reported to be capable of the simultaneous production of steatohepatitis (NAFLD) and preclinical atherosclerosis within a period of 6 months (Garcia-Rivera et al., 2014; Madrigal-Perez et al., 2015).

The mice were kept in cages, with a maximum of 5 mice per cage. Light and temperature were controlled and the animals had access to food and water ad *libitum*. The mice were fed the diet for a period of 6 months, after which they were decapitated. Samples of the testis, liver,

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Table 1. Main Clinical Characteristics of Human Patients.

Clinical characteristic	*N* = 29
Age mean (years)	45.3 ± 9.4
Age range (years)	31–60
BMI	29.0 ± 8.9
Healthy weight	34.5%
Overweight	34.5%
Obesity	31.0
Diabetes	41.4
High blood pressure	20.7
Hyperlipidemia	17.2
Metabolic syndrome[a]	31.0
Smoking (%)	13.8

Note. Percentages or averages and standard deviation are shown. [a]Diagnosed in accordance with the guidelines of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults "ATP III." BMI = body mass index.

Table 2. DGEL According to the Presence or Absence of Relevant Clinical or Morphologic Characteristics in Human Patients and According to the Group in Mice.

Characteristic (*n*)[a]	DGEL		
Human patients	Absence	Presence	*p*
Type 2 diabetes (12)	63.1±16	64.7±15.2	.79
High blood pressure (6)	66.0±14	51.2±16.1	.08
Obesity (9)	59.1±17	66.2±14.9	.27
Steatosis[b] (14)	55.0±15.5	71.0±14.6	.02[c]
Liver inflammation[d] (12)	62.7±17.0	60.6±13.9	.76
Atherosclerosis[e](15)	60.0±14.1	72.9±14.0	.03[c]
Age above 50 years (12)	65.7±11.0	60.7±21.8	.43
MS[f] (9)	63.3±15.3	65.3±17.7	.78
Mouse model group	Control	NAFLD	
	12.3±8.2	32.2±14.1	.000[c]

Note. [a](*n*): Individuals with presence of the characteristic of a total sample of 29 human patients. [b]Steatosis in more than 20% of the liver tissue. [c]Statistically significant. [d]Liver inflammation in 33% or more of the liver tissue. [e]Grade IV atherosclerosis, or higher. [f]Metabolic syndrome. DGEL = degree of germinal epithelial loss; MS = metabolic syndrome.

and thoracic aorta were extracted and processed for their analysis. Whole blood samples were collected and biochemical analyses were performed. The protocols for experiments on animals were approved by the Research Ethics Committee of the School of Medicine of the *Universidad de Colima*, Mexico. The animals were manipulated according to institutional guidelines, in addition to the Mexican Official Norm regulating laboratory animal use (NOM-062-ZOO-1999) and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences.

Human Study

A parallel study was conducted to carry out the association analysis. Deceased patients that underwent autopsy at the Pathology Department of the *Hospital Universitario "Dr. José Eleuterio González"* in Monterrey, Mexico, within the time frame of January 2014 to December 2015 were studied. The simultaneous analysis included 29 men with the following inclusion criteria: age >30 years old with completed medical records, especially those patients with analysis of histologic tissue samples of the thoracic aorta, right lobe of the liver, testis, as well as the cause of death related to an acute event that did not affect the morphology of the tissues analyzed. Sixty-nine percent of the patients died from complications from acute cerebral edema secondary to trauma, 21% from acute infection of the central nervous system or lung, and 10% from hypovolemic and hemorrhagic shock. Patients with cancer, alcoholism, drugs uptake, autoimmune diseases, chronic kidney disease, liver trauma, acute or chronic infections affecting the liver were excluded. The clinical data of the patients were obtained from their medical records. All the subjects were having outpatient family medicine, internal medicine, or geriatric consultations before presenting with the event causing their deaths. The diagnoses of type 2 diabetes mellitus (DM), high blood pressure, and obesity (body mass index ≥30) were confirmed by medical specialists from those departments. The main clinical characteristics of the studied patients are shown in Table 1. The individuals were divided into two groups, depending on the presence or absence of one of the vascular comorbidities that make up MS or on the histologic alterations of NAFLD or aortic atherosclerosis (see Table 2). The Health Research Ethics Board of the School of Medicine of the *Universidad Autónoma de Nuevo León* approved that part of the study.

Histopathologic Analysis

All of the tissue specimens were fixed, processed, and embedded in paraffin to form blocks. Histologic sections of 5 microns were prepared and stained with hematoxylin and eosin, according to the standard staining technique. The Masson trichrome stain was used for evaluating fibrosis. Tissue samples of the liver, thoracic aorta, and testis from both the animal model and the human patients were included and analyzed. The evaluations of the slices were carried out through images taken with an Axiocam MRC-5 model digital camera (Zeiss®, Germany) attached to an AxioPlan 2 M model bright field optical microscope (Zeiss, Germany) with a motorized stage and A-plan ×5 and ×20 objective (total magnification ×50 for the aorta and ×200 for the liver). Images of the entire sample surfaces were scanned using MosaiX and

Autofocus modules. All the shots were taken under the same conditions of light and exposure. The analyses were done in a blinded manner by one pathologist.

The evaluation of germinal epithelial loss was carried out in the previously described manner. Ten randomly selected, essentially round cross sections of seminiferous tubules from each sample were classified into one of eight different grades, indicating the relative severity of seminiferous epithelial abnormalities. Briefly, grade 0—normal intact seminiferous epithelium; grade 1—seminiferous epithelium with pyknotic germ cells and desquamation or focal vacuolation; grade 2—seminiferous epithelium between grades 1 and 3; grade 3—seminiferous epithelium with pre-meiotic germ cells and Sertoli cells; grade 4—Sertoli cells only; grade 5—no seminiferous epithelium, leaving only the basement membrane; grade 6—seminiferous tubule with sperm stasis, sperm granuloma, or mineralization; grade 7—fibrosis of the seminiferous tubule. A weight between 0 and 1, to reflect the relative absence of germ cells, was assigned to each grade—0, 1/4, 2/4, 3/4 to grades 0, 1, 2, 3, respectively, and 4/4 to grades 4–7. The degree of germinal epithelial loss (DGEL) was calculated by multiplying the percentage of tubules in each grade by the respective assigned weight and summing the products (Higuchi, Palmer, Gray, & Veeramachaneni, 2003).

Steatosis was considered in relation to the percentage of liver tissue with fat accumulation. Three cross-sectional liver slices were analyzed, two from the right lobe (central and external third region). The percentage of liver tissue with inflammation was evaluated by functional histologic zones, according to the oxygen supply (zone 1 encircles the portal tracts where the oxygenated blood from the hepatic arteries enters; zone 3 is located around the central veins, where oxygenation is poor; zone 2 is located between zones 1 and 3). There were four categories with respect to the percentage of tissue presenting with inflammatory infiltrate: none, mild, moderate, and severe (0%, up to 33%, 34%–66%, and more than 66%, respectively) (Haddad, Vallerand, Brault, Spenard, & Haddad, 2011).

To evaluate atherosclerosis, only the slice qualitatively showing the lesion with the highest grade of disease, according to the Stary classification (grades I–VI) (Stary, 2000), was selected and a blinded analysis was carried out by one pathologist. The same slice was quantitatively evaluated by measuring the intima-media thickness (from the interior edge of the endothelium to the exterior edge of the middle layer). This was done at eight equidistant sites per section, selected through systematic uniform random sampling, regardless of the presence or absence of atherosclerotic lesions at the measuring site (Garcia-Rivera et al., 2014; Madrigal-Perez et al., 2015).

Data Analysis

The comparative analysis of continuous clinical data between groups was carried out using the Student's *t*-test for the numerical data with normal distribution and the Mann–Whitney *U* test for the qualitative data or the numerical data that did not have normal distribution. The categorical variables were compared using the Fisher's exact test. A Pearson product-moment correlation test was done to evaluate the correlation between pairs of clinical or histologic variables in the study groups. The association between any of the MS vascular comorbidities, age, steatosis, liver inflammation, or atherosclerosis, and the risk for severe germinal epithelial loss (equal to or greater than a 66% loss) was estimated by odds ratios (OR) and 95% confidence intervals (CI) (Crosstabs procedure) using the Mantel–Haenszel test. Statistical analysis was performed with SPSS v. 20 software (IBM, Armonk, NY) and the statistical power of the case-control human study (Table 5) was computed by an on-line power calculator (http://sampsize.sourceforge.net/iface/s3.html#ccp) developed by Philippe Glaziou. The estimated statistical power for the association between steatosis (≥20% of liver cells with fat deposition) and severe degree of germinal epithelial loss (≥66%) was 85.3%, using the following parameters: OR = 8; exposed controls = 27%; one-sided alpha risk =5%; controls/case ratio = 1.07, number of cases = 14 and number of controls = 15. p values < .05 were considered statistically significant.

Results

Table 2 reports that the presence of liver steatosis was the only metabolic or morphologic variable that caused a significant difference in the DGEL, in both the animal model and in humans (see Figures 1 and 2). In humans, having advanced atherosclerosis (Stary scale grade IV or higher) was a factor that significantly increased the DGEL. Age above 50 years or having MS, diabetes mellitus, or high blood pressure did not significantly increase the DGEL.

Tables 3 and 4 report that liver steatosis, again, was the only factor statistically correlated with DGEL in the animal model, as well as in humans. Other variables correlated with the DGEL were the grade of atherosclerosis in humans, and liver inflammation in the animal model. Other variables in humans that were correlated with each other were: aortic wall thickness with liver inflammation; atherosclerosis grade with age; and diabetes mellitus with the percentage of steatosis and with high blood pressure. Aortic wall thickness was correlated with the percentage of steatosis and liver inflammation in the animal model.

To estimate the degree of association between the risk factors and germinal epithelial loss, the human patients were divided into two groups: cases (severe germinal

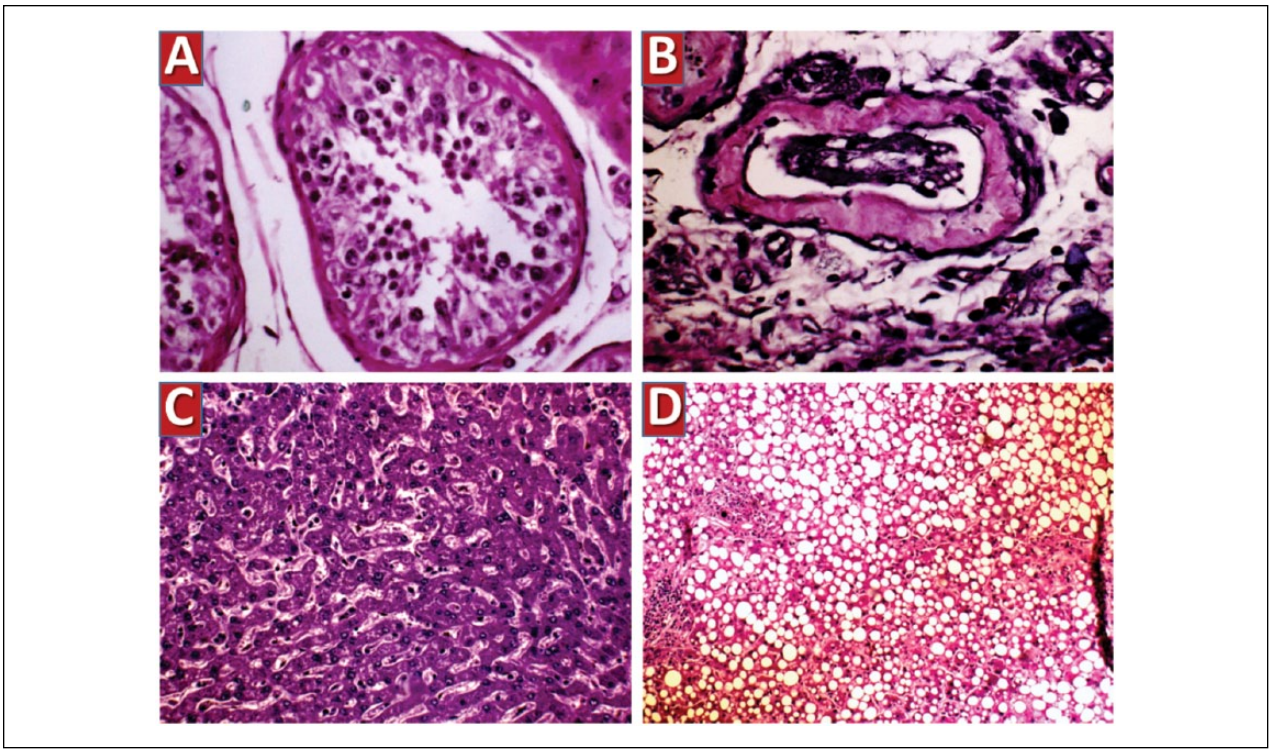

Figure 1. Histologic images of human liver and testicular tissues. A and C are tissues from a patient with seminiferous tubules with conserved germinal epithelium (mild germinal epithelial loss -A-) showing the liver tissue with normal characteristics, and C) the absence of steatosis and inflammatory cells. B and D are images from another patient showing B) a seminiferous tubule that has severe germinal epithelial loss, and D) severe liver steatosis and inflammatory cell foci.

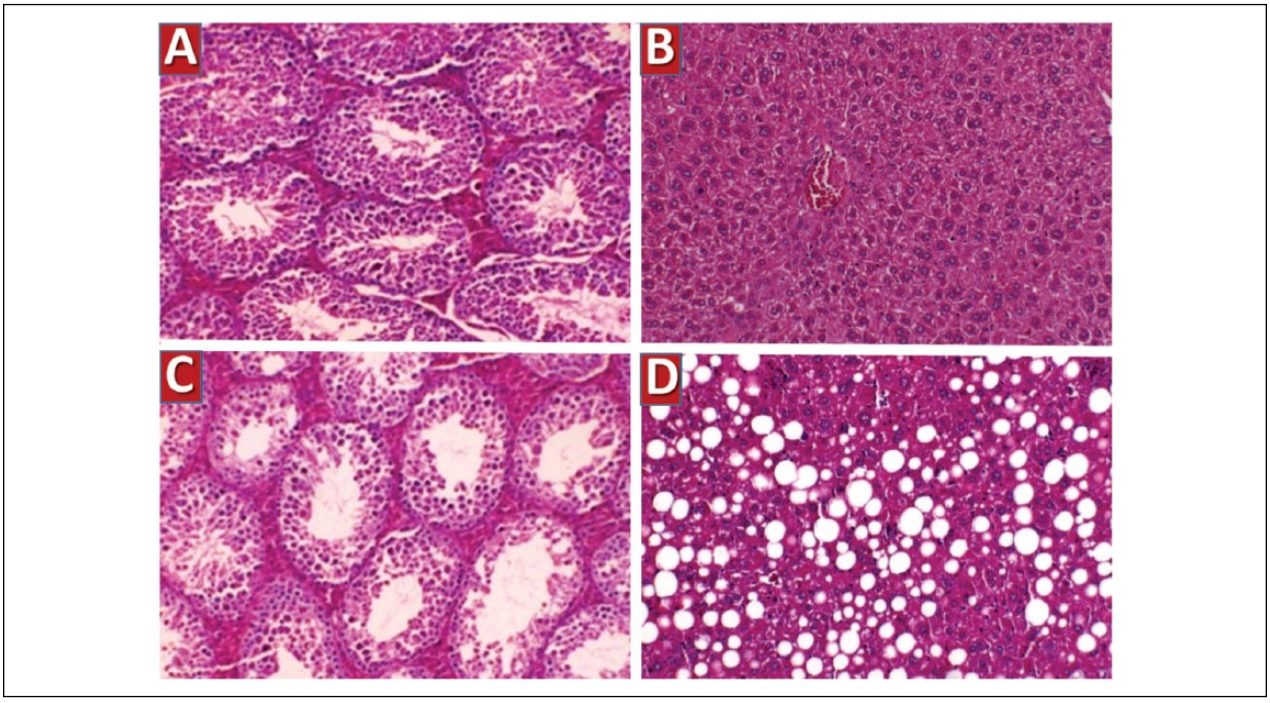

Figure 2. Histologic images of liver and testicular tissues from the mouse model. A and B are tissues from a mouse fed a balanced diet showing A) undamaged germinal epithelium in the seminiferous tubules and B) normal liver tissue characteristics. C and D are tissues from a mouse fed a high-fat diet showing C) a seminiferous tube with moderate germinal epithelial loss and reduced thickness, and D) a liver with severe steatosis (micro and macrodrip pattern) and inflammatory cell foci.

Table 3. Correlation Between the Relevant Clinical and Morphologic Characteristics in the Human Patients. The Correlation Coefficient and *p* Value are Shown.

	Age	BMI	DM	HBP	TAWT	Stary	Steato	Inflam	DGEL
DGEL	−0.01	0.13	0.08	−0.26	−0.27	.43[a]	.50[a]	0.17	1.00
	(0.97)	(0.52)	(0.69)	(0.19)	(0.26)	(0.04)	(0.01)	(0.44)	
Inflam	0.32	0.12	0.28	−0.02	.56[a]	0.36	0.29	1.00	0.17
	(0.13)	(0.58)	(0.19)	(0.91)	(0.01)	(0.12)	(0.16)		(0.44)
Steato	−0.14	0.27	0.50[a]	−0.19	0.24	0.35	1.00	0.29	.50[a]
	(0.50)	(0.20)	(0.01)	(0.36)	(0.34)	(0.13)		(0.16)	(0.01)
Stary	.49[a]	0.20	0.34	0.54[a]	0.24	1.00	0.35	0.36	0.43[a]
	(0.02)	(0.34)	(0.10)	(0.01)	(0.28)		(0.13)	(0.12)	(0.04)
TAWT	0.33	0.12	0.03	−0.09	1.00	0.24	0.24	0.56[a]	−0.27
	(0.13)	(0.61)	(0.90)	(0.68)		(0.28)	(0.34)	(0.01)	(0.26)
HBP	0.28	0.09	−0.01	1.00	−0.09	0.54[a]	−0.19	−0.02	−0.26
	(0.14)	(0.63)	(0.95)		(0.68)	(0.01)	(0.36)	(0.91)	(0.19)
DM	0.23	−0.13	1.00	−0.01	0.03	0.34	.53[a]	0.28	0.08
	(0.23)	(0.49)		(0.95)	(0.90)	(0.10)	(0.01)	(0.19)	(0.69)
BMI	−0.16	1.00	−0.13	0.09	0.12	0.20	0.27	0.12	0.13
	(0.39)		(0.49)	(0.63)	(0.61)	(0.34)	(0.20)	(0.58)	(0.52)
Age	1.00	−0.16	0.23	0.28	0.33	0.48[a]	−0.14	0.32	−0.01
		(0.39)	(0.23)	(0.14)	(0.13)	(0.01)	(0.50)	(0.13)	(0.97)

Note. [a]Statistically significant. DGEL = degree of germinal epithelial loss; BMI = body mass index; HBP = high blood pressure; DM = type 2 diabetes mellitus; TAWT = thoracic aortic wall thickness; Stary = degree of atherosclerosis according to Stary's classification; Steato = liver steatosis (% of cells with fat deposition); Inflam = % of liver tissue with inflammation.

Table 4. Correlation Between the Relevant Clinical and Morphologic Characteristics in the Animal Model. The Correlation Coefficient and *p* Value are Shown.

	CW	Glycemia	TAWT	Stary	Steato	Inflam	DGEL
DGEL	0.34	−0.15	0.13	−0.17	.58[a]	0.47[a]	1.00
	(0.11)	(0.49)	(0.48)	(0.38)	(0.001)	(0.01)	
Inflam	0.26	−0.25	0.35[a]	−0.11	0.90[a]	1.00	0.47[a]
	(0.21)	(0.23)	(0.04)	(0.54)	(0.000)		(0.01)
Steato	0.34	−0.31	0.45[a]	0.09	1.00	0.90[a]	.58[a]
	(0.10)	(0.13)	(0.01)	(0.61)		(0.000)	(0.001)
Stary	0.22	0.33	0.29	1.00	−0.09	−0.11	−0.17
	(0.29)	(0.11)	(0.13)		(0.61)	(0.54)	(0.38)
TAWT	0.13	−0.34	1.00	0.29	0.45[a]	0.35[a]	0.13
	(0.52)	(0.09)		(0.13)	(0.01)	(0.04)	(0.48)
Glycemia	0.10	1.00	−0.34	0.33	−0.31	−0.25	−0.15
	(0.64)		(0.09)	(0.11)	(0.13)	(0.23)	(0.49)
CW	1.00	0.10	0.13	0.22	0.34	0.26	0.59
		(0.64)	(0.52)	(0.29)	(0.10)	(0.34)	(0.11)

Note. [a]Statistically significant. DGEL = degree of germinal epithelial loss; CW = corporal weight; Glycemia = blood sugar levels 2 hr after the intraperitoneal administration of 3g/kg of weight of glucose; TAWT = thoracic aortic wall thickness; Stary = degree of atherosclerosis according to Stary's classification; Steato = liver steatosis (% of cells with fat deposition); Inflam = % of liver tissue with inflammation.

epithelial loss equal to or greater than 66%) and controls (germinal epithelial loss below that of the cases). Table 5 reports that in a raw analysis, steatosis and atherosclerosis increased the risk for presenting with severe germinal epithelial loss 8 and 6-fold, respectively. Moderate-to-severe inflammation of the liver, age above 50 years, or MS or any of its components (obesity, DM, or HBP) were not associated factors. When the analysis adjusting the variable of atherosclerosis or steatosis was performed, steatosis prevailed as the only significant risk factor, increasing the risk 12-fold for a severe DGEL.

Discussion

NAFLD was identified to be the main factor associated with severe germinal epithelial loss. This was demonstrated in an animal model and in a data analysis of human patients. It is striking that age and MS or its components were not

Table 5. Relevant Clinical or Morphologic Characteristics and Their Associations with a Severe DGEL in Human Patients.

Variable	OR	Raw 95% CI	*p*	OR	95% CI	*p*	OR	95% CI	*p*
				Atherosclerosis-adjusted[a]			Steatosis-adjusted[a]		
Steatosis	8.0	[1.2, 51.5]	.03	12.5	[1.2, 128.9]	.03	–	–	–
Athero	6.0	[1.0, 35.3]	.04	–	–	–	2.5	[0.3, 21.4]	.38
MS	1.3	[0.2, 7.5]	.74	0.7	[0.1, 6.8]	.78	0.2	[0.01, 6.0]	.24
Age ≥50 years	1.6	[0.3, 8.2]	.52	0.8	[0.1, 6.6]	.86	1.8	[0.3, 12.8]	.54
Obesity	0.8	[0.2, 3.9]	.78	1.5	[0.2, 12.1]	.67	0.5	[0.1, 3.8]	.53
DM	3.0	[0.6, 14.6]	.17	1.2	[0.1, 9.1]	.86	0.5	[0.1, 4.9]	.58
HBP	0.25	[0.02, 2.8]	.26	0.1	[0.01, 2.2]	.15	0.1	[0.01, 2.9]	.21
Inflam	1.4	[0.3, 7.7]	.67	0.9	[0.1, 7.7]	.97	0.3	[0.03, 3.6]	.37

Note. Severe DGEL: ≥66%; Steato: ≥20% of liver cells with fat deposition; Athero: degree of atherosclerosis ≥IV, according to Stary's classification; Obesity: body mass index ≥30; [a]Controlling other risk factors using a Mantel–Haenszel test. DM = type 2 diabetes mellitus; DGEL = degree of germinal epithelial loss; Inflam: >33% of liver tissue with inflammation; MS = metabolic syndrome; HBP = high blood pressure; OR = odds ratios; 95% CI: confidence intervals.

associated or correlated with germinal epithelial damage. This last finding concurs with results from a study by Ventimiglia et al. (2016), in which they concluded that different anomalies related to germinal epithelium function were not associated with MS. Nevertheless, there are controversial reports on this (Leisegang et al., 2016).

It has recently been suggested that NAFLD is not only a liver-specific disease, but also an early mediator of systemic diseases (Fotbolcu & Zorlu, 2016). Compared with patients without NAFLD, those with the disease exhibit increased liver-related complications and liver-related mortality, as well as an increased risk for developing type 2 diabetes, cardiovascular disease (CVD), chronic kidney disease, an increased risk for postoperative complications after major liver surgery, and an increased risk for developing certain malignancies, including primary liver cancer and colorectal cancer (Lonardo, Sookoian, Chonchol, Loria, & Targher, 2013; Adams, Anstee, Tilg, & Targher, 2017). Ours is the first study to report that the testes (germinal epithelium) are one of the organs damaged by NAFLD's multisystemic effect. Previous studies on animal models reported that certain liver alterations affected semen parameters. It was reported that paternal NAFLD in rats did not influence sperm morphology by decreasing testicular testosterone synthesis (Li et al., 2013). A study on the European eel (*Anguilla anguilla*) stated that the liver appears to play a role in determining sperm volume, through the production of certain fatty acids (Baeza et al., 2015).

NAFLD could cause damage to the germinal epithelium in numerous ways. In a recent meta-analysis of 27 studies, Polyzos et al. (2011) reported that lower circulating adiponectin levels were observed in patients with simple steatosis, compared with controls. Adiponectin has anti-inflammatory properties and its deficiency can trigger an increase in the production of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 (Kaser et al., 2015), and interleukin-1 alpha (IL-1 α) (Lonardo et al., 2013), among others. Physiologically, the immunosuppressive testicular microenvironment protects germ cells from immune attack, whereas under inflammatory conditions, tolerance is disrupted and immune cells and their mediators respond to germ cell self-antigens, inducing damage of the germinal epithelium (Pérez et al., 2012; Pérez et al., 2013). TNF-α and IL-6, both of which are elevated in NAFLD, have an essential role in testicular damage under conditions of inflammation. *In vitro* experiments reported that IL-6 induced germ cell apoptosis (Rival, Theas, Guazzone, & Lustig, 2015) and *in vivo* experiments induced focal inflammatory cell infiltration and germ cell sloughing in adjacent seminiferous tubules associated with alterations of tight junction protein expression and distribution (Pérez et al., 2012; Pérez et al., 2013). TNF-α, IL-1 α, and IL-1 β cytokines in the male reproductive tract (testis, epididymis, and sperm) may have certain physiologic functions. When the levels of these cytokines are higher than normal, as seen in conditions of inflammation, TNF-α, IL-1 α, and IL-1 β cytokines levels become very harmful to sperm production (Azenabor, Ekun, & Akinloye, 2015). High levels of TNF-α induced germ cell apoptosis *in vitro*. Neutralization of TNF-α with etanercept, a protein that mimics the inhibitory effects of soluble TNF receptors, reverted this apoptotic effect (Scallon et al., 2002). Concomitant with high levels of proinflammatory cytokines in the testicular microenvironment, nitric oxide (NO) production by macrophages increases. *Ex vivo* experiments have reported that NO released by testicular macrophages in rats with orchitis induced germ cell apoptosis. The presence of a competitive NOS inhibitor, L-NAME, reduced NO production by these cells and the percentage of seminiferous tubules with apoptotic germ cells (Pérez et al., 2012; Pérez et al., 2013).

Inflammation is also associated with oxidative stress. In NAFLD, histopathologic disease severity significantly correlated with oxidative stress parameters (Köroğlu et al., 2016). In addition, epidemiologic studies regarding male infertility have revealed that more infertile men suffer from acute or chronic inflammation of the genitourinary tract, which often occurs without any symptoms. The inflammatory reactions within the male genital tract are inevitably connected to oxidative stress. Oxidative stress is harmful, especially to spermatozoa, because it damages sperm DNA and causes sperm apoptosis (Azenabor et al., 2015). In animal models, drugs that reduce oxidative stress, such as doxycycline, have been reported to prevent testicular damage caused by a high-fat diet (Delgado-Enciso et al., 2014). Elevated oxidative stress parameters at the systemic level caused by NAFLD could be another cause of damage to the germinal epithelium. It is striking that in the analysis of the histologic alterations of the liver, steatosis was more relevant for presenting with germinal epithelial loss than the liver inflammation itself. The pathophysiology of NAFLD is complex and other mechanisms could be involved in testicular damage.

Age is an important factor in testicular health. However, the present study results did not correlate age with the DGEL. Studies on animals have reported that individuals with short life expectancies present with inflammatory, oxidative, and apoptotic testicular processes. In contrast, longevity confers anti-inflammatory, anti-oxidant, and anti-apoptotic capacities on the adult testis. Studies on humans have also reported that comorbidities and obesity strongly contribute to late-onset hypogonadism (Basaria, 2013). This concurs with the results of this study, in the sense that patients with NAFLD have a shorter life expectancy and a systematic inflammatory state (Matzkin et al., 2016), accompanied with accelerated testicular damage. This suggests that testicular damage is more related to the state of health of the liver and/or systemic inflammation, than to patient age.

In the present study, atherosclerosis was also a parameter associated with severe germinal epithelial loss. That association was not statistically significant when the adjustments for the presence of steatosis were made, supporting the idea that NAFLD simultaneously participates in the genesis of atherosclerosis, as well as in testicular damage, and that as a result, there is a noncausal association between atherosclerosis and testicular damage. The current study identified a significant correlation between steatosis/liver inflammation and the grade of atherosclerosis and/or aortic wall thickness. Specifically related to this, there is evidence to support the association of NAFLD with subclinical atherosclerosis that is unrelated to traditional risk factors and metabolic syndrome (Oni et al., 2013; Ampuero, Gallego-Durán, & Romero-Gómez, 2015).

The findings of the present study could be useful in the study of male fertility. Evaluating liver health is not a traditional approach in the management of patients presenting with fertility problems. Nevertheless, the first strategies recommended for improving fertility correctly include measures that reduce NAFLD symptoms. Weight reduction through diet and exercise and the taking supplements, such as glutathione, omega-3, selenium, and zinc, are supported in the medical literature and have positive effects on male fertility (Yao & Mills, 2016), as well as on NAFLD (Cave et al., 2007; Guo, Liong, So, Fung, & Tipoe, 2015; de Castro & Calder, 2017; Chen et al., 2013; Trappoliere et al., 2005). Some experimental therapies that have been independently tested for NAFLD (Wong et al., 2016) or fertility have also reported improvement benefits for both pathologies. These include vitamin E (Salas-Huetos, Bulló, & Salas-Salvadó, 2016) pioglitazone (Meneses et al., 2016), and pentoxifylline (Feyli, Ghanbari, & Keshtmand, 2017) therapies, but their results are still limited. New preventive and therapeutic perspectives in relation to fertility could involve strategies used in the approach to NAFLD, and vice versa. At this point, it is important to take into consideration that the degree of germinal epithelial loss that could be associated with clinical infertility remains unknown. Studies that associate testicular morphological variables with the fertility of individuals are limited, even in animal models. A previous report in rats in which dipentyl phthalate (toxic substance for the germinal epithelium) was administrated showed that it is required >50% of the seminiferous tubules are degenerated to cause a significant loss of fertility (reduction in pregnancies in their partners) compared to a control group (Lindström, Harris, Ross, Lamb, & Chapin, 1988). The degeneration of 20%–50% of the seminiferous tubules did not reduce the ability of the animals to generate pregnancies in their partners (Lindström et al., 1988). We believe that very important germinal epithelial losses are required to generate affectations in male fertility, without being able to determine a loss percentage associated with such affectation. Undoubtedly, further studies are required to generate more data about these observations, as well as assessing its clinical implications in patients.

A limitation of the present study was its small sample size, which could be the reason age or MS or its components were not associated with germinal epithelial damage. Damage to the germinal epithelium undoubtedly has a multifactorial origin and it is likely that MS and age participate to some degree in its pathophysiology. Nevertheless, these findings suggest that NAFLD could play an important role, because despite our small sample, it was strongly associated with DGEL in both the human and animal model studies. Future studies are needed to confirm these findings. Another characteristic of the present

study was that patients with severe germinal epithelial damage were evaluated and this should be taken into account in future comparisons.

In conclusion, the results presented herein suggest that NAFLD was a strongly associated factor with the severity of germinal epithelial damage. Additionally, the testis was identified as a probable target organ for damage caused by the disease. These findings suggest that NAFLD interferes more with testicular damage than other characteristics, such as diabetes, MS or age >50 years old, therefore, these results could lead for developing new strategies focus on the management of men with fertility problems. Further studies are necessary to confirm these findings.

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Authors' Contributions

Study Concept and Design: Ivan Delgado-Enciso, Alejandrina Rodriguez-Hernandez, Laura E. García-Labastida; Data collection: Uriel A. Lopez-Lemus, Alejandro Garcia-Rivera, Violeta M. Madrigal-Perez, Alejandro D. Soriano-Hernandez, Iram P. Rodriguez-Sanchez, Elizabeth Sanchez-Duarte, Raquel Garza-Guajardo, Oralia Barboza-Quintana, Ariana Cabrera-Licona; Statistical analyses and data interpretation: Margarita L. Martinez-Fierro, Jose Guzman-Esquivel; Critical revision of the manuscript: Jose Guzman-Esquivel, Gabriel Ceja-Espiritu.

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References

Adams, L. A., Anstee, Q. M., Tilg, H., & Targher, G. (2017). Non-alcoholic fatty liver disease and its relationship with cardiovascular disease and other extrahepatic diseases. *Gut*, *66*(6), 1138–1153. doi:10.1136/gutjnl-2017-313884

Ampuero, J., Gallego-Durán, R., & Romero-Gómez, M. (2015). Association of NAFLD with subclinical atherosclerosis and coronary-artery disease: meta-analysis. *Revista Española de Enfermedades Digestivas*, *107*, 10–16.

Azenabor, A., Ekun, A. O., & Akinloye, O. (2015). Impact of inflammation on male reproductive tract. *Journal of Reproduction & Infertility*, *16*, 123–129.

Baeza, R., Mazzeo, I., Vílchez, M. C., Gallego, V., Peñaranda, D. S., Pérez, L., & Asturiano, J. F. (2015). Relationship between sperm quality parameters and the fatty acid composition of the muscle, liver and testis of European eel. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *181*, 79–86.

Basaria, S. (2013). Reproductive aging in men. *Endocrinology and Metabolism Clinics of North America*, *42*, 255–270.

Cave, M., Deaciuc, I., Mendez, C., Song, Z., Joshi-Barve, S., Barve, S., & McClain, C. (2007). Nonalcoholic fatty liver disease: Predisposing factors and the role of nutrition. *The Journal of Nutritional Biochemistry*, *18*, 184–195.

Chen, Y., Dong, H., Thompson, D. C., Shertzer, H. G., Nebert, D. W., & Vasiliou, V. (2013). Glutathione defense mechanism in liver injury: Insights from animal models. *Food and Chemical Toxicology*, *60*, 38–44.

de Castro, G. S., & Calder, P. C. (2017). Non-alcoholic fatty liver disease and its treatment with n-3 polyunsaturated fatty acids. *Clinical Nutrition*, *37*(1), 37–55.

Delgado-Enciso, I., García-Rivera, A., Madrigal-Pérez, M. V., Rodriguez-Hernandez, A., Lugo-Radillo, A., Galvan-Salazar, H. R., … Guzman-Esquivel, J. (2014). Protective effect of doxycycline on germinal epithelial loss caused by a high-fat diet. *International Urology and Nephrology*, *46*, 895–899.

Fan, Y., Liu, Y., Xue, K., Gu, G., Fan, W., Xu, Y., & Ding, Z. (2015). Diet-induced obesity in male C57BL/6 mice decreases fertility as a consequence of disrupted blood-testis barrier. *PLoS One*, *10*(4), e0120775.

Feyli, S. A., Ghanbari, A., & Keshtmand, Z. (2017). Therapeutic effect of pentoxifylline on reproductive parameters in diabetic male mice. *Andrologia*, *49*(1), e12604. doi:10.1111/and.12604

Fotbolcu, H., & Zorlu, E. (2016). Nonalcoholic fatty liver disease as a multi-systemic disease. *World Journal of Gastroenterology*, *22*(16), 4079–4090.

Garcia-Rivera, A., Madrigal-Perez, V. M., Rodriguez-Hernandez, A., Martinez-Martinez, R., Martinez-Fierro, M. L., Soriano-Hernandez, A. D., … Delgado-Enciso, I. (2014). A simple and low-cost experimental mouse model for the simultaneous study of steatohepatitis and preclinical atherosclerosis. *Asian Journal of Animal and Veterinary Advances*, *9*, 202–210.

Guo, R., Liong, E. C., So, K. F., Fung, M. L., & Tipoe, G. L. (2015). Beneficial mechanisms of aerobic exercise on hepatic lipid metabolism in non-alcoholic fatty liver disease. *Hepatobiliary & Pancreatic Diseases International*, *14*, 139–144.

Haddad, Y., Vallerand, D., Brault, A., Spenard, J., & Haddad, P. (2011). NCX 1000 alone or in combination with vitamin E reverses experimental nonalcoholic steatohepatitis in the rat similarly to UDCA. *International Journal of Hepatology*, *2011*, 1–12.

Han, T. S., & Lean, M. E. (2016). A clinical perspective of obesity, metabolic syndrome and cardiovascular disease. *JRSM Cardiovascular Disease*, *5*, 204800401663337.

Higuchi, T. T., Palmer, J. S., Gray, L. E., & Veeramachaneni, D. N. R. (2003). Effects of dibutyl phthalate in male rabbits

following in utero adolescent or postpubertal exposure. *Toxicoloy Sciences*, *72*, 301–313.

Kaser, S., Moschen, A., Cayon, A., Kaser, A., Crespo, J., Pons-Romero, F., … Tilg, H. (2015). Adiponectin and its receptors in non-alcoholic steatohepatitis. *Gut*, *54*, 117–121.

Köroğlu, E., Canbakan, B., Atay, K., Hatemi, Đ., Tuncer, M., Dobrucalı, A., … Şentürk, H. (2016). Role of oxidative stress and insulin resistance in disease severity of non-alcoholic fatty liver disease. *The Turkish Journal of Gastroenterology*, *27*, 361–366.

Leisegang, K., Bouic, P. J., & Henkel, R. R. (2016). Metabolic syndrome is associated with increased seminal inflammatory cytokines and reproductive dysfunction in a case-controlled male cohort. *American Journal Reproductive Immunology*, *76*, 155–163.

Li, Y., Liu, L., Wang, B., Xiong, J., Li, Q., Wang, J., & Chen, D. (2013). Impairment of reproductive function in a male rat model of non-alcoholic fatty liver disease and beneficial effect of N-3 fatty acid supplementation. *Toxicology Letters*, *222*, 224–232.

Lindström, P., Harris, M., Ross, M., Lamb, J. C., & Chapin, R. E. (1988). Comparison of changes in serum androgen binding protein with germinal epithelial damage and infertility induced by di-n-pentyl phthalate. *Fundamental and Applied Toxicology*, *11*, 528–539.

Lonardo, A., Sookoian, S., Chonchol, M., Loria, P., & Targher, G. (2013). Cardiovascular and systemic risk in nonalcoholic fatty liver disease: Atherosclerosis as a major player in the natural course of NAFLD. *Current Pharmaceutical Design*, *19*, 5177–5192.

Madrigal-Perez, V. M., García-Rivera, A., Rodriguez-Hernandez, A., Ceja-Espiritu, G., Briseño-Gomez, X. G., … Delgado-Enciso, I. (2015). Preclinical analysis of nonsteroidal anti-inflammatory drug usefulness for the simultaneous prevention of steatohepatitis, atherosclerosis and hyperlipidemia. *International Journal of Clinical and Experimental Medicine*, *8*, 22477–22483.

Masarone, M., Federico, A., Abenavoli, L., Loguercio, C., & Persico, M. (2014). Nonalcoholic fatty liver: Epidemiology and natural history. *Reviews on Recent Clinical Trials*, *9*, 126–133.

Matzkin, M. E., Miquet, J. G., Fang, Y., Hill, C. M., Turyn, D., Calandra, R. S., … Frungieri, M. B. (2016). Alterations in oxidative, inflammatory and apoptotic events in short-lived and long-lived mice testes. *Aging (Albany NY)*, *8*, 95–110.

Meneses, M. J., Bernardino, R. L., Sá, R., Silva, J., Barros, A., Sousa, M., … Alves, M. G. (2016). Pioglitazone increases the glycolytic efficiency of human sertoli cells with possible implications for spermatogenesis. *International Journal of Biochemistry & Cell Biology*, *79*, 52–60.

Murillo-Zamora, E., García-Ceballos, R., Delgado-Enciso, I., Garza-Guajardo, R., Barboza-Quintana, O., Rodríguez-Sánchez, I. P., & Mendoza-Cano, O. (2002). Regional-level estimation of expected years of life lost attributable to overweight and obesity among Mexican adults. *Global Health Action*, *9*, 31642.

Oni, E. T., Agatston, A. S., Blaha, M. J., Fialkow, J., Cury, R., Sposito, A., … Nasir, K. (2013). A systematic review: Burden and severity of subclinical cardiovascular disease among those with nonalcoholic fatty liver; should we care? *Atherosclerosis*, *230*, 258–267.

Pérez, C. V., Sobarzo, C. M., Jacobo, P. V., Pellizzari, E. H., Cigorraga, S. B., Denduchis, B., & Lustig, L. (2012). Loss of occludin expression and impairment of blood-testis barrier permeability in rats with autoimmune orchitis: Effect of interleukin 6 on sertoli cell tight junctions. *Biology of Reproduction*, *87*, 122.

Pérez, C. V., Theas, M. S., Jacobo, P. V., Jarazo-Dietrich, S., Guazzone, V. A., & Lustig, L. (2013). Dual role of immune cells in the testis: Protective or pathogenic for germ cells? *Spermatogenesis*, *3*, e23870.

Polyzos, S. A., Toulis, K. A., Goulis, D. G., Zavos, C., & Kountouras, J. (2011). Serum total adiponectin in nonalcoholic fatty liver disease: A systematic review and meta-analysis. *Metabolism*, *60*, 313–326.

Rival, C., Theas, M. S., Guazzone, V. A., & Lustig, L. (2006). Interleukin-6 and IL-6 receptor cell expression in testis of rats with autoimmune orchitis. *Journal of Reproductive Immunology*, *70*, 43–58.

Salas-Huetos, A., Bulló, M., & Salas-Salvadó, J. (2017). Dietary patterns, foods and nutrients in male fertility parameters and fecundability: A systematic review of observational studies. *Human Reproduction Update*, *23*(4), 371–389. doi:10.1093/humupd/dmx006

Scallon, B., Cai, A., Solowski, N., Rosenberg, A., Song, X. Y., Shealy, D., & Wagner, C. (2002). Binding and functional comparisons of two types of tumor necrosis factor antagonists. *Journal Pharmacology Experimental Therapeutics*, *301*, 418–426.

Stary, H. C. (2000). Natural history and histological classification of atherosclerotic lesions: An update. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *20*, 1177–1178.

Trappoliere, M., Tuccillo, C., Federico, A., Di Leva, A., Niosi, M., D'Alessio, C., … Loguercio, C. (2005). The treatment of NAFLD. *European Review for Medical and Pharmacological Sciences*, *9*, 299–304.

Ventimiglia, E., Capogrosso, P., Colicchia, M., Boeri, L., Serino, A., Castagna, G., … Salonia, A. (2016). Metabolic syndrome in white European men presenting for primary couple's infertility: investigation of the clinical and reproductive burden. *Andrology*, *4*, 944–951.

Wainwright, P., & Byrne, C. D. (2016). Bidirectional relationships and disconnects between NAFLD and features of the metabolic syndrome. *International Journal of Molecular Sciences*, *17*, 367.

Wong, V. W., Chitturi, S., Wong, G. L., Yu, J., Chan, H. L., & Farrell, G. C. (2016). Pathogenesis and novel treatment options for non-alcoholic steatohepatitis. *The Lancet Gastroenterology & Hepatology*, *1*, 56–67.

Yao, D. F., & Mills, J. N. (2016). Male infertility: Lifestyle factors and holistic, complementary, and alternative therapies. *Asian Journal of Andrology*, *18*, 410–418.