Proteomics in seminal plasma as predictor of successful sperm retrieval in infertile azoospermic men

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1. INTRODUCTION

The proteomes of a cell or tissue (either in normal or pathological state) are known to have a more dynamic nature than their genomic frameworks. Although massive advances have been achieved in our understanding of the genomic causes of pathophysiological diseases, there are still considerable gaps in our knowledge that hinder the identification of suitable strategies for early diagnosis of disease and development of effective treatments. Based on recent studies, it is plausible that a proteomic approach to disease investigations may overcome some of these limitations (Hanash, 2003), by enabling an interrogation that includes changes in protein expression and post-translational modifications. The potent role of proteomics as a reliable predictor has been reported in many studies especially at the level of tissue or the whole cell. In addition, the application of proteomics to biological fluids has allowed the discovery of biomarkers for diagnosis and early detection of illnesses. Both of these proteomic approaches were considered as main prospects to design a new study for the development of novel protein biomarkers of male infertility, via an investigation of the seminal plasma from infertile patients with azoospermia. Couples are referred to as being infertile when they are unsuccessful at being able to reproduce after a year of unprotected intercourse. Infertility occurs in nearly 15% of the general population and its climbing rate highlights the need for infertility treatments (Leung *et al*., 2018). It is known that the male factor contributes in part or whole to 50% of total cases of infertility (Leung *et al*., 2018).

Azoospermia is one of the reasons that males can be infertile, and is defined as zero spermatozoa in the semen following at least two assessments by microscopy. Clinically, azoospermia is divided into two major pathological forms, non-obstructive azoospermia (NOA) and obstructive azoospermia (OA). If the cause is due to a physical obstruction in the male reproductive tract, commonly congenital or acquired defects in the epididymis or vas deferens, it is classified as OA. In contrast, NOA refers to the inability of the testis to make sperm, and this pathology has been categorized into several sub-groups based on various perturbations in the testis. These sub-groups include spermatogenic arrest (SA) or also called maturation arrest, mixed testicular atrophy (MA) otherwise known as hypospermatogenesis,

Sertoli cell-only syndrome (SCO) and Klinefelter's syndrome. The definition of each subgroup is shown in the Table 1-1.

For each azoospermia sub-group, assisted reproductive technology (ART) has different solutions. A key concern is that azoospermic men constitute 1% of the general population but the only diagnostic tool available for the clinician is histology following testis biopsy, which is an extremely invasive surgical procedure (McLachlan *et al*., 2007; Dohle *et al*., 2012; Gudeloglu and Parekattil, 2013) (Figure 1.1). Hence there is a high necessity to find a non-invasive diagnostic method, but despite many efforts, this goal has yet to be achieved (Barceló *et al*., 2018; Wu *et al*., 2016 and for reviews see Kovac *et al*., 2013). Tissues and samples for a non-invasive test could include whole semen, spermatozoa, or seminal plasma, and a number of studies have now applied high throughput mass spectrometry-based proteomics to these sources (for reviews see Milardi *et al*., 2013 and Camargo *et al*., 2018).

Human seminal plasma has a protein concentration of 35-55mg/ml and is a rich, easily accessible and promising source of potential biomarkers (Pilch and Mann 2006). Up till 2017, seminal plasma has been reported to contain 4188 proteins secreted by testis and epididymis (10%), prostate (20%) and seminal vesicles plus Cowper's glands (70%) (Camargo *et al*., 2016). Additionally, of these 4188 proteins, 2020 proteins are considered as redundant proteins and 2168 are non-redundant (Gilany *et al*., 2015). Seminal plasma proteins have been observed are mainly involved in protein binding (~40-50%) and catalytic activity (~29-39%). From the view point of localization (cytoplasmic or extracellular), seminal plasma proteins are mostly cytoplasmic (58.7%) such as proteins found in the cytoskeleton and the endomembrane system or in vesicles. The next main group are extracellular proteins (~21.2%) (Rolland *et al.,* 2013, Camargo *et al.,* 2018). Information for the rest of seminal plasma proteins regarding the function and localization is still unclear. However, different references concluded that seminal plasma lacks nucleic acid-binding proteins, transcription regulators, and membrane receptors and channels (Pilch and Mann, 2006; Milardi *et al*., 2012).

To design the backbone of this study, three important viewpoints: proteomics, azoospermia and seminal plasma have been considered. In the first part of this study, the seminal plasma proteomes of two azoospermic subgroups were compared by mass spectrometry. The two

subgroups were i) men with mixed testicular atrophy, from whom advanced spermatids had been successfully retrieved at biopsy for ART, and ii) men with Sertoli cell-only syndrome, from whom no spermatids were present for retrieval. For doing the biopsy a combined trifocal and microsurgical testicular sperm extraction was used and this biopsy technique is known as the best technique for testicular sperm retrieval in low-chance NOA (Marconi *et al*., 2012).

The hypothesis to be tested was that analysis of differentially expressed proteins between these two groups could uncover biomarker(s) useful for the non-invasive diagnosis of the presence of advanced spermatids in the testis of NOA patients. The mass spectrometry-based comparison of these seminal plasma samples provided a list of proteins that were differentially expressed, from which three proteins were selected for further validation by independent methods, including immunohistochemistry (IHC), Western blotting, and enzyme-linked immunosorbent assay (ELISA). The candidate proteins chosen were superoxide dismutase 1 (SOD1), superoxide dismutase 3 (SOD3) (both from the same protein family) and Galectin-3-binding protein (LGALS3BP).

In the second part of the study, the existing data came from a previous work in the department of urology, pediatric urology and andrology, (Justus Liebig University, Giessen, Germany) using the method of two-dimensional gel electrophoresis (2-DE) was used for follow-up assessments. Previously, with the method of 2-DE, seminal plasma proteome of normal men before and after vasectomy was compared and a list of significant proteins was obtained (unpublished data). While men after vasectomy have a mimic of OA, in the current study a comparison was done between the groups of OA and NOA by using the method of Western blotting for one candidate protein. This candidate protein was Cysteine-Rich Secretory Protein-1 (CRISP1) and it was chosen for further validation because it had shown the totally absence in the seminal plasma after vasectomy. In continue, CRISP1 also was compared among the various sub-groups of NOA (mixed testicular atrophy (MA), spermatogenesis arrest (SA), Sertoli cell-only syndrome (SCO) and patients with Klinefelter's syndrome).

This Introduction will now focus on the character and function of the selected candidate proteins. Firstly, the SOD family of enzymes will be considered, and subsequently information about the CRISP1 will be presented (see Section 1.3). Finally, in the section of 1.4 of introduction, the available literature for LGALS3BP will be reviewed.

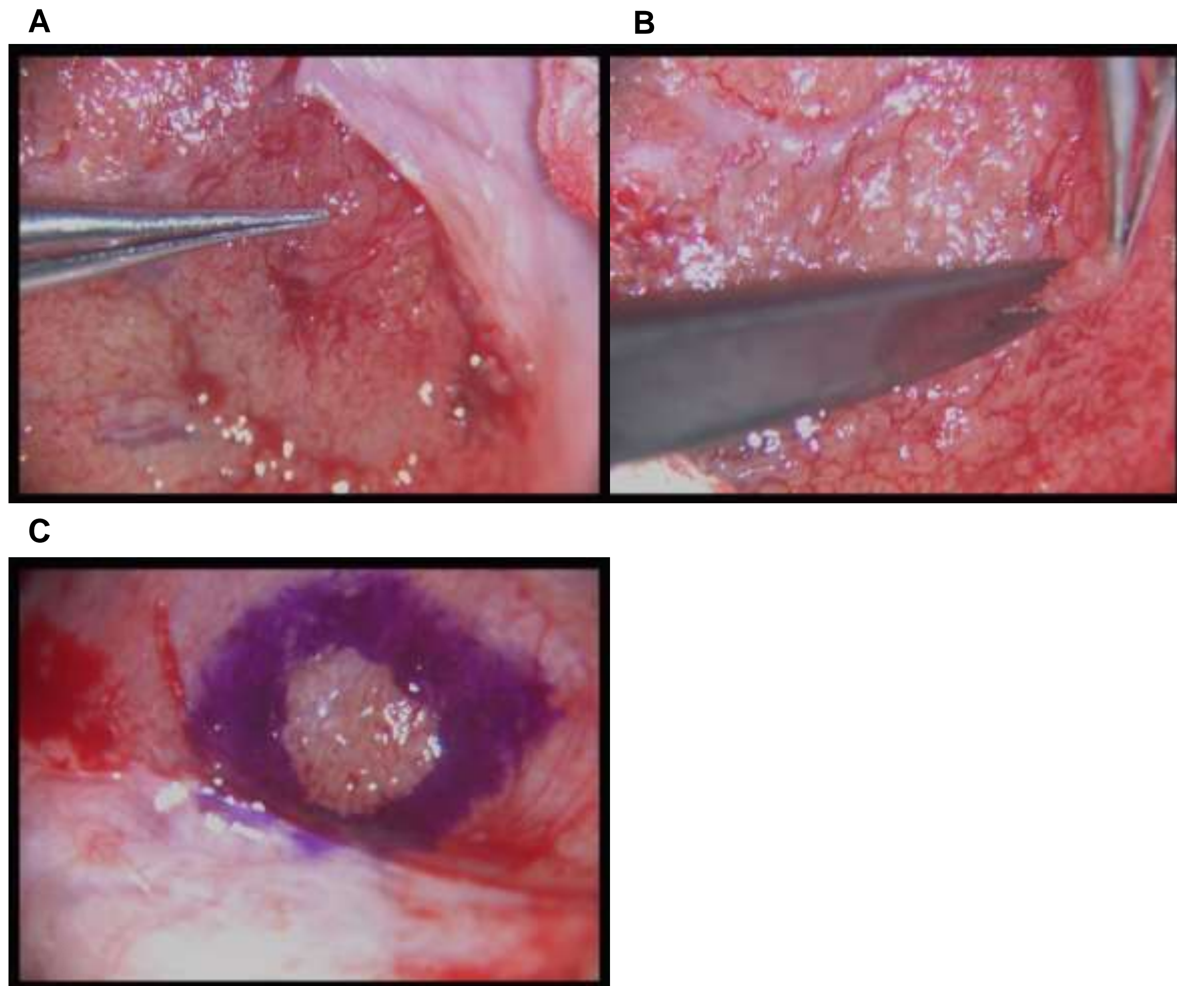


Figure 1-1:

The procedure of micro dissection TESE (M-TESE) for a patient with non-obstructive azoospermia (NOA) who underwent a testicular biopsy.

A: M-TESE: overview after midline incision and exposure of testicular tissue; B: Micro dissection of enlarged testicular tubules with higher diameters representing regular spermatogenesis; C: Area of enlarged tubules artificially marked with purple dye

Table 1-1: Definition of various subgroups of the non-obstructive azoospermia

	NOA subgroups	Definition
1	**Spermatogenic arrest or maturation arrest**	Men, who had either severe oligospermia (partial arrest) or azoospermia (complete arrest), normal testicular volume, and depending on the etiology normal, high, or low levels of gonadotropins. Level of interruption has seen in the differentiation of germinal cells of specific cellular type, which elicits an altered spermatozoa formation. The arrest is most frequently observed at primary spermatocyte level. Maturation arrest is subcategorized into early maturation arrest, in which only spermatogonia or spermatocytes are found, and late maturation arrest, in which spermatids are detected without spermatozoa. Although spermatozoa are not visualized on histopathological evaluation, some men with maturation arrest may have small foci of spermatozoa in the testis (Weedin *et al.*, 2011).
2	**Mixed testicular atrophy or hypospermatogenesis**	Men, who have a synchronous occurrence of both seminiferous tubules containing all germ cell types or Sertoli cell-only tubules, in variable proportions. In tubules containing germ cells, different types of abnormalities in spermatogenesis may be seen. There is a chance for presence of adult spermatids in the biopsy, even in small numbers (Cerilli *et al.*, 2010).
3	**Sertoli cell-only syndrome (also known as Del Castillo syndrome or germ cell aplasia)**	Men, who have a condition of the testis in which only Sertoli cells line the seminiferous tubules. Absent spermatogenesis despite the presence of both Sertoli and Leydig cells (McLachlan *et al.*, 2007; Stouffs *et al.*, 2016).
4	**Klinefelter's syndrome**	Men, who have symptoms that result from two or more X chromosomes (47, XXY). Small testicles, reduced number of intratubular germ cells, some tubules are Sertoli cell-only, tubular sclerosis, Leydig cell nodules and rarely-seen foci of focal spermatogenesis. In fact, Klinefelter's syndrome is a type of hypospermatogenesis, but is separated into its own group because of the chromosomal abnormality (Bearelly and Oates, 2019).

1.1. REACTIVE OXYGEN SPECIES

The human male reproduction system, like all other systems of the mammalian body, has been equipped with highly developed enzymes in order to fight invading chemical species termed Reactive Oxygen Species ”ROS”. The toxicity of these species is expressed with “oxidative stress” and the cell defending agents are antioxidants. The mechanism of pathogenesis is initiated by increased ROS levels, and two different possibilities are considered as causes. First, an uncontrolled increase in the products of oxidation pathways and second, a decrease in antioxidant defense mechanisms, however, the possibility that both likely contribute together also has been proved (Gharagozloo *et al*., 2016).

During the procedure of oxidative phosphorylation, cells obtain their required energy by oxygen consumption. This process results in the generation of some highly reactive and short-lived metabolites such as reactive oxygen species at the end of this chemical reaction. Meanwhile, reactive nitrogen species (RNS) also remain at the end of similar biochemical pathways. The main content of these metabolites are free radicals such as the hydroxyl radical ion (OH·), ionic species such as superoxide anion (O2·−) and nitric oxide (NO·), or peroxynitrite (ONOO2) which is a combined molecule of O2·− and NO·, and finally neutral but reactive molecules such as hydrogen peroxide (H2O2) (O’Flaherty, 2014a). These molecules can attack and damage all other macromolecules including lipids, proteins, and nucleic acids. ROS has been linked to a number of pathologies and autoimmunity, and leading the cell to undesirable apoptosis (Schieber and Chandel, 2014).

Some metal ions like divalent iron and copper ions are also a suitable target for free radicals by entering Haber–Weiss and Fenton's reactions, and this combination can provide an attacking reaction accompanied by enhanced ROS generation (more information in section 1.2.) (Valko *et al*., 2005).

As mentioned earlier, oxidative stress results from an imbalance between ROS components and antioxidants (Agarwal *et al*., 2014). The interaction among ROS components and antioxidants should stay in the balance and oxidative stress cannot be omitted totally from the live body. It is because there are positive roles for oxidative stress. It is well known that ROS can have beneficial aspects, especially in signaling pathways for anticancer strategy, stem-cell renewal, longevity and aging-related disease tolerance (Gorrini *et al*., 2013; Yan,

2014; Schieber and Chandel, 2014). This theme continues into reproduction, because a low level of free radicals is critical for spermatozoa to be able to conjunct with and fertilize an ovum. Generation of free radicals occurs in mammalian sperm cells when they are under capacitation conditions. In this stage, some free radicals, including superoxide, H2O2, and nitric oxide are produced by spermatozoa to induce a successful acrosome reaction (Aitken *et al*., 1998; O'Flaherty and De Souza, 2011; De Lamirande and O'Flaherty, 2008; O'Flaherty, 2015).

Antioxidant enzymes (superoxide dismutase, peroxiredoxins, thioredoxins, thioredoxin reductases, and glutathione s-transferases) are mainly dealing with ROS, whilst other non-enzyme-antioxidants like some vitamins or minerals are also involved. The human male reproduction system has a good source of antioxidant enzymes, which coordinately work to assure the normal function of stress oxidative protection. Several papers have been published to clarify the function of antioxidant enzymes in the male reproductive system (O'Flaherty, 2014b; for reviews see Wagner *et al*., 2017; for reviews see Alahmar, 2019; O'Flaherty, 2019).

In this Ph.D. project, it has been focused on the very important and high impact antioxidant family confronted with oxidative stress, namely, the superoxide dismutase family. Some researchers believe the dysfunction of this family of enzymes is the major culprit for damaging spermatozoa (Alvarez *et al*., 1987; O'Flaherty, 2014b). To this end, several key findings that demonstrate the role of this family in the male reproductive system are examined in more detail in the following sections.

1.2. The Superoxide Dismutase (SOD) family in the male reproductive system: Location and biochemistry

The scientific knowledge about superoxide dismutase was started with discoveries of McCord and Fridovich (1969). These authors discovered the enzymatic activity of superoxide dismutase, after first having described the SODs as a group of metalloproteins (McCord and Fridovich, 1969, 1988). The family of superoxide dismutase is comprised of three SOD isoforms that are present in aerobic cells. Superoxide dismutase 1 (SOD 1) is a copper-zinc SOD (Cu-Zn SOD or SOD1) located in the cytosol and also in the mitochondrial

intermembrane space (Lindsey *et al.*, 2011). This isoform was the first one found (McCord and Fridovich, 1969). The SOD1 gene is located on chromosome 21 (21q22.1) and the expressed protein is a dimer molecule (Human PubMed, Gene ID: 6647, https://www.ncbi.nlm.nih.gov/gene/6647).

Superoxide dismutase 2 (SOD 2) was discovered in 1973 and the protein was originally known as a manganese SOD (Mn-SOD or SOD2) (Weisiger and Fridovich, 1973). This protein is only found in mitochondria and the SOD2 gene is located on chromosome 6 (6q25.3). SOD2 protein forms as a tetramer molecule (Human PubMed, Gene ID: 6648 https://www.ncbi.nlm.nih.gov/gene/6648).

The third isoenzyme of SOD family was named superoxide dismutase 3 (SOD3) or secreted and extracellular SOD (EC-SOD) (Marklund, 1982). The SOD3 gene is on chromosome 4 (4p15.3-p15.1), and similar to SOD2 is expressed as a tetramer protein (Human PubMed, Gene ID: 6649, https://www.ncbi.nlm.nih.gov/gene/6649). SOD3 has copper and zinc in its reactive center, as observed for SOD1.

The SOD members convert the superoxide anion ($O2\cdot-$), which is a moderately reactive oxygen species, into a strong and highly reactive one, namely, H2O2 ($2O2\cdot- + 2H+ \rightarrow H2O2 + O2$). The mechanism appears strange and raises the question as to why the superoxide dismutase family intensifies the potential activity of ROS components, rather than removing them? The answer can be found regarding the content of other antioxidant enzymes (e.g. peroxiredoxin 6) for which H2O2 produced by SODs is considered a better substrate for removal.

However, produce a suitable substrate for the other antioxidant enzymes is not the single duty of the family of superoxide dismutase. They are responsible for avoiding the organization of the hydroxyl radical (HO·), as well. The mechanism of this reaction can be explained with two steps. In the first step, via the Haber–Weiss reaction, divalent iron is oxidized by H2O2 to produce a ferric molecule (trivalent iron), hydroxyl radical and a hydroxide ion ($Fe2+ + H2O2 \rightarrow Fe3+ + HO\cdot + OH-$). In the second step, via Fenton's reaction, the trivalent iron, is reacted with another H2O2 molecule ($Fe3+ + H2O2 \rightarrow Fe2+ + HOO\cdot + H+$) thus forming divalent iron, a hydroperoxyl radical and a proton (Halliwell, 2012). SOD enzymes rapidly

react the O2·− with free hydroxyl radical molecules formed by the Haber-Weiss reaction to prevent them staying free (Kehrer, 2000). The free hydroxyl radical has a high affinity to react with lipids and arrange lipid peroxidation. The plasma membrane of human spermatozoa is composed of numerous complex lipid molecules, hence both intracellular and extracellular SODs are needed to prevent the formation of hydroxyl radicals and potential destruction of the cell (Alvarez and Storey, 1984; Agarwal *et al*., 2012). In this regard, the ratio of SODs activity will compare first by considering the spermatozoa as a single cell (intracellular) and second by considering the spermatozoa in reaction with seminal plasma (extracellular).

As human spermatozoa have a very small amount of cytoplasm, it can be presumed that there is limited intracellular protection against ROS by SOD1 (Cu-Zn SOD). This subject was proven by two different investigations (Aitken *et al*., 1994; Aitken *et al*., 1996), and subsequently it has been shown that the contributions of SOD2 and SOD3 are also negligible (Peeker *et al*., 1997).

However, despite the limited amounts of SODs in spermatozoa, more recent investigations have shown in terms of enzyme activity that SOD1 is present to a much greater extent than either SODs 2 or 3 (Kobayashi *et al*., 1991; Peeker *et al*., 1997).

In older references a noticeable enzyme activity for SODs in the mitochondria of spermatozoa was reported (Saaranen *et al*., 1989). It was strange when the plentiful occurrence of mitochondria in spermatozoa is considered. However, recently the dual role of mitochondria to produce ROS and in parallel to defend against free radicals in spermatozoa has become more apparent (Zhang *et al*., 2009). The current understanding is that mitochondria are the main source of ROS in spermatozoa (Koppers *et al*., 2008). Excessive ROS can initiate the mitochondria-dependent apoptotic signaling pathway in spermatozoa, which is proven in oligospermia and asthenospermia (Zhang *et al*., 2014).

SOD activity in extracellular fluids, for example in seminal plasma, can be readily measured by a colourimetric assay (Arthur and Boyne, 1985; Gavella *et al*., 1996a). Both SOD1 and SOD3 are present in high activity in seminal plasma (75% and 25%, respectively), at a level 20 fold greater than in human blood plasma (Peeker *et al*., 1997). It has been proposed that

this high extracellular SOD activity in seminal plasma can compensate for the lower amounts of SODs in spermatozoa (O’Flaherty, 2014b).

The role of SODs in capacitation has been clearly demonstrated. Superoxide dismutase blocks the capacitation procedure in spermatozoa. Superoxide anion ($O2^{\cdot -}$) and nitric oxide ($NO^{\cdot}$) are both known as second messengers (after cAMP) involved in signal transduction cascade to initiate sperm capacitation (Herrero *et al*., 2000; Jin and Yang, 2017). SOD enzymes block the sperm capacitation via their effect on the superoxide anion ($O2^{\cdot -}$) but not on nitric oxide ($NO^{\cdot}$) (De Lamirande *et al*., 2009). However, the relative contribution of each SOD isoform (1, 2 or 3) to sperm capacitation remains elusive and requires further study. Maybe new information, which shows that extracellular actions of SODs have the ability to block sperm capacitation, potentially give a clue (O'Flaherty, 2015). This fact, besides the confirming that these ROS forms ($O2^{\cdot -}$ and $NO^{\cdot}$) are produced at the level of the sperm plasma membrane (O'Flaherty, 2015), also suggests that SOD3 (as a secreted and extracellular enzyme) may contribute to extracellular SOD activity with respect to sperm capacitation. More explanation is that secreted SOD3 from spermatozoa to the seminal plasma can accumulate close to the membrane of spermatozoa and influence the capacitation from outside (extracellular environment).

SOD1 and SOD3 in semen have been reported to originate via contributions from spermatozoa and apocrine secretions from the prostate gland epithelium, although these are not necessarily the sole sources (Aumuller and Seitz, 1990; Peeker *et al*., 1997). For example, SOD protein has been found in a search of secreted epididymal proteins, although the individual isoforms were not distinguished (Dacheux *et al*., 2006). Interestingly, there are two animal case reports about boar and goat spermatozoa that show completely opposite results regarding the activity of SOD1 in spermatozoa, when traveling along the epididymis from the caput to the cauda (Park *et al*., 2012; Rana *et al*., 2016). The boar research shows an increasing in the SOD1 activity (and also the protein level of SOD1) from 41.9% (in the caput) to 46.7% (in the corpus) and finally to 72.5% (in the cauda) through the epididymis. Simply it can be explained that the ratio of SOD1 protein increases 1 to 1.11 to 1.73fold over those 3 segments. Epididymal maturation of boar spermatozoa was suggested to be the cause of such a drastic increment (Park *et al*., 2012). The goat research inversely shows a significant

decrease ($P < 0.05$) of SOD1 activity from the caput to cauda during epididymal transit in parallel with increases in SOD1 activity in the luminal fluid of the epididymis (Rana *et al*., 2016). Despite these contradictory results, it can be concluded that the epididymal epithelium is a source of SODs in seminal plasma. There is no similar investigation on human or even mice models to record the changes in SOD quantity or activity in spermatozoa during epididymal maturation.

Interestingly, SOD3 (EC-SOD) has been found to have a high affinity for heparin, and in the human body the main portion of SOD3 is found to be attached to heparin sulfate on cell surfaces and in tissue interstitial matrix (Karlsson and Marklund, 1989; Sandström *et al*., 1993; Karlsson *et al*., 1994). In contrast, SOD3 in seminal plasma has a low affinity for heparin and this fact causes a weak linkage of SOD3 to spermatozoa and other cell surfaces, for example in the vagina. It is thought that the heparin-binding domain of SOD3 has a high susceptibility for proteolytic cleavage by a trypsin-like prostate-specific enzyme that is present at a very high concentration in seminal plasma (Peeker *et al*., 1997).

SOD enzyme activity has roles elsewhere in the male reproductive tract. It is known that erectile dysfunction is linked to ROS accumulation in the corpus cavernosum of the penis, and similarly the superoxide anion has been shown to be a major player (Alan *et al*., 2010, Silva *et al*., 2013). In an animal model of erectile dysfunction via surgical cavernous nerve injury, a significant increase of SOD enzyme activity was demonstrated (Wang *et al*., 2015). Hence it appears that SOD enzyme activity and ROS may be important in erectile dysfunction. However, a continuing theme in both this field and much of the SOD/ROS literature in general is that most studies have primarily focused on SOD enzyme activity, and the relative contributions of the SOD isoforms remains to be determined.

1.2.1. Superoxide Dismutase (SOD) family and sperm parameters

The superoxide dismutase family of proteins have functions as a primary enzymatic antioxidative defense in the male reproductive system, and much effort has focused on their correlation with semen parameters. It is estimated that a high percentage of male infertilities are due to motility impairments (otherwise known as asthenozoospermia) yet this condition remains poorly understood in terms of the latest biochemical, molecular and genomic analysis methods (Bonanno *et al*., 2016). Previous reports show a significant relationship

exists between the motility of spermatozoa and SOD activity from different sources including human semen, seminal plasma, and spermatozoa themselves. A positive significant relationship between superoxide dismutase and the motility of spermatozoa in human seminal plasma was published more than 35 years ago (Nissen and Kreysel, 1983). It was discussed that a positive correlation between SOD activity in seminal plasma and motility has been occurred due to the prevention of lipid peroxidation of the sperm membrane by SOD. Since then, further positive associations between seminal SOD activity, motility and sperm count have been documented, as well as significant negative associations to abnormal morphology (Kobayashi *et al*., 1991; Shiva *et al*., 2011; Atig *et al*., 2012; Yan *et al*., 2014; Atig *et al*., 2017).

In keeping with these relationships, a high negative correlation exists between SOD activity and malondialdehyde (MDA) in seminal plasma of infertile patients (Tavilani *et al*., 2008; Chyra-Jach *et al*., 2018; for reviews see Huang *et al*., 2018). MDA is known as the most important marker of lipid peroxidation (LPO), and high amounts of seminal MDA suggests that excessive membrane lipid peroxidation might be responsible for abnormal sperm motility. However, this hypothesis is not accepted by everyone and it was explained that the decreased motility is not related to LPO, and "the unbalance of the redox mechanism" that favors oxidative stress was introduced as the main reason (Micheli *et al*., 2016). Also, some scientists postulated a multifactorial etiology to relate sperm motility and SOD activity. For example, Siciliano *et al*., hypothesized a limited influence of SOD deficiency on sperm motility. This group demonstrated that SOD activity in seminal plasma of normal viscosity from asthenozoospermic men was similar to fertile controls. In contrast, SOD activity was significantly lower than controls for asthenozoospermic men with a hyper viscosity semen, suggesting that SOD deficiency may occur in conjunction with other ejaculate pathologies (Siciliano *et al*., 2001). Conflicting results have also been reported for other sperm parameters (morphology and concentration) (Kobayashi *et al*., 1991; Macanovic *et al.,* 2015). These kinds of controversy and the high variability of results between SOD activity and sperm parameters in seminal plasma, strongly indicate that the "activity" of superoxide dismutase in seminal plasma cannot be a useful factor to predict human male fertility, and other factors with proper stability are required. In this regard, it is likely that knowledge about the protein concentrations of individual SOD family members will be a useful factor in the

replacement of their enzymatic activity. For example, a recent study did not observe any correlation between SOD activity and sperm parameters in seminal plasma, but did detect a positive correlation between protein concentration of SOD2 and sperm count, and SOD1 expression and progressive motility and sperm morphology (Macanovic *et al*., 2015).

1.2.2. Superoxide dismutase family: Lessons from animal models

Animal models have used two different approaches to investigate the connection between superoxide dismutase family and the quality of sperm, seminal plasma, or other parts of the reproductive system of male animals. The first approach is to discover more about the molecular details of SOD functions in these tissues. The second approach has used animal models with the purpose of testing new drugs and supplements that target the antioxidant properties of this enzyme family. For example Gharagozloo *et al*., used a tissue-specific animal knockout model of oxidative stress to test the efficacy of a newly designed antioxidant supplement with constituents including folic acid, lycopene, selenium, vitamin C, vitamin E and zinc. The mouse Gpx5 knockout was nullified for the H2O2-scavenging activity of glutathione peroxidase 5 in the epididymis. The effectiveness of the new supplement was investigated by quantitative real-time PCR for mRNA accumulation of extracellular superoxide dismutase (Sod3), catalase and glutathione S-transferase in cauda epididymal extracts. While a significant increase ($p \leq 0.05$) was seen for transcripts of catalase and glutathione S-transferase, in contrast, it was disclosed that none of those antioxidant elements had significantly affected Sod3 expression (Gharagozloo *et al*., 2016). More studies are needed to find the specific antioxidants among the dietary molecules such as vitamin C, E and carotenoids, which can works synergistically with SOD family members.

To determine the impact of the superoxide dismutase family on reproduction and fertility, various studies have assessed either the loss of Sod activity by global Sod gene knockout in mice, or the overexpression of Sod genes as powerful tools to study the metabolic functions of these enzymes (Table 1-2) (Lei *et al*., 2016). The absence of Sod3 is without effect on the lifespan of mice. Null Sod3 males and females mice not only develop normally to fertile age but also, after mating, have a normal number and size of litters (Sentman *et al*., 2006). However, Sod3-null mice show a potential to be overweight.

Overexpression of Sod3 has detrimental outcomes for many organs including brain, heart and vascular system as well as immune system (Oury *et al*., 1992). However, the effects of Sod3 overexpression on the male reproduction system have not yet been reported.

Sod1 null female mice are totally infertile and their embryos die 10 days after pregnancy. Ovarian dysfunction that results in decreased progesterone secretion has been reported as the main reason for this infertility (Ho *et al*., 1998; Matzuk *et al*., 1998; Noda *et al*., 2012). Determining the fertility situation of Sod1 null male mice is controversial. Initial studies from three independent laboratories showed that Sod1 null male mice are fully fertile (Ho *et al*., 1998; Matzuk *et al*., 1998; Tsunoda *et al*., 2012).

Interestingly, a more recent study rejected all previous results about the fertility of (Sod1−/−) male mice (Garratt *et al*., 2013). It was clarified that the fertility status of Sod1 null male mice is associated with the female strain used in the experiment. The normal fertility of (Sod1−/−) male mice had been reported by simply pairing with female mice of the same strain (C57BL/6) and checking for litters. However, when male (Sod1−/−) mice were mated with females from a different genotype (CBA strain), they were almost completely infertile (Garratt *et al*., 2013). The infertile phenotype included significantly reduced sperm motility ($p=0.003$) and velocity path ($p=0.001$) compared to wild type Sod1 male mice (Sod1+/+). Moreover it was shown that, with the exception of a minor smaller testis mass for Sod1 null male mice ($p=0.039$), there were no differences in the weights of the other reproductive organs, namely, seminal vesicles and epididymis (Garratt *et al*., 2013). Regarding the inconsistent results of mating male mice with females of different genetic backgrounds, the cause remains to be determined. However documents show a different reproductive physiology between those two female mice strains (C57BL/6 versus CBA), which it seems significantly effects the litter size of Sod1 null male mice (Haig, 2003).

The other fact, which has been confirmed by several studies is related to the ~25% reduction of lifespan for Sod1 null male mice (Sod1−/−) from almost 30months to 22months in comparison with wild type mice (Zhang *et al*., 2017). Presumably this points towards regulation of apoptosis by SODs in key tissues.

Sod1 heterozygote mice (Sod1+/−) are fertile in both male and female and it seems there is a dose-response relationship between the levels of Sod1 and normal pathophysiology of mice

and indeed just one wild type allele of Sod1 is sufficient for normal breeding (Lei *et al*., 2016).

Male mice overexpressing Sod1 appear normal, but these animals exhibit certain phenotypes such as thymic abnormalities, enhanced apoptosis of bone marrow cells, diminished serotonin uptake in platelets, all of which are phenotypes also found in patients with Down's syndrome (Lei *et al*., 2016). As already mentioned, the location of the Sod1 gene is on chromosome 21 and therefore it is not surprising that similar symptoms have been reported for mouse models that overexpress Sod1 compared to mouse models of Down's syndrome caused by trisomy of chromosome 21 (Peled-Kamar *et al*., 1995).

Sod2 null mice (Sod2−/−) is known as a neonatal lethal phenotype. Mice with this genetic ablation exhibit severe neurodegeneration and cardiovascular damage, and die between 1 to 24 days after birth (Lebovitz *et al*., 1996; Huang *et al*., 2001). The variable longevity is related to the genotype of mice, and no abnormality in the genital tract was seen in these animals (Calogero *et al*., 2017). More interestingly, a normal folliculogenesis and production of viable offspring has been reported for Sod2 null female mice (Sod2−/−) when it's neonatal ovaries were transplanted to the bursa of wild type hosts. This result suggests that the Sod2 enzyme is dispensable in terms of ovarian function (Matzuk *et al*., 1998).

As observed for the Sod1 heterozygote mice, both male and female Sod2 heterozygote mice (Sod2+/−) are fertile (Strassburger *et al*., 2005). In contrast, Sod2 male mice with overexpression and high levels of Sod2 (3.9 fold extra level of Sod2 in the testis) are associated with a reduced body size and infertility. The authors have argued that Sod2 overexpression is destructive especially for Leydig cell mitochondrial development resulting in arrested sperm production. The toxic effect of extra H2O2 molecules (which are the main products of superoxide dismutase) in testicular cells may also play a role (Calogero *et al*., 2017). Decreased fertility was also reported for female transgenic mice with Sod2 overexpression due to inhibition of ova release (Raineri *et al*., 2001).

Notably, mice carrying a double knockout of both Sod1 and Sod3 (Sod1−/− plus Sod3−/−) are fertile (Sentman *et al*., 2006; Tsunoda *et al*., 2012). Although does not exist a study to show the reproductive outcomes of double knockout mice for Sod1 and Sod2 but exists one in Caenorhabditis elegans (sod-1−/− plus sod-2−/−) (Sakamoto and Imai, 2017). The

approach of this study to function of SOD family in spermatozoa is interesting because the scavenging role of SODs has not been considered versus the positive affect of their product (which is H2O2) on spermatozoa has been noticed. It was found the double mutant of (sod-1−/− plus sod-2−/−) in C. elegans causes sperm activation defects. In addition it was tried to activate the sperm by adding "Pronase" (a commercially available mixture of proteases) to (sod-1−/−) and (sod-2−/−) single mutants and finally it was revealed that sod-2 and its product (H2O2) is required for sperm activation of C. elegans (Sakamoto and Imai, 2017).

In comparative terms, SOD activity in semen from donkeys and stallions has been found to be 35fold- and 10fold- greater respectively than in human semen, whereas in contrast SOD activity in male birds like the cock was found to be 15 fold less than human (Mennella and Jones, 1980). Spermatozoa from boars have provided an interesting model for investigators in the area of antioxidant enzymes. On the basis of several studies, the semen of boars is highly sensitive to reactive oxygen species (ROS) due to very high unsaturated fatty acid concentrations in the boar sperm plasma membrane, that result in a low cholesterol/phospholipid ratio (De Leeuw *et al*., 1991; Cerolini *et al*., 2000; Zhang *et al*., 2016). SOD family become manifest to be a major antioxidant enzyme in boar seminal plasma, which could protect boar sperm from the noxious effects of ROS (Kowalowka *et al*., 2008).

The motility and fertilizing capacity of frozen-thawed boar sperm have been found to be significantly lower than that of semen stored as the liquid (Holt, 1997; Yi *et al*., 2004). This difference occurs because during preservation in vitro, ROS is generated by spermatozoa and applies an oxidative stress on the sperm plasma membrane (Agarwal *et al*., 2008). These conditions underscore the necessity for the inclusion of antioxidant supplements to reduce the impact of oxidative stress (Michael *et al*., 2009) and regarding a stepwise investigation, superoxide dismutase family (SOD) is introduced as a suitable antioxidant candidate. Indeed, this fact has been proved by demonstrating that the addition of SOD to the storage semen (directly) or freezing medium as a supplement improves the quality of frozen-thawed boar semen by reducing ROS generation during cryopreservation (Roca *et al*., 2005; Vallorani *et al*., 2010). Moreover, improvement the different criteria of boar spermatozoa under the

Table 1-2: Fertility status of various transgenic models for Sod family in male and female mice.

	Male Mice		**Female Mice**	
Transgenic Status	**Fertility Status**	**Reference(s)**	**Fertility Status**	**Reference(s)**
Sod1 (-/-)	1) Infertile 2) Fertile	1) Garratt *et al*., 2013 2) Ho *et a*l., 1998 Matzuk *et al*., 1998 Tsunoda *et al*., 2012	Infertile	Ho *et a*l., 1998 Matzuk *et al*., 1998 Noda *et al*., 2012
Sod1 (+/-)	Fertile	Lei *et al*., 2016	Fertile	Lei *et al*., 2016
Sod2 (-/-)	Dead	Lebovitz *et al*., 1996 Huang *et al*., 2001	Dead	Lebovitz *et al*., 1996 Huang *et al*., 2001
Sod2 (+/-)	Fertile	Strassburger *et al*., 2005	Fertile	Strassburger *et al*., 2005
Sod3 (-/-)	Fertile	Sentman *et al*., 2006	Fertile	Sentman *et al*., 2006
Sod3 (+/-)	No information	.....	No information	.....
Sod1 (-/-) + **Sod3 (-/-)**	Fertile	Sentman *et al*., 2006 Tsunoda *et al*., 2012	Fertile	Sentman *et al*., 2006 Tsunoda *et al*., 2012
Sod1 (-/-) + **Sod2 (-/-)**	No information	.....	No information	.....
Sod2 (-/-) + **Sod3 (-/-)**	No information	.....	No information	.....
Sod1 overexpression	Fertile	Lei *et al*., 2016	Fertile	Lei *et al*., 2016
Sod2 overexpression	Infertile	Calogero *et al*., 2017	Reduced	Calogero *et al*., 2017
Sod3 overexpression	No information	.....	No information	.....

effects of SOD have been proved (Zhang *et al*., 2016). Those different criteria are sperm motility, acrosome integrity, plasma membrane integrity, total antioxidant capacity (TAC), malondialdehyde (MDA) content and H2O2 content during liquid preservation (at special temperature: 17°C). The optimum concentration of SOD as an extra supplement during the liquid preservation on boar also has been reported almost 200 U/mL (Zhang *et al*., 2016).

Herein it is important to explain the concept of “total antioxidant capacity” or TAC. TAC is a combination of antioxidant activities of all enzymatic antioxidants and non-enzymatic-antioxidants including ascorbic acid, α-tocopherol, β-carotene, reduced glutathione, uric acid, bilirubin, albumin, ceruloplasmin, and ferritin in the body fluids. Moreover, TAC can be measured by a TAC assay and has been reported by many studies instead of measuring individual components, as it is believed that this method gives more relevant biological information (Agarwal *et al*., 2016). For example, the TAC of different body fluids from infertile men, including seminal plasma, has been published (Josarayi *et al*., 2017; Kumar *et al*., 2017; Yousefniapasha *et al*., 2015).

However, the addition of SOD-like activity as a cryopreservation extender for stallion sperm yielded completely opposite results (Baumber *et al*., 2005a). No improvement in motility, the integrity of acrosome, vitality, or mitochondrial membrane potential of stallion spermatozoa were observed after thawing, whilst an increased level of spermatozoal DNA fragmentation was documented (Baumber *et al*., 2005a). It was concluded that the additional oxidative stress caused by the generation of H2O2 by the SOD-like activity was responsible. As next step, this group determined the activity of superoxide dismutase in different parts/tissues of horse reproductive system including spermatozoa, seminal plasma, testis, epididymis, bulbourethral gland, prostate, vesicular gland, and ampulla. Although ampulla and prostate had the highest SOD activity, it was not significantly different than other tissues (Baumber and Ball, 2005b). However, their results clarified a high SOD enzyme activity for equine seminal plasma versus spermatozoa.

1.2.3. Superoxide Dismutase (SOD) family in male infertility: Results from clinical investigations

It is now recognized that human seminal plasma is comprised of at least 2545 different proteins (Sharma *et al*., 2013), although newer reports suggest that up to 10,000 expressed proteins may be present (Gilany *et al*., 2015). Meanwhile few reliable biomarker proteins have been found in seminal plasma that are useful in the diagnosis of human infertility (Liu *et al*., 2004; Rodríguez-Martínez *et al*., 2011; Sharma *et al*., 2013). However, it is clear that changes in the oxidative stress mechanism including antioxidant enzymes and ROS contribute to the etiology of male infertility (Agarwal *et al*., 2009; Gharagozloo and Aitken, 2011; Sharma *et al*., 2013). Several studies have hypothesized a difference in superoxide dismutase enzyme activities in seminal plasma between fertile and infertile men but the results are controversial (Table 1-3). For example, a significant reduction in total SOD activity was observed in a comparison of seminal plasma from oligozoospermic and normospermic patients, although this study did not quantify individual SODs (Tamilselvan *et al*., 2015). This outcome was comparable to previously published studies, although they had some different conditions. For example, a lower SOD activity was also reported for whole semen (seminal plasma + spermatozoa) from oligoasthenozoospermic patients versus normospermic (Murawski *et al*., 2007) (Table 1-3). In other infertility subgroups, namely, both asthenozoospermic- and oligoasthenoteratozoospermic- patients, decreased levels of seminal plasma SOD activity were also observed compared to normozoospermic men (Atig *et al*., 2012; Hosen *et al*., 2015). More studies are needed to show how, and via which mechanisms, that the shortage of SOD enzymes affects sperm in these patients. Examples of possible causes could be inheriting a poor cytoplasm with a low level of SOD during spermiogenesis, or dysfunction of mitochondria to generate sufficient enzyme, or deactivated enzymes.

Table 1-3: Different reports for whole SOD-activities in different infertile men

Infertile Study Group	Sample Type	Whole SOD Enzymes Activity	Reference
Oligozoospermia	spermatozoa	Higher	Sinha et al., 1991
Oligozoospermia asthenozoospermia	spermatozoa	Higher	Zalata et al., 1995
Infertile men (No specific type)	spermatozoa	Higher	Aitken et al., 1996
Oligozoospermia	spermatozoa	Higher	Gavella et al., 1996(b)
Oligozoospermia Teratozoospermia Oligoteratozoospermia Asthenoteratozoospermia Oligoasthenoteratozoospermia	spermatozoa	Higher	Sanocka et al., 1997
Oligozoospermia	Seminal plasma	Higher	Sanocka et al., 1997
Oligozoospermia Azoospermia	Seminal plasma	Higher	Sakamoto et al., 2008
Asthenozoospermia	spermatozoa	lower	Sanocka et al., 1997
oligoasthenozoospermia	Semen Seminal plasma	lower	Murawski et al., 2007
Oligoasthenoteratozoospermia	Seminal plasma	lower	Atig et al., 2012
Infertile men (No specific type)	Seminal plasma	lower	Hosen *et al.*, 2015
Infertile (positive antisperm antibody)	Seminal plasma	lower	Zhao *et al.*, 2015
Oligozoospermia	Seminal plasma	lower	Tamilselvan et al., 2015
Asthenozoospermia	Seminal plasma	lower	Atig et al., 2017
Infertile men (No specific type)	Spermatozoa Seminal plasma	No difference	Zini et al., 1993
Oligoasthenozoospermia	Spermatozoa Seminal plasma	No difference	Hsieh et al., 2002
Asthenozoospermia Asthenoteratozoospermia Oligoasthenoteratozoospermia	Seminal plasma	No difference	Khosrowbeygi et al., 2007

At about this time, a number of groups compared spermatozoa from infertile oligozoospermic men with normozoospermic men (Sinha *et al*., 1991; Zalata *et al*., 1995; Gavella *et al*., 1996(b), Aitken *et al*., 1996; Sanocka *et al*., 1997). These studies collectively concluded that high SOD activity in seminal plasma from infertile patients was due to sperm immaturities or defects of sperm development. Hence it is interesting to compare the SOD activity of spermatozoa and seminal plasma among six different groups of infertile men (oligozoospermia, asthenozoospermia, teratozoospermia, oligoteratozoospermia, asthenoteratozoospermia and oligoasthenoteratozoospermia) and normal men (Sanocka *et al*., 1997). The level of SOD activity in spermatozoa was elevated in all sperm pathologies except for asthenozoospermia. As it was mentioned earlier in this section, the results came from different studies are controversial. While In the paragraph above, references showed reduced SODs activities are associated with altered spermatogenesis, whereas in the other references the argument is the reverse (enhanced SODs activities are associated with altered spermatogenesis). Further research is needed to sort this situation out.

Here, the question is, by a definitive increase of SOD to protect the cells from oxidative stress, how they achieve the paradoxical effect? A possible answer is that excess superoxide dismutase may be an advantage for protecting the cell from oxidative stress if accompanied by “H2O2 scavenger partner” enzymes such as peroxiredoxins, glutathione peroxidase or catalase (Gavriliouk and Aitken, 2015). Otherwise, these excess SODs simply will convert all short-lived superoxide ions (O2·−) to dangerous long-lived hydrogen peroxides (H2O2), which can attack to all vital parts of spermatozoa, including mitochondria, plasma membrane and DNA in the sperm nucleus (Gavriliouk and Aitken, 2015).

To compound the problems introduced by data variability in the analysis of SOD activity and male infertility (see above), a third group of scientific papers has reported a non-significant correlation between SOD activity and semen quality (Zini *et al*., 1993; Hsieh *et al*., 2002; Khosrowbeygi *et al*., 2007). Interestingly, the study population in this third group covered several different infertile patient types such as asthenozoospermic, asthenoteratozoospermic, oligoasthenoteratozoospermic, oligoastenozoospermic and azoospermic, all compared with normozoospermic men.

Recently, a Chinese research group examined SOD activity in infertile patients with the positive antisperm antibody (AsAb) in their seminal plasma. They compared 40 infertile patients with positive AsAb with 40 fertile males and found a significant ($P < 0.01$) decrease in the SOD level in seminal plasma of the infertile patients. It was notable that at the same time, a parallel decrease of acrosomal enzyme activity in spermatozoa of patients with positive AsAb was also observed (Zhao *et al*., 2015).

Changes in the amount of SOD1 protein expression have been examined during the protein profiling of sperm and seminal plasma between men with and without varicocele (Al-Huwaizi *et al*., 2016). When an abnormal enlargement of the pampiniform venous plexus occurs in the scrotum, the resulting disorder is named varicocele, and is relatively common in 19-41 % of men with primary infertility (i.e. couples who have not become pregnant >1 year unprotected intercourse) and 45-81 % of men with secondary infertility (couples who have been able to get pregnant at least once, but unable to repeat it) (Cocuzza *et al*., 2008). Initial studies used two-dimensional gel electrophoresis and found SOD1 protein expression to be significantly reduced in the spermatozoa of infertile men complaining of varicocele (Hosseinifar *et al*., 2013). Moreover, significantly decreased SOD1 protein in both seminal plasma and spermatozoa from varicocele patients has been reported elsewhere (Al-Huwaizi *et al*., 2016). Following treatment of the same patients with varicocelectomy, SOD1 levels in spermatozoa were significantly greater than prior to surgery. Moreover, SOD1 levels in the treated men were similar to a normal control group (Hosseinifar *et al*., 2014). It was concluded that high-grade varicocele influences sperm protein expression by increasing testicular temperature. In support of this result, the effect of heat on seminal plasma SOD activity was recently measured in high risk men like bicycle athletes. The results strongly proved ($p < 0.008$) a significant decrease in seminal SOD activity in male road cyclists, and interestingly it remained low after 30 days of recovery (Maleki *et al*., 2014).

More generally, it is known that physical activity increases oxygen consumption 10-15 fold over common consumption and hence, a higher oxidative stress and production of free radicals is expected in athletes (Nenad *et al*., 2011). A study that measured SOD activity in athletes versus non-athletes showed a significantly greater level of SOD activity in the blood plasma of athletes (Nenad *et al*., 2011). To determine whether any correlation exists between

seminal SOD activity and physical exercise, the seminal SOD activity was measured in three groups of healthy men with different levels of physical fitness, representing elite athletes, recreationally active men, and inactive men. The results showed a higher SOD activity in the recreationally active men, and additionally this group had better semen parameters. Elite athletes had lower SOD activity than recreationally active men plus having a lower semen quality (Hajizadeh Maleki *et al*., 2013). A low semen quality among high-intensity training athletes has been proven previously (Vaamonde *et al*., 2009).

Sperm DNA fragmentation as a subclass subject under the umbrella of sperm chromatin damage has always been a fascinating topic for scientists in the area of oxidoreductase systems. Nowadays, excess ROS is known to be a major cause of sperm DNA damage in comparison with other factors such as recombination defects during spermatogenesis, dysfunction of nucleases and topoisomerases, and finally an imbalance in the ratio of protamine 1 to protamine 2 (Aktan *et al*., 2013; Aitken *et al*.,2014; Savadi-Shiraz *et al*., 2015). It is considered widely plausible that SOD activity has a significant inverse correlation with sperm DNA fragmentation (Bojar *et al*., 2013). Indeed, all available evidence supports this hypothesis, as decreased SOD activity in seminal plasma is associated with increased sperm DNA fragmentation (Tartibian and Maleki, 2012; Yan *et al*., 2014; Wdowiak *et al*., 2015; Atig *et al*., 2017).

1.2.4. Superoxide dismutase family and ART: Significance in assisted reproductive technologies

Assisted reproductive technologies (ART) can be considered as the greatest achievement in the field of reproduction biology. IVF (In vitro fertilization) and ICSI (Intracytoplasmic sperm injection) are the two most common methods used in assisted reproductive technologies (Gupta *et al*., 2010). However, in many cases, the technique of IUI (Intrauterine insemination) is the primarily applied method of ART consultants to infertile couples owing to ease of use and treatment costs (Tremellen *et al*., 2008). Statistical information estimates that 15% of couples worldwide are unsuccessful in achieving an ongoing pregnancy after at least 12 months of regular unprotected sexual intercourse (Zegers-Hochschild *et al*., 2009). Within this group, 25% have idiopathic infertility meaning that no causative factor for their infertility can be discerned (Sharlip *et al*., 2002). Since the birth of the world’s first in vitro

fertilization baby, Louise Brown, in the UK, a multitude of factors have been recognized as positive and negative effectual elements on ART (Kamel, 2013; Lampiao, 2012). Amongst these factors, it is well known that enhanced antioxidant capacity and decreased ROS levels are two main variables that can be manipulated to improve the quality of in vivo fertilization and ART outcomes (Agarwal *et al*., 2014).

While the contribution of both types of antioxidants (antioxidant enzymes and non-enzyme components) to male infertility has been well studied (as discussed above), only a limited number of clinical trials have investigated the effect of antioxidant enzymes in assisted reproductive technologies.

Strong evidence now proves that several environmental factors are potential sources of ROS that can cause exposure of spermatozoa to oxidative stress during the preparation of seminal plasma for ART (Tremellen *et al*., 2008; Agarwal *et al*., 2014). Those environmental items include visible light, composition of culture media, pH and temperature, oxygen concentration, ART technique, cryopreservation (freeze/thawing) and even centrifugation (Agarwal *et al*., 2014). Meanwhile, very few studies have tried to measure the level of SOD enzymes in response to these environmental items, or to correlate SOD expression with ART outcomes in seminal plasma or spermatozoa. One good example examined the effects of antioxidant enzyme supplementation in human semen samples undergoing cryopreservation (Rossi *et al*., 2001). They found no significant change in progressive motility with adding SOD or catalase alone, while in a mixture of both enzymes, a significant improvement in sperm parameter recovery was seen.

Unexpectedly, in this regard, the contribution of the female reproductive system was greater than males when correlations were sought with ART mechanisms or results and antioxidant enzymes. Particularly for the SOD family, correlations have been reported between the members of this enzymatic family and female reproductive components such as endometrial fluid, peritoneal fluid, and follicular fluid, as well as reproductive tissues and serum (Santulli *et al*., 2013; Santulli *et al*., 2015; Gupta *et al*., 2014). For example, a significant difference between SOD activity and total antioxidant capacity in endometrial secretions has been documented between three different groups of patients representing ongoing pregnancy, miscarriage and failed IVF cycles (Rahiminejad *et al*., 2016). Moreover, a higher amount of

antioxidant enzymes such as superoxide dismutase, catalase or total antioxidant capacity and lower levels of oxidative stress markers, such as lipid peroxidation in the endometrial secretions, were associated with successful IVF outcome (Rahiminejad *et al.*, 2016). On the other hand, there is no evidence to show the influence of SOD family activity or concentration on the results of ICSI, IVF or IUI.

It is understood that cancer itself causes an increase in ROS levels (Tong *et al.*, 2015). In this regard, the association of testicular tumors with testicular oxidative imbalance has been reported (Baumber *et al.*, 2000; Agarwal and Allamaneni 2005a). Recently, studies to investigate SOD content in patients with testicular cancer have been undertaken, whereas previous reports mostly examined the role of the SOD family in prostate cancer (Li *et al.*, 2016). A comparison of SOD activity in seminal plasma found no difference between seminoma and healthy control men despite a significant increase in oxidative damage in the former group, thought possibly due to an insufficient number of patients in the study (Sposito *et al.*, 2017). It seems that more investigations are required regarding testicular cancer and the SOD enzymes. Moreover, no study has yet been published that investigates the relationship(s) between SOD family members and cancers of other parts of the male reproductive tract (e.g. epididymis or seminal vesicles).

1.2.5. Current and future perspectives

As published evidence frequently reports the pathophysiological role of oxidative stress in damaged spermatozoa in almost 30-80% of male infertility cases, it is not surprising to see increased scientific attention in the field of “male reproduction antioxidant enzymes” (Aktan *et al.*, 2013). Superoxide dismutase enzymes are well-known elements in antioxidant protection and are therefore centrally involved, but our understanding of their functions in male fertility remains incomplete. The literature is full of studies with conflicting results about the impact of SOD family activity on semen parameters like sperm motility and count. However their involvement with other important parameters such as sperm morphology and morphology impairment in severe teratozoospermic patients has yet to be studied. Due to the specific localization of SOD family members in different parts of spermatozoa (SOD1 in the cytoplasm and mitochondria, SOD2 in mitochondria and SOD3 in extracellular environment

that surrounds all sperm) (see Section 1.2), there may be correlations between abnormal morphology and the function of SOD enzymes. Another important reason to associate sperm morphology and SOD enzymes could be the effective role of SOD on sperm capacitation and the acrosome reaction. The normal morphology of the sperm head is a critical factor to progress a successful acrosome reaction (Magli *et al*., 2012). As mentioned previously in this review, SOD family play a role to block the capacitation. Hence interaction mechanisms between these three factors (sperm shaping, acrosome membrane integrity and SOD function) together need more investigations.

Mitochondria are oxidant producers and effect on sperm by its endogenous ROS production and the same time is an antioxidant producer via releasing the antioxidant enzymes. The coexistence of two SOD family members (SOD1 and SOD2) in mitochondria has provided an interesting area for male infertility researchers. In particular, the functional significance of SOD1 in the mitochondrial intermembrane space is unknown, as is its contribution to the mitochondrial membrane potential (MMP). MMP is a key parameter to evaluate the function of mitochondria (Sakamuru *et al*., 2016), and is related to the mitochondrial electron transport chain that creates an electrochemical gradient through a series of redox reactions to drive the synthesis of ATP. In spermatozoa, loss of MMP and the subsequent decrease in cellular energy is known to be related to decreased sperm motility and also with the outcome of in vitro fertilization (Marchetti *et al*. 2012; Condorelli *et al*. 2012). In addition, low MMP has been associated significantly with high ROS production in spermatozoa (Wang *et al*., 2003). Nonetheless, no scientific evidence is available to show any association between SOD1 content or activity, and MMP in spermatozoa. Therefore, it is necessary to investigate the physiology of mitochondria in reaction with all SOD enzymes. Results of this kind of investigation can consequently be related to semen analysis parameters, especially to sperm motility (because of the role of mitochondria in the provision of energy) and also sperm vitality (because of the role of mitochondria in regulating apoptosis) (Srivastava *et al*. 2016).

Male adolescents may be an important target group in which to study the function of SOD family, with little to no information currently available that examines seminal SOD content or activity. Semen analysis is not enough predictor of failure or success in fertilization opportunities in the future of adolescents. Where the semen content of a young boy is

reported as lacking sperm, an additional test to show the ability of antioxidants to preserve sperm function for future fertility could be useful. Adolescents also are an advisable group to check their seminal SOD content from another important perspective and that is physical activity. Due to adolescents for involving the sports activities, it will be helpful for their fertility status in future to know about the seminal antioxidant index and consultation to choose a suitable sport. Additionally, more studies are also required in the group of elite athletes in relation to the reduced SOD activity in their semen, as well as the low quality of their seminal parameters. For example, the analysis of SOD content / activity in their spermatozoa, separately from semen and seminal plasma, has not yet been performed. Moreover, there is no evidence in the group of men with high physical activity (or in male mice or other animal models) to show that the use of SOD-supplements (as antioxidant supplements) can improve seminal SOD content / activity or increase other semen parameters.

Reliable antioxidant supplements could be an acceptable defensive strategy against ROS, not only for human cases, but also for other mammals or other species. According to the level of superoxide dismutase detected in spermatozoa, supplements that directly contain SOD or treatments to increase the production of this enzyme family are expected from pharmacology scientists. For the first suggestion, it is unquestionable that the protein concentrations of all SOD members in semen, seminal plasma or spermatozoa are needed. As discussed in previous sections of this review, there is a lack of scientific publications to report the superoxide dismutase protein concentration in different parts of the reproductive system. For the second suggestion, more attempts are needed, as this strategy has been previously trialled with SOD3 with no success (see Section 1.2.2) (Gharagozloo *et al*., 2016).

Experiments aimed at finding a suitable dosage of SOD family to be used as a supplement could benefit from attention to the subject of total antioxidant capacity (TAC). Earlier in this review the concept of TAC was explained. Although numerous studies have reported the TAC of seminal plasma, to the best of our knowledge there is no evidence to show the specific contribution of SOD family enzymes to whole seminal TAC in different groups of infertile men.

Successful outcomes of ART is one the most important aims in the field of reproductive biology. With available scientific evidences the role of ROS and antioxidants to get succeed in ART is large enough to do not ignore (Agarwal *et al*., 2017). However from the perspective of ART in combination with SOD family there is no study to show the positive or negative influence of SOD-treated spermatozoa in semen or in the sperm preparation culture medium. It can be reasonable to find any links between SOD enzymes and some important subsequent harmful circumstances like increasing rates of miscarriage, birth defects, metabolic disease, and also dangerous mutations in the offspring (Aitken *et al*., 2009; Chabory *et al*., 2009; De Iuliis *et al*., 2009; Lane *et al*., 2014; Gharagozloo *et al*., 2016).

1.3. Cysteine-rich secretory protein 1 (CRISP1)

The gene of CRISP1 (Cysteine-rich secretory protein-1) in the human is located in chromosome 6 (6p12.3). CRISP1 is a member of glycoprotein superfamily of CRISPs and was identified as its first member in 1976 (Cameo and Blaquier, 1976). The other members are CRISP2, CRISP3 and CRISP4. This family is highly evolutionarily conserved and contains 16 conserved cysteines, of which 10 are located in the C-terminal sequence region. This region contains both a Cysteine-Rich Domain (CRD) and a hinge that connects it to the plant Pathogenesis Related-1 (PR-1) domain located in the N-terminus of the molecule (Guo *et al*., 2005; Gibbs *et al*., 2008). These domains have different functions, as the N-terminal PR-1 domain is implicated in membrane interaction and protease activity, whilst the C-terminal region possesses ion channel regulatory properties (Guo *et al*., 2005).

There is a difference between the molecular structure of CRISPs and other proteins that contain a similar large number of cysteine residues, because most in the latter case are involved in disulfide bond formation via the oxidation of cysteines to form cystine. In contrast, the CRISP family members retain a high number of native (unoxidised) cysteine residues, separate to the cystine residues involved in disulfide bonds (Osipov *et al*., 2005).

1.3.1. Cysteine-rich secretory protein 1 (CRISP1) in male reproductive system

CRISPs are known to have a role in the procedure of mammalian fertilization and their presence has been reported in the testis, epididymis, sperm and seminal plasma. CRISP1 is mostly found as a secreted protein from the epithelial cells of epididymis in an androgen-

respond manner, which loads to the surface of spermatozoa during its travel through the epididymis (Kohane *et al*., 1980; Cohen *et al*., 2000b). In particular it has been reported that CRISP1 plays a role in sperm maturation, capacitation and the acrosome reaction (egg-sperm fusion) with the binding to zona pellucida (Rochwerger *et al*., 1992; Cohen *et al*., 2000a; Busso *et al*., 2007). Whereas maturation confers upon sperm the ability to recognize and fertilize the egg, capacitation prepares sperm for both undergoing the acrosome reaction, which takes place in the head, and developing a strong flagellum-movement known as hyper activation, which takes place in the tail.

There are several evidences have revealed that for sperm capacitation the procedure is started by mainly activation of the cAMP/PKA-dependent and increase two further things. Increasing the rate of protein tyrosine phosphorylation and increasing the intracellular Ca2+ concentrations (Visconti *et al*., 1995; Ruknudin and Silver, 1990). Despite many attempts regarding to know how this increase interacts to induce acrosome reaction and hyper activation, details are still under investigation. On the base of special structure of the CRISP1 molecules, evidences support the potencial of CRISP1 to be involved in different stages of fertilization (Da Ros *et al*., 2015). This special structure refers to the CRISP1 molecules, which can have a charachter as a loosely-bound molecules or tightly-bound molecules. The loosely-bound molecules work as a decapacitating factor regulating protein tyrosine phosphorylation and the progesterone-induced acrosome reaction (Roberts *et al*., 2003). For the tightly-bound molecules is considered having two roles. First in the sperm-zona pellucida interaction and the second in the gamete fusion via its interaction with complementary sites localized in the zona pellucida and the oolema (Busso *et al*., 2007).

With considering the available evidences, it is obvious that CRISP1 participates in gamete fusion with having an important role in sperm- zona pellucida binding. But when the knockout mise were developed for lacking CRISP1, they were totally fertile without significant difference to normal control mice (Weigel Muñoz *et al*., 2018). It was argued that the members of CRISP family (including CRISP1, CRISP2, CRISP3 and CRISP4) have a high sequence homology and they support the function of each other. In other words, there is a kind of compensatory homeostasis among homologous CRISPs (Weigel Muñoz *et al*., 2019).

In the female gametes, CRISP1 protein is shown exists in the cumulus cells that surround the egg. In cumulus cells, the sperm hyper activation is modulated and it seems this happens via its ability to regulate CatSper channels. CatSper channels are essential for male fertility. In this channels, which are the cation channels of sperm (Ca2+ channel) developes the sperm hyper activation (Ernesto *et al*., 2015).

1.4. Galectin-3-binding protein (LGALS3BP)

Lectin galactoside-binding soluble 3 binding protein (LGALS3BP) is also known as 90K, M2BP, gp90, CyCAP, BTBD17B, MAC-2-BP, and TANGO10B. However, mostly in the references a shorted version as "Galectin 3 binding protein" is used as the protein name.

The LGALS3BP gene in the human contains 6 exons and has been located on chromosome 17 (17q25.3) (Human PubMed, https://www.ncbi.nlm.nih.gov/gene/3959). The LGALS3BP protein is comprised of subunits that form homodimers and homomultimers. The multimers form ring-like structures with a diameter of 30 to 40 nanometers. The unglycosylated form interacts with PDE4DIP isoform 13/MMG8/SMYLE; this interaction may connect a pericentrosomal complex made of AKAP9, CDK5RAP2, EB1/MAPRE1 and PDE4DIP, to the gamma-tubulin ring complex (gamma-TuRC) to promote microtubule assembly and acetylation (Bouguenina *et al*., 2017). LGALS3BP is ubiquitously expressed by keratinocytes and fibroblasts and detected in body fluids such as semen, milk, serum, tears, saliva and urine. It was seen that LGALS3BP has been elevated in the blood serum of patients with malignancy and in patients infected by HIV (human immunodeficiency virus). LGALS3BP is a virus-induced protein and to be involved in immune responses via lymphokine-activated killer cell cytotoxicity and natural killer cells. Moreover LGALS3BP leading to interferons and pro-inflammatory production (Xu *et al*., 2019).

It has been shown LGALS3BP binds to the one member of the galectins, namely galectin-3 (LGALS3). The galectins are a superfamily of proteins that bind specifically to β-galactoside sugars, such as N-acetyllactosamine (Galβ1-3GlcNAc or Galβ1-4GlcNAc), which can be bound to proteins by either O-linked or N-linked glycosylation. The other name for galectins is "S-type lectins" and it is because of their dependency on disulfide bonds for stability and carbohydrate binding. Fifteen galectins have been found in mammals, which are encoded by

the genes of LGALS. Among the galectins 1 to 15 only galectin -1, -2, -3, -4, -7, -8, -9, -10, -12 and -13 have been identified in human. Galectins are soluble proteins with both extracellular and intracellular functions and this is unlike the other of lectins, which they are membrane bound. LGALS found primarily in the nucleus, cytosol and extracellular matrix or in circulation. Most of the galectins must be secreted but till now the mechanism of secretion is not clarified. Classical secretion for LGALS is improbable because this mechanism needs a special signaling pathway/elements, and those typical signal peptides do not exist. The non-classical mechanisms of secretion is possible but still remain to discover (Barondes. 1988).

1.4.1. Galectin-3 (LGALS3)

Because galectin-3 (LGALS3) has been shown to be the main interacted protein with LGALS3BP, here is describing its features and functions.

Galectin-3 is a protein with approximately 30 kDa weight and is encoded by a single gene located on chromosome 14(q21- q22) (Human PubMed). Galectin-3 is expressed in the extracellular space, cell surface, cytoplasm, mitochondrion and nucleus (Carvalho *et al.*, 2014).

The structure of the molecule of galectin-3 is a chimera. For superfamily of galectins, three different forms of structures (namely, dimeric, tandem and chimera) have been explained. Only galectin-3 has a chimera structure and for this reason, it is unique among the members of the superfamily of galectins. In the structure of galectin-3, there is a domain with a function of carbohydrate-recognition-binding domain (CRD). This domain does not particularly belong to galectin-3 and all the other galectins also possess that. CRD is formed by around 130 amino acids. Via CRD, galectins can specifically bind to beta-galactosides and also a long non-lectin domain. More details about the structure of galectin-3 can be explained with its monomeric form up to a pentameric form. For multimeric forms, galectin-3 can associate via the non-lectin domain into multivalent complexes. This structure is the basis of the formation of an adhesive character in galectin-3, which lets this molecule bridging between cells or cells and the extracellular matrix.

The high affinity of galectin-3 has been proved for binding to beta-galactosides. With this connection, galectin-3 gets involved in many functions, which can be listed as: cell-cell

adhesion, cell-matrix interactions, macrophage activation, cell growth and differentiation, cell cycle, angiogenesis, metastasis, apoptosis.

In the male reproductive system, it is known that galectin-3 is a secretory lectin in human seminal plasma. In the initial studies, it was demonstrated that there is a strong galectin-3 immunoreactivity in the acrosomal region of ejaculated and capacitated spermatozoa. This immunoreactivity was much stronger than that of epididymal spermatozoa. Therefore it was tried to find a source of galectin-3 to release in seminal plasma and it was found galectin-3 can originate from seminal plasma-derived extracellular vesicles. Those galectin-3 molecules in the next step means during post-testicular maturation can be transferred to the sperm surface. They play an important role later and in the time of fertilization for strong binding between spermatozoa and zona pellucida (Mei *et al*., 2019).

1.4.2. LGALS3BP in male reproductive system

In the male reproductive tract, the expression of LGALS3BP was previously identified in the whole male genital tract. It was found in the human seminal plasma (Fung *et al*., 2004; Tsuruya *et al*., 2006), prostate secretions, prostate cancer cells (Bair *et al*., 2006) and in the mouse testis (Sasaki *et al*., 1998). Regarding the multiple functions of LGALS3BP in human's semen, it has been reported that seminal LGALS3BP are associated with semen liquefaction, sperm motility, angiogenesis in the female reproductive tract and finally as a pro-inflammatory agent (Kovak *et al*., 2014). Results of a recent study showed that LGALS3BP interacts with albumin. On the other hand albumin has been known as a molecule involved in sperm capacitation and acrosome reaction. Therefore those mentioned roles can also related to LGALS3BP, indirectly. Moreover, LGALS3BP has been found to have interaction with complement factor D (CFD). CFD is an activator of the immune system in the female reproductive tract (Viana *et al*., 2018).

1.5. Hypothesis and aim

The hypothesis of this study was to identify biomarker(s) in the seminal plasma, which predicts the presence of spermatids in the testis of men with non-obstructive azoospermia. It was hypothesized those seminal biomarkers can provide non-invasive diagnosis tools for the prediction of the presence of advanced spermatids in the testis of NOA patients and therefore exempt them from an unnecessary invasive diagnosis surgery.

This study aimed to compare the protein expression patterns in the seminal plasma from men with a positive sperm retrieval at M-TESE (men with mixed-testicular atrophy (MA) versus patients with a negative sperm retrieval (men with Sertoli cell-only syndrome (SCO).

In the first part of the study, a mass spectrometry-based comparison of seminal plasma samples provided a list of proteins that were differentially expressed. Superoxide dismutase 1 (SOD1), superoxide dismutase 3 (SOD3) and Galectin-3-binding protein LGALS3BP were three proteins selected for further validation by independent methods, including immunohistochemistry (IHC), Western blotting, and enzyme-linked immunosorbent assay (ELISA). In addition, the quantitative status of protein LGALS3BP was examined for an extra NOA group means patients with Klinefelter’s syndrome. Histopathological diagnosis of testis biopsies from patients with Klinefelter’s syndrome shows both patterns of SCO and MA and it was the aim to see the protein quantification in both subgroups of this category.

In the second part of the study and on the basis of existing data, came from a previous work using the method of two-dimensional gel electrophoresis (2-DE) the follow-up assessments were performed. Previously, a list of significant proteins was obtained (unpublished data) with the method of 2-DE from the comparison of seminal plasma proteome related to normal men before and after vasectomy. Cysteine-Rich Secretory Protein-1 (CRISP1) was the candidate protein selected from the list for further validation by Western blotting and IHC. CRISP1 is supposed to be an epididymal protein and it was the aim to see the protein quantitation status in the seminal plasma of men with testicular impairment histology. Various sub-groups of NOA patients were studied, including men with mixed testicular atrophy (MA), spermatogenesis arrest (SA), Sertoli cell-only syndrome (SCO) and patients with Klinefelter’s syndrome.

2. MATERIALS AND METHODS

2.1. STUDY SAMPLES

Human semen ejaculates were collected by masturbation from 74 infertile-azoospermic men who were referred for treatment to the Department of Urology at the University Hospital Giessen (UKGM), which is associated with the Justus Liebig University of Giessen, Germany. Blood serum was also obtained from all patients. Seminal plasma and serum samples were also collected from 23 healthy fertile volunteer men. Seminal plasma was isolated from whole semen samples by centrifugation at 11,200 g (10,000 rpm) for 5 minutes at 25 °C. Serum was isolated from whole blood samples (after clotting) by centrifuging at 1000 g (3000 rpm) for 10 minutes at 25 °C.

To verify azoospermia, at least two semen analyses were performed by experienced laboratory technicians according to World Health Organization criteria (WHO 2010). Karyotype analysis was performed in all azoospermic patients and 14 patients were diagnosed as having Klinefelter syndrome (47, XXY). Besides, with applying the fructose test 8 patients were diagnosed as having obstructive azoospermia (OA=8).

Patients with non-obstructive azoospermia underwent a diagnostic testicular biopsy, using a combination of trifocal TESE and microdissection TESE (M-TESE) (Marconi *et al*., 2012).

Testicular biopsies were immediately fixed by immersion in Bouin's solution and embedded in paraffin. For histological evaluation, slides were prepared and evaluated by score count analysis as detailed elsewhere (Bergmann and Kliesch, 2010). Two groups of azoospermia testicular biopsies were Sertoli cell-only syndrome (SCO, n=23) and spermatogenic arrest (SA, n=6) and they had a negative (unsuccessful) sperm retrieval. The next group of biopsies was mixed testicular atrophy (MA, n=23) and they had a positive (successful) sperm retrieval. Histological evaluation for the group of patients with Klinefelter syndrome revealed that they have two different histological patterns (Klinefelter syndrome with SCO pattern, n=7 and Klinefelter syndrome with MA pattern, n=7).

This study was approved by the human ethics committee of both contributed universities: The Justus Liebig University, Giessen, Germany and Monash University, Melbourne,

Australia (IRTG-62280541). All men gave informed consent to use their whole semen (volunteers) and leftover semen (patients) for the study.

2.2. MASS SPECTROMETRY AND DATA ANALYSIS OF SEMINAL PLASMA PROTEINS

Protein concentrations were determined by the BCA method (Pierce, Rockford). Equal amounts of seminal plasma from control, SCO and MA men (n = 8/group) were prepared for mass spectrometry analysis using the FASP protein digestion method as previously described (Wiśniewski *et al.,* 2009) with the following modifications. Protein material was reduced with Tris-(2-carboxyethyl) phosphine (TCEP, 10 mM final concentration). Eluates were digested with sequence-grade modified trypsin Gold (Promega) (1 µg) in 50 mM ammonium bicarbonate (NH_4HCO_3) and incubated overnight at 37 °C. Peptides were then eluted with 50 mM NH_4HCO_3 in two 40 µL sequential washes and acidified in 1% formic acid (final concentration).

Peptides (2µl) were separated by reverse-phase chromatography on a 1.9 µm C18 fused silica column (I.D. 75 µm, O.D. 360µm x 25cm length) packed into an emitter tip (Ion Opticks, Australia), using a nano-flow HPLC (M-class, Waters). The HPLC was coupled to an Impact II UHR-QqTOF mass spectrometer (Bruker, Bremen, Germany) using a CaptiveSpray source and nanoBooster at 0.20Bar using acetonitrile. Peptides were loaded directly onto the column at a constant flow rate of 400 nL/min with buffer A (99.9% Milli-Q water, 0.1% formic acid) and eluted with a 90 min linear gradient from 2 to 34% buffer B (99.9% acetonitrile, 0.1% formic acid). Mass spectra were acquired in a data-dependent manner including an automatic switch between MS and MS/MS scans using a 1.5 second duty cycle and 4 Hz MS1 spectra rate followed by MS/MS scans at 8-20 Hz dependent on precursor intensity for the remainder of the cycle. MS spectra were acquired between a mass ranges of 200–2000 m/z. Peptide fragmentation was performed using collision-induced dissociation (CID).

Raw files consisting of high-resolution MS/MS spectra were processed with MaxQuant (version 1.5.3.0) for feature detection and protein identification using the Andromeda search engine (Cox *et al*., 2011). Extracted peak lists were searched against the UniProtKB/Swiss-Prot *Homo* sapiens database and a separate reverse decoy database to empirically assess the

false discovery rate (FDR) using strict trypsin specificity allowing up to 2 missed cleavages. The minimum required peptide length was set to 7 amino acids. In the main search, precursor mass tolerance was 0.006 Da and fragment mass tolerance was 40 ppm. The search included variable modifications of oxidation (methionine), amino-terminal acetylation, the addition of pyroglutamate (at N-termini of glutamate and glutamine) and a fixed modification of carbamidomethyl (cysteine). The “match between runs” option in MaxQuant was used to transfer identifications made between runs on the basis of matching precursors with high mass accuracy (Cox *et al.*, 2008). PSM and protein identifications were filtered using a target-decoy approach at an FDR of 1%. Protein identification was based on a minimum of two unique peptides.

2.3.IMMUNOHISTOCHEMISTRY

Slides with two 5µm testicular biopsy sections were deparaffinized in xylene and rehydrated in graded ethanol/water washes. Slides were then boiled (sodium citrate (0.1 M, pH 6.0), cooled (20min) and washed in phosphate-buffered saline (PBS, 0.02M, 3x5min). Endogenous peroxidases were blocked with 3% H_2O_2 (30min) followed by 5% bovine serum albumin (BSA) containing 0.1% Triton X-100 (20min). Primary antibodies [SOD1, rabbit polyclonal diluted 1:2500 (cat.no. HPA001401, Sigma-Aldrich), SOD3, rabbit polyclonal diluted 1:100 (cat.no. HPA042110, Sigma-Aldrich), CRISP1 (A), rabbit polyclonal diluted 1µg/ml (cat.no. LS-C81749, LifeSpan BioScience), CRISP1 (B), rabbit polyclonal diluted 1:10000 (cat.no. LS-C320601, LifeSpan BioScience)] in 5% BSA/PBS were incubated overnight at 4C, followed by goat anti-rabbit IgG-HRP secondary antibody (diluted 1:800, cat.no. A9169; Sigma-Aldrich, 1hour, room temperature). DAB (3, 3′-Diaminobenzidine tetrahydrochloride) chromagen (cat.no. D3939; Sigma-Aldrich) was then applied (1-5min) followed by Mayer's haematoxylin (cat.no. 2E-038; WALDECK) for 10 seconds. All samples were mounted with glycerol gelatin (cat.no. 1092420100; Merck) and observed with the Leica DM750 microscope.

2.4. PROTEIN ASSAY

The protein concentrations of all seminal plasma and blood serum samples were determined using a Pierce BCA protein assay kit (cat.no. 23225; Thermo Scientific), with absorbance measured on the plate reader.

2.5. ELISAS

SOD1 and SOD3 concentrations in seminal plasma and serum samples were measured using specific solid phase sandwich enzyme-linked immunosorbent assay (ELISA) kits (SOD1: cat.no. ab202410; Abcam, SOD3: ab119520; Abcam) according to the manufacturer's instructions. All samples were assayed using multiple dilutions (SOD1: 1:10 to 1:40, SOD3: seminal plasma; 1:4000-1:8000, serum; 1:100-1:200) with duplicate wells per dilution. Absorbance was measured at 450nm in an ELISA plate reader (Multiskan Go, N13135; Thermo Scientific). Intra- and inter- assay coefficients of variation, based on the repeated measurement of a pooled seminal plasma sample, were 10.1% and 15% respectively for SOD1, and 3.4% and 17.6% for SOD3.

2.6. WESTERN BLOT ANALYSIS

Seminal plasma samples were diluted to reach the desired concentration, which already had been established by performing serial dilutions. Afterwards diluted seminal plasma samples were boiled (5mins at 100C) in reducing sample buffer (Bolt LDS sample buffer, Invitrogen) and applied to precast polyacrylamide gradient gels (Bolt 4%-12% Bis-Tris gels electrophoresis, cat.no. NW04125BOX; Invitrogen), prior to electrophoresis at 200V for 30 minutes at room temperature using Bolt MES tank buffer (Invitrogen). For SOD1 detection, replicate samples at total protein concentrations of 26µg and 13µg were loaded, whilst for SOD3, total protein concentrations were 75µg and 37.5µg. For analysis the protein of LGALS3BP, replicate samples at total protein concentrations of 3µg and 1.5µg were loaded. The designed study plan for the protein of CRISP1 was to do analysis by the method of Western blotting with two different anti CRISP1-antibodies (antibody A and antibody B). Each antibody recognized the different epitope of the protein. No overlap exist between two epitopes.

Anti CRISP1 (A) detected epitope between 63-112 amino acids and anti CRISP1 (B) immunised epitope between 162-191 amino acids.
For CRISP1 (A) detection, replicate samples at total protein concentrations of 100µg and 50µg were loaded, whilst for CRISP1 (B), total protein concentrations were 50µg and 25.5µg. A molecular weight standard ladder was included (PageRuler Prestained Protein Ladder, cat.no. 26616; ThermoFisher Scientific). Subsequently, separated proteins were blotted onto 0.45µm PVDF membrane (Immobilon-P, cat.no. IPVH00010; Merck) at 30V constant voltage for 16-18 hours at 4C, using a wet/tank electroblotting system (Mini Trans-Blot Cell, Bio-Rad) with Tris/glycine/methanol tank buffer.

Nonspecific binding sites on membranes were blocked for 1 hour with 1% BSA (w/v) in TBS-T buffer (Tris, NaCl, KCl, pH 7.9, 0.05% Tween 20). Membranes were then probed with the same SOD primary antibodies as used for immunohistochemistry (above), (SOD1 1:2000 1hour, SOD3 1:250 2.5hour) at room temperature, then washed in TBS-T and probed with goat anti-rabbit IgG-HRP secondary antibody (1:100,000) for 1hour at room temperature.

For LGALS3BP, membranes were probed with the primary antibody of anti LGALS3BP, mouse polyclonal diluted 1:1000 (cat.no. ab67353, Abcam) for 1hour at room temperature. In the next step membranes washed in TBS-T and probed with goat anti-mouse IgG-HRP (cat.no. ab205719, Abcam) secondary antibody (1:100,000) for 1hour at room temperature.

Membranes for CRISP1 were probed with the same CRISP1 primary antibodies as used for immunohistochemistry (above), (CRISP1 (A) 1µg/ml 1hour, CRISP1 (B) 1:10000 1hour) at room temperature, then washed in TBS-T and probed with goat anti-rabbit IgG-HRP secondary antibody (1:100,000) for 1hour at room temperature.

Blots were visualized by chemiluminescence procedures (Lumi-Light Plus Western Blotting Substrate, cat.no. 12015196001; Merck) according to the manufacturer's recommendations. Western blot images were quantified using Image Lab software (version 1.1.1 5, Bio-Rad), and each blot was performed twice.

2.7. TWO-DIMENSIONAL GEL ELECTROPHORESIS

Comparative proteome analysis of seminal plasma was performed in 10 men requesting vasectomy. Approval of the study obtained from the institutional review board (institutional review board number: 32/11). Written informed consent was obtained from all participants. Sampling was done before vasectomy and six weeks after vasectomy. Protein quantification was performed using a two-dimensional (2D) Quant kit (GE Healthcare). The laboratory procedure performed for this part of the study was established and published previously by a collaboration between the Department of Urology, Pediatric Urology and Andrology and the Institute of Biochemistry at Justus Liebig University, Giessen, Germany (Pilatz *et al*., 2014).

100 µl of each seminal plasma samples containing 500 µg protein were diluted in 780 µl lysis buffer. Regarding isoelectric focusing, IPG-strips (pH 3-10 nL; GE Healthcare) were rehydrated at the temperature of 20°C with the protein extract. Technical replicates of 260 µl of protein extract were applied on three strips (Pixton *et al*., 2004; Secciani *et al*., 2009) and isoelectric focusing was performed for 32.05 kilovolt-ampere (kVA). After focusing, the IPG-strips were equilibrated for 10 minutes in 2 ml equilibration stock solution of ESS (ESS contains 6M urea, 0.1 mM EDTA (Sigma-Aldrich), 0.01 % bromophenol blue (Serva), 50 mM Tris-HCl (Roth) with pH 6.8 and 30% glycerol). At the next step, the IPG-strips were equilibrated for 15 minutes in 2 ml ESS I (10 ml ESS combined with 200 mg SDS (Roth) and 100 mg DTT (GE Healthcare)) followed by 15 minutes in ESS II (10 ml ESS combined with 200 mg SDS and 480 mg iodoacetamide (Sigma-Aldrich)). Protein separation in the second dimension was done by electrophoresis on 12.5% SDS polyacrylamide gels (Laemmli, 1970). Electrophoresis was performed in a Hoefer 600 system with the following program: 15 minutes at 60 mA/gel and 5 hours at 110 mA at 25°C. The fixation of gels was performed with a solution of 40% ethanol plus 10% acetic acid and later gels were stained overnight with flamingo (Bio-Rad Laboratories). A Typhoon 9200 laser scanner (GE Healthcare) with an excitation of 532 nm using an emission 555 BP filter was used for gel image detection. Afterward, all gel images were analyzed with PdQuest software (Bio-Rad Laboratories). Target spots were excised with the ExQuest spot cutter, and proteins were digested with trypsin on a liquid-handling robot system (MicroStarlet; Hamilton Robotics). For protein identification matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry was performed on an Ultraflex I TOF/TOF mass

spectrometer (Bruker Daltonics) equipped with a nitrogen laser and a LIFT-MS/MS (tandem mass spectrometry) facility. The instrument was operated in the positive-ion reflectron mode using 2.5-dihydroxybenzoic acid (Bruker Daltonics; Billerica) and methylendiphosphonic acid (Sigma-Aldrich) as the matrix. Sum spectra consisting of 200-400 single spectra were acquired. Regarding process the data and to control the instrument software was used with details of: the Compass 1.1 software package consisting of FlexControl 2.4, FlexAnalysis 3.0 and BioTools 3.0 (Bruker Daltonics). Protein identification was performed by MASCOT peptide mass fingerprint search using the human IPI database (Mascot 2.4; MatrixSciences) (v. 3.87; 91464 sequences; 36355611 residues). For the search, a mass tolerance of 75 ppm (parts per million mass accuracy) error was allowed, and carbamidomethylation of cysteine as global modification and oxidation of methionine as variable modification were used. The identified proteins were categorized according to biological function and subcellular localization via using the information available at the UniProt Knowledgebase.

2.8. STATISTICAL ANALYSIS

Results are shown as mean ± standard deviation (SD) of the number of assays indicated in each case. Statistical analyses were performed using GraphPad Prism software (v7.01), with data distribution analyzed by the Kolmogorov-Smirnov normality test. Differences between experimental groups were analyzed by means of one-way ANOVA, student t-test and the post-hoc Tukey’s test. A p-value of < 0.05 was considered statistically significant.

3. RESULTS

3.1.SOD1 AND SOD3

3.1.1. Demographic data of samples

The average patient age was 36.2 ± 6.1 years old (Mean ± SD, n=23) in the group of mixed testicular atrophy and 33.9 ± 2.9 years old in the group of Sertoli cell-only syndrome. Fertile-normozoospermic men were chosen as the control group and their average age was 38.0 ± 4.2 years old. There were no significant differences among the groups with respect to the mean age ($p>0.05$). The average level of seminal leucocytes in the patient and normal groups were less than one million white blood cells per milliliter (Note: The World Health Organization (WHO) defines the normal white blood cell concentration in the semen as anything less than one million per milliliter). Other parameters of semen analysis for the control group is presented in the Table 3-1 and are all in accordance with the standard range defined by WHO.

Table 3-1: Semen analysis parameters in the study groups (Mean ± SD)

Groups Parameters	Mixed testicular atrophy, n=23	Sertoli cell-only syndrome, n=23	Fertile-normozoospermic (Control), n=15	pValue
Volume of Semen (ml)	4.08±1.8	3.42±1.4	3.04±1.5	p > 0.05
pH of Semen	7.5±0.2	7.5±0.2	7.6±0.2	p > 0.05
Sperm Concentration (Mio/ml)	0.00	0.00	108.1±51.7	p < 0.05
Motility (a+b+c)	-	-	54.67±15.3	-
Sperm Vitality (%)	-	-	75.4±12.9	-
Sperm Normal Morphology	-	-	8.6±5.3	-

3.1.2. Comparison of seminal plasma protein content resulted from mass spectrometry between the group of mixed testicular atrophy and Sertoli cell-only syndrome

Label-free liquid chromatography-mass spectrometry revealed a total of 1354 proteins that were identified in the seminal plasma samples from the two NOA patient groups, mixed testicular atrophy and Sertoli cell-only syndrome and the group of normal-fertile men. The ratio comparison of MA/SCO is important to my thesis because that any protein which can distinguish between these two groups could be a useful marker for prognosis of M-TESE success. Differentially expressed proteins were defined to have at least a fold-change >2.0 with significance $p<0.05$. 61 proteins were identified (Figure 3-1) and Table 14-1 (appendix). Among the significantly decreased proteins, two proteins from the same family, SOD1 and SOD3 were selected for further validation by Western blotting, immunohistochemistry and ELISA. Both SOD1 and SOD3 were downregulated by more than 3 fold, and SOD3 exhibited the greatest fold down-regulation of the 61 differentially-expressed proteins (Figure 3-1). The other member of the superoxide dismutase family, SOD2, was also found in these seminal plasma patient groups, but was present at equivalent concentrations (Table 3-2).

Table 3-2: Mean fold-change and significance level of SOD family isoforms identified by MS in seminal plasma between the groups of mixed testicular atrophy and Sertoli cell-only syndrome (n=8/group)

SOD family isoforms	Fold	*P* Value
SOD3	0.16	0.003
SOD1	0.31	0.02
SOD2	0.99	Not Significant

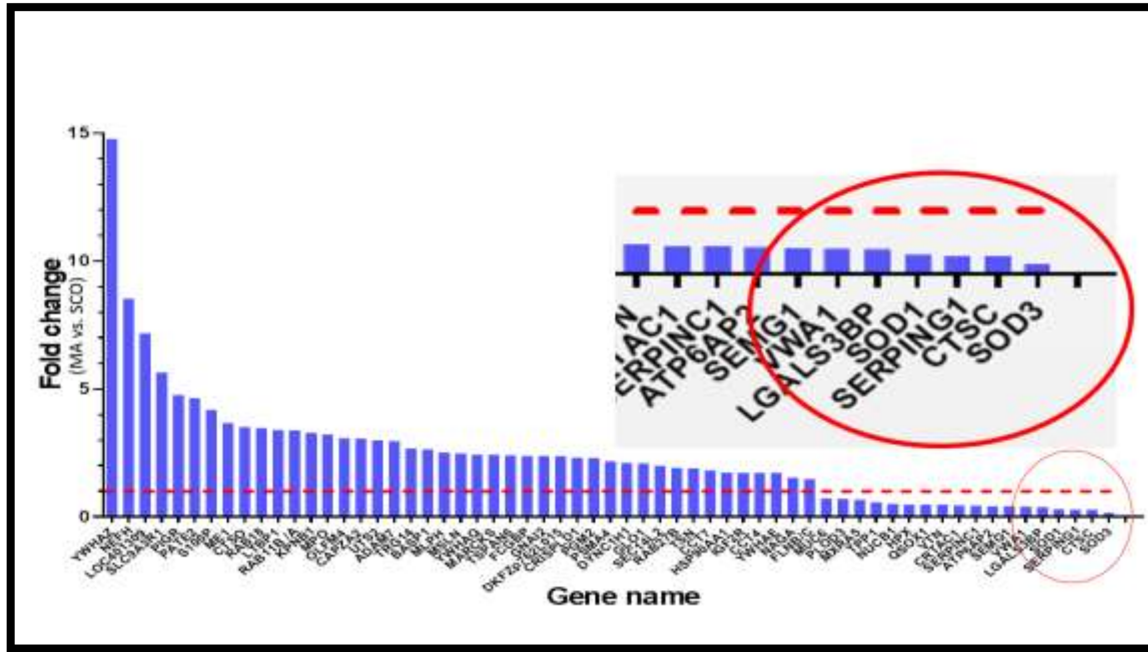


Figure 3-1: 61 proteins were identified by mass spectrometry for the comparison of seminal plasma samples from patients with mixed testicular atrophy versus Sertoli cell-only syndrome azoospermia. Differentially expressed proteins were defined to have at least fold-change >2.0 with significance p<0.05. The location of candidate proteins SOD1 and SOD3 has been magnified in the insert. All gene name and fold information is presented in the appendix Table 14-1.

3.1.3. Immunohistochemistry

In normal human testis tissue, SOD3 staining was observed predominantly in Sertoli cells (Figure 3-3), whereas SOD1 staining was seen strongly stained in spermatogonia (Figure 3-2). Testis biopsies from azoospermic-patient groups were subjected to evaluation of spermatogenesis and classified according to the overall histopathological diagnosis (Histopathological diagnosis is the study and diagnosis of diseases of the tissues, and involves examining tissues and/or cells under a microscope). Histological evaluation was performed according to Bergmann & Kliesch 2010. Samples were classified as intact spermatogenesis, quantitatively reduced spermatogenesis (hypospermatogenesis or mixed trescicular atrophy), obstructive azoospermia (OA), maturation arrest at the level of spermatogonia, spermatocytes or spermatids and Sertoli cell-only syndrome. In the testis of azoospermic patients, SOD1 and SOD3 staining was present, however variable. SOD1 and SOD3 staining in SCO biopsies from various patients were uniform in their staining pattern.

In contrast, the mixed atrophy samples were very heterogeneous with some containing only single tubules with preserved spermatogenesis and other samples containing more or less only those tubules. No significant histological differences were found.

More explanation is that the phrase of uniform staining pattern refers to samples (of patients) with intact spermatogenesis where antibodies were tested on. For example, staining was uniform for SOD3 in Sertoli cells and SOD1 in spermatogonia. Samples with impaired spermatogenesis like patients with mixed testicular atrophy that antibody reaction failed therefore showed staining as heterogeneous.

3.1.4. Protein concentration in blood serum and seminal plasma

The total protein concentration of seminal plasma and blood serum samples was compared among various patient and normal groups (mixed testicular atrophy, Sertoli cell-only syndrome and normal-fertile groups). These are different SP samples than those used for MS. These are a validation set samples. In addition, those seminal plasma and blood serum samples are matched. For seminal plasma samples, no significant differences were found, although ~2-fold variation occurs within each group (Figure 3-4 A). Mean protein concentrations (mg/ml) are MA: 25.0, SCO: 25.1, normal-fertile: 21.3 (Table 3-3). Protein concentrations of blood serum showed a significant difference among all groups in comparison to each other (Figure 3-2 B).

Table 3-3: Statistics measurements for total protein concentrations of seminal plasma (SP) and blood serum (BS) for various groups in the study for SOD family. SD: standard deviation, CV: coefficient of variation

	group mean (mg/ml)		Group SD		CV (%)	
	SP	BS	SP	BS	SP	BS
mixed testicular atrophy (n=15)	25	97	6,62	16,78	27	17,39
Sertoli cell-only syndrome (n=15)	25,1	83	7,52	14,43	30	17,31
Fertile-normozoospermic (n=15)	21,3	70	5,88	7,10	28	10,08

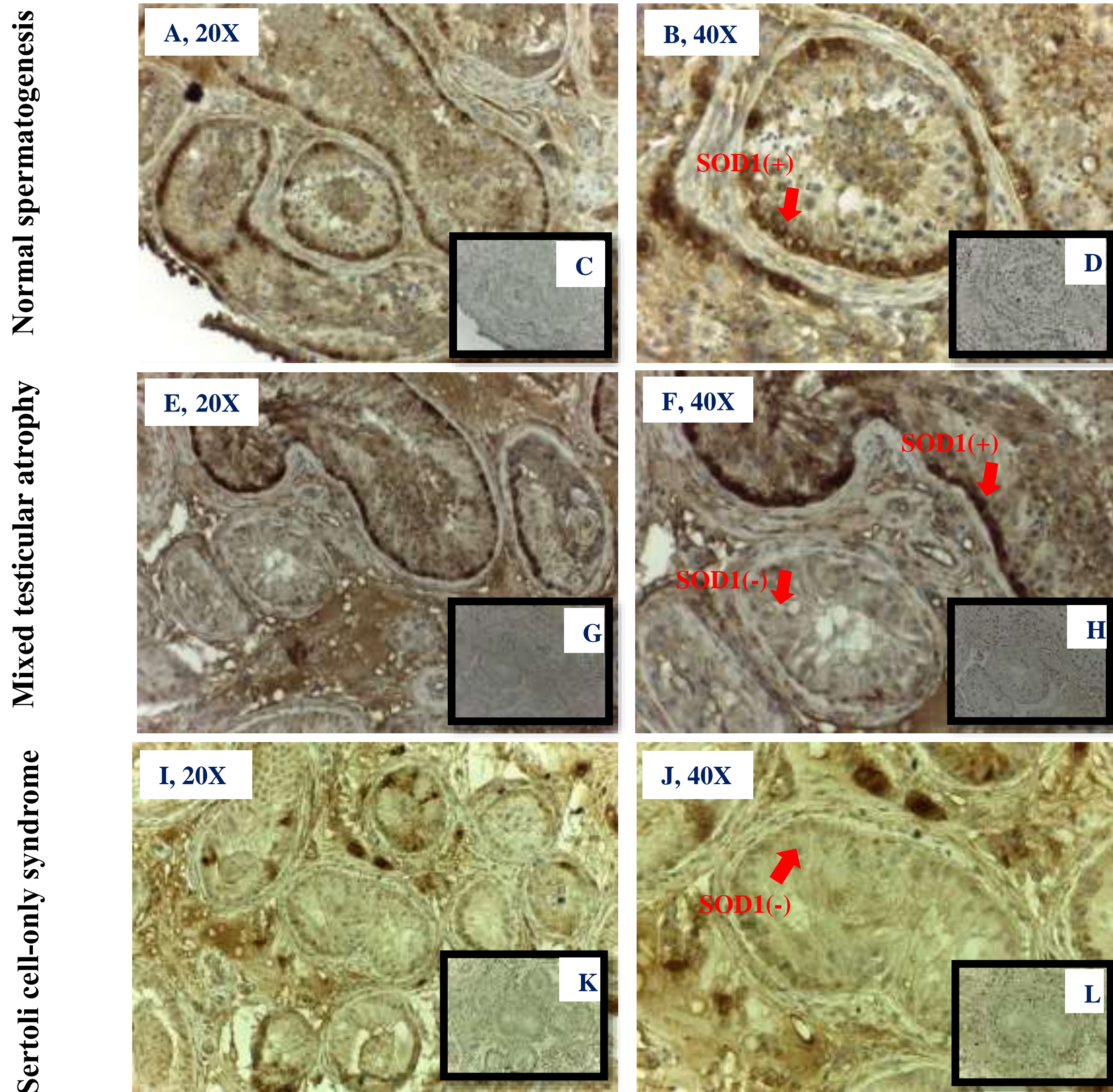


Figure 3-2: Immunohistochemistry (IHC) staining for SOD1.

SOD1 staining was predominantly in spermatogonia in normal testis tissue (A and B, negative controls have been showen in C and D). SOD1 immunorecactivity in the mixed testicular atrophy testis was seen in a variable patterns. Beside the stained spermatogonia, some seminiferus tubuls are unstained or impaired staining (E and F, negative controls have been showen in G and H). SOD1 immunorecactivity in the testis tissue of Sertoli cell-only syndrome patients was missed because of the lacking the spermatogonia (I and J, negative controls have been showen in K and L).

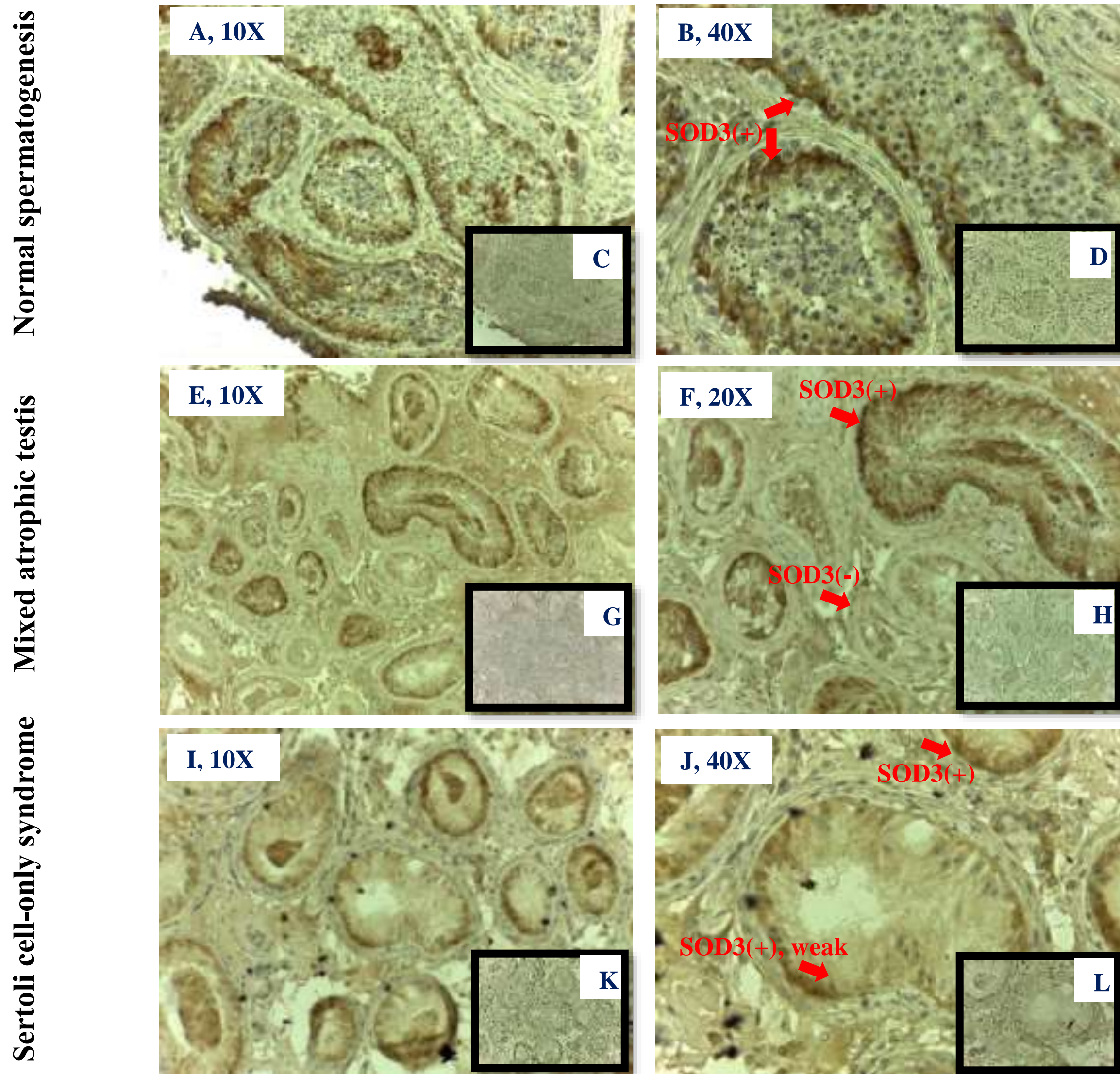


Figure 3-3: Immunohistochemistry (IHC) staining for SOD3.

SOD3 staining was observed predominantly in Sertoli cells in normal testis tissue. (A and B, negative controls have been showen in C and D). SOD3 immunorecactivity in the mixed atrophic testis was seen in a variable patterns. Positive staining and negative (impaired staining) were seen in the different seminiferous tubules (E and F, negative controls G and H). SOD3 immunorecactivity in the testis tissue of Sertoli cell-only syndrome patients was seen positively stained with anti SOD3 but with difference impact. Some seminiferous tubules are stained as sharp as normal tissues. While in some other SCO tubuls stainings are seen very weakly (I and J, negative controls K and L)

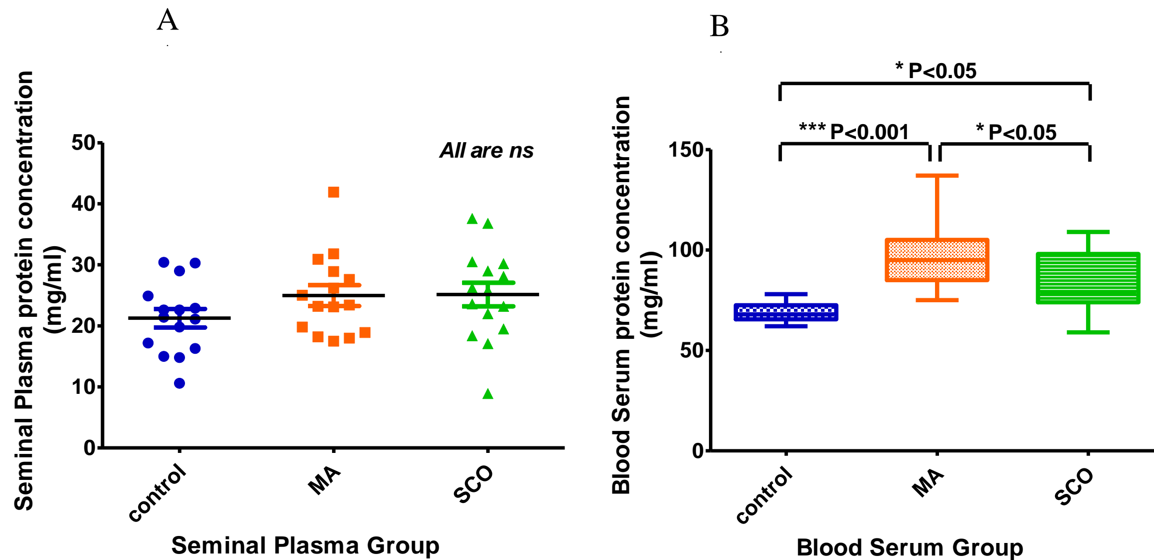


Figure 3-4: Total protein concentrations of seminal plasma and blood serum.

No significant difference was seen for total protein concentration of seminal plasma among various patient and normal groups (mixed testicular atrophy, Sertoli cell-only syndrome and normal-fertile groups) (A, Mean, SD, n=15, One-way ANOVA, P>0.05,).

In contrast, a significant difference was seen for total protein concentration of blood serum among various patient and normal groups (B, Mean, SD, n=15, One-way ANOVA, P<0.05).

3.1.5. SOD quantitation by ELISA in seminal plasma and blood serum

The protein concentration of SOD1 was decreased (p<0.05) in seminal plasma from men with mixed testicular atrophy and men with Sertoli cell-only syndrome compared to the control groups. There was no significant difference in SOD1 protein concentration between the two patient groups: “MA versus SCO” (Figure 3-5 A).

SOD3 immunoreactivity in seminal plasma from men with Sertoli cell-only syndrome was 2.3 fold lower than controls (p<0.05) whilst seminal plasma from men with mixed testicular atrophy was 4.6 fold lower (p<0.0001) than control. A significant (p<0.05) difference was also seen for SOD3 between SCO and MA patient groups (Figure 3-5 B). The SOD3 MA/SCO ratio was 0.49 (p<0.05) in comparison to the mass spectrometry ratio of 0.16 (p<0.01). Hence the ELISA data for SOD3 supports the mass spectrometry data that showed decreased SOD3 protein expression in seminal plasma from the MA patient group (Figure 3-5 B).

The protein concentration of the SOD1 and SOD3 in the blood serum samples in matching with seminal plasma samples were then investigated using the same ELISAs. There was neither significant difference in both SOD1 and SOD3 protein concentration between the two patient groups: “MA versus SCO” nor between patient groups and the group of control (Figure 3-6 A and Figure 3-6 B). With comparing the relative protein concentration of SOD1 in blood serum and seminal plasma a strong concentration gradient was noticeable. More explanation that there was a SOD1 protein concentration around ~4ng/mg in blood serum of control group and instead around ~180ng/mg in seminal plasma. Same is true for the relative protein concentration of SOD3 in blood serum and seminal plasma (around ~2ng/mg in blood serum of control group and instead around ~280ng/mg in seminal plasma). Up to my knowledge, it is the first time that the protein concentration of SOD1 and SOD3 in the seminal plasma is reported and therefore there is no other reference for comparing. However, in one available reference, SOD1 protein concentration in blood serum from control group reported close to my results and it proves my job (Peng *et al*., 2016).

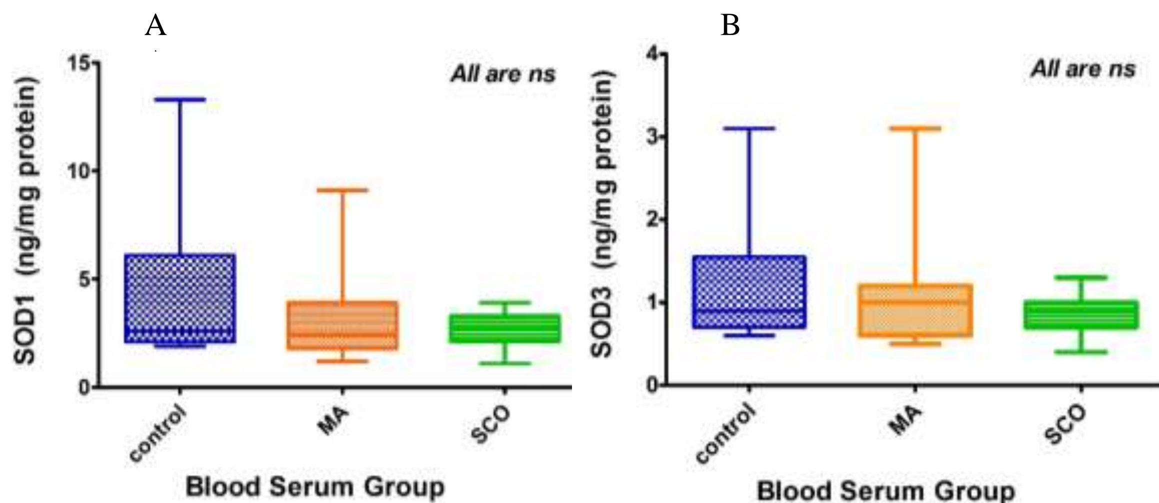


Figure 3-5: SOD1 and SOD3 protein concentrations of blood serum analysed by ELISA.
SOD1 and SOD3 were compared among various patient and normal groups (mixed testicular atrophy, Sertoli cell-only syndrome and normal-fertile groups, n/group=15). No significant relationship were seen among the studied groups for both of proteins of SOD1 and SOD3 (A and B).

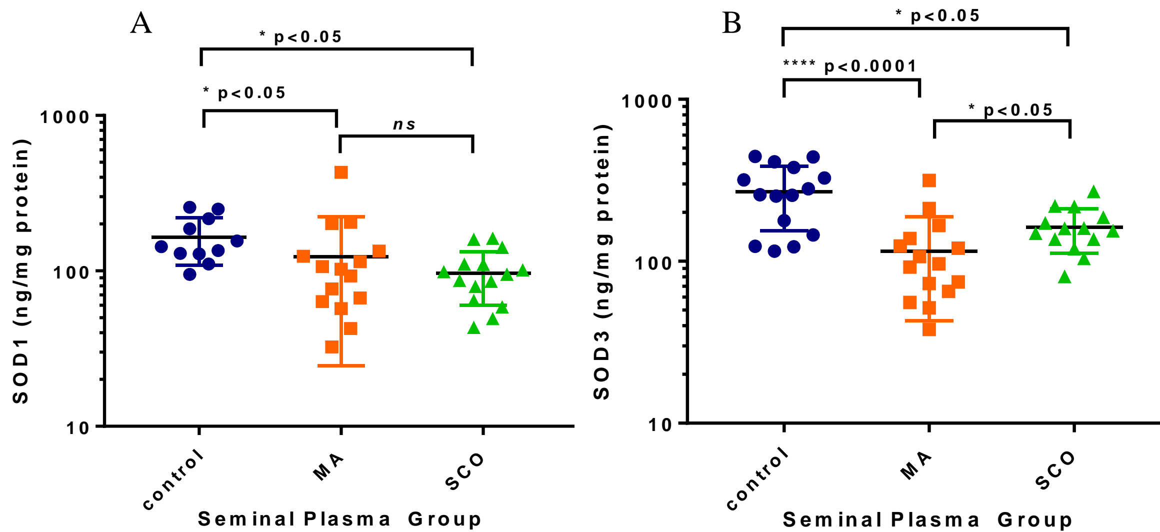


Figure 3-6: SOD1 and SOD3 protein concentrations of seminal plasma analysed by ELISA.

SOD1concentration was compared among various patient and normal groups (mixed testicular atrophy (n=15), Sertoli cell-only syndrome (n=14) and normal-fertile groups (n=11)). Significant difference were seen between the patient groups and controls. No significant relationship was found between two patient groups (MA versus SCO) (A). SOD3 concentration was compared among various patient and normal groups (mixed testicular atrophy (n=15), Sertoli cell-only syndrome (n=14) and normal fertile groups (n=15)). Significant differences were seen between the patient groups and controls. A significant difference was also seen for SOD3 between SCO and MA patient groups (B).

3.1.6. Western blot Analysis

A serial dilution of seminal plasma protein concentration conducted for both proteins of SOD1 and SOD3 with the purpose of creating the best possible standard curve. The pool seminal plasma which used for creating the standard curve was a mixture of 5 seminal plasma belongs to 5 normal fertile men. The samples used for Western blot analysis are from the same patients as used for ELISA.

For SOD1 the serial dilution started from the highest amount of protein 220µg and end in a 64-fold down dilution of protein concentration around 3µg. The antibody against the SOD1 protein gave a single band corresponding to the predicted size in 20kD. After generating the linear standard curve, two followed concentrations, 26µg and 13µg, which they had the best

band quality selected as standard concentrations for repeating in all further Western blots and in order to use in relative quantitation analysis. It means for all samples this two followed concentration was prepared and loaded into the gel. In continue, for analyzing the bands, all bands were relatively quantified by considering the band volume of standard protein. At the end the result presented as “min relative quantitation”. Comparisons of the SOD1 concentrations was significant between the azoospermic patient groups and normal-fertile group ($p<0.01$). However a significant difference was not found between mixed atrophy and SCO azoospermic groups.

For SOD3 the serial dilution started from the highest amount of protein 220µg and end in a 16-fold down dilution of protein concentration around 13µg. The antibody against the SOD3 protein gave a sharp single band corresponding to the predicted size in 35kD. Some other pale bands also was seen as non specific bands, which were not surprising and already were reported by the reference of the antibody (https://www.proteinatlas.org/ENSG00000109610-SOD3/antibody). After generating the linear standard curve, two followed concentrations, 75µg and 37µg, which they had the best band quality and also had located accurately in along the standard curve selected as standard concentrations for repeating in all further Western blots and in order to use in relative quantitation analysis.

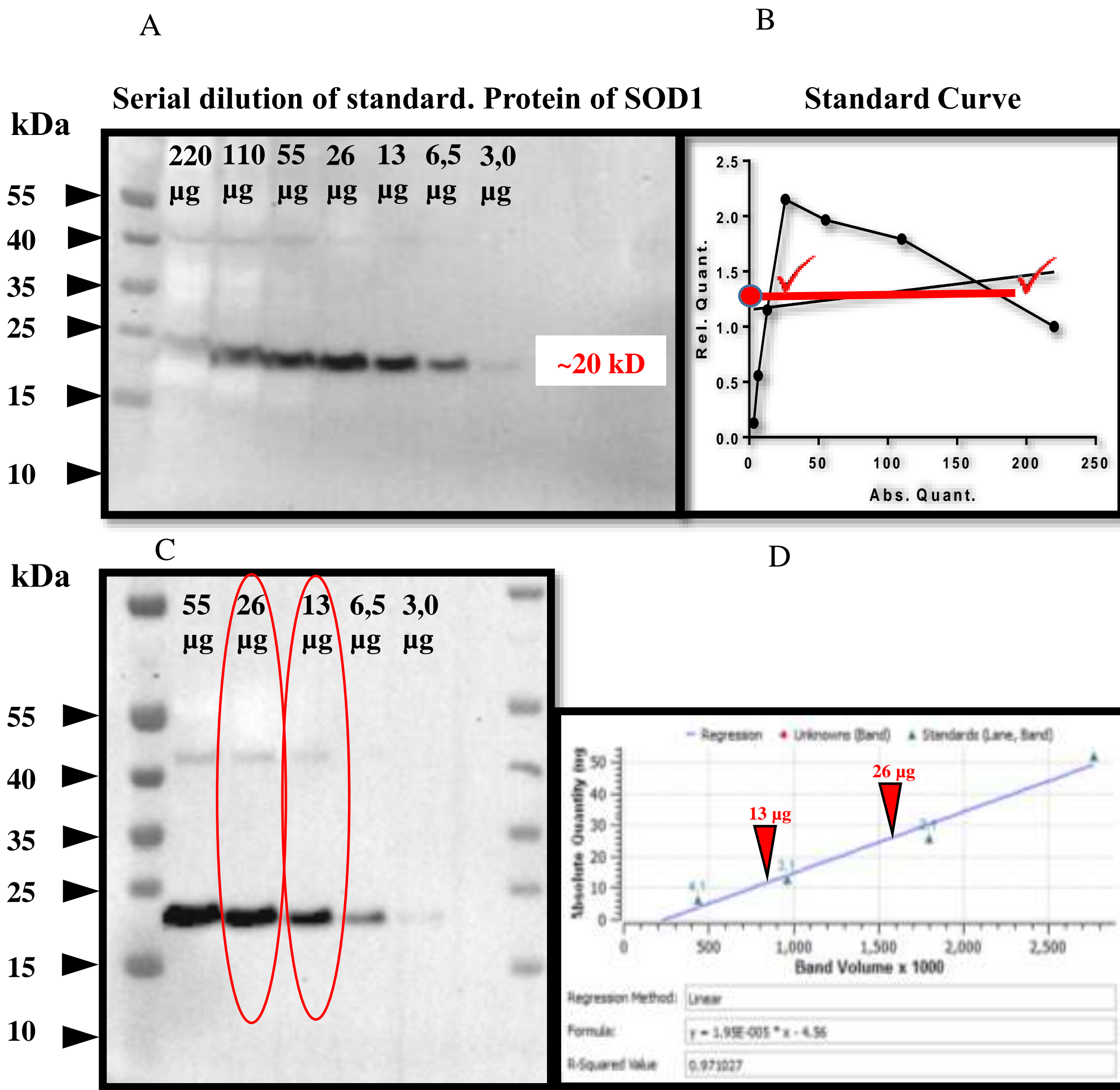


Figure 3-7: The flow of the Western blotting setup for the protein of SOD1 is summarized here.

Serial dilution of protein concentration for the protein of SOD1 including 220µg, 110µg, 55µg, 26µg, 13µg, 6,5µg, 3µg. Anti SOD1 showed a single band in 20kD (A). The standard curve was linear for protein concentrations in the middle of serial dilutions (B). 26µg and 13µg as two following concentrations, selected as standard concentrations for applying in all Western blots to provide a basic standard for final quantitation analysis (C). The location of standard proteins on the linear standard curve, based on their densitometry for absolute quantity (D).

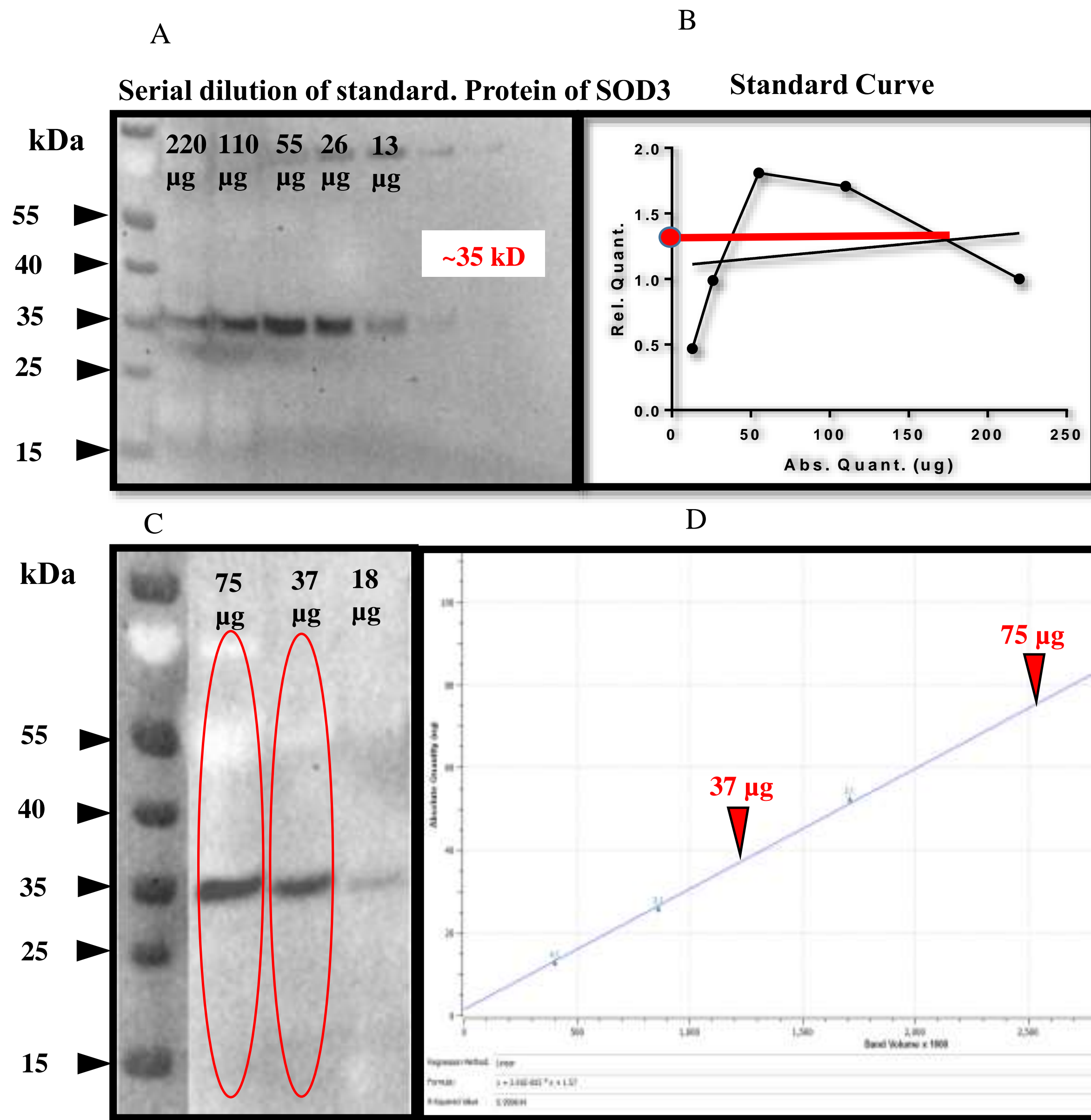


Figure 3-8: The flow of the Western blotting setup for the protein of SOD3 is summarized here.

Serial dilution of protein concentration for the protein of SOD3 including 220µg, 110µg, 55µg, 26µg, 13µg. Anti SOD3 showed a single band in 35kD (A). The standard curve was linear for protein concentrations in the middle of serial dilutions (B). 75µg and 37µg as two following concentrations, selected as standard concentrations for applying in all Western blots to provide a basic standard for final quantitation analysis (C). The location of standard proteins on the linear standard curve, based on their densitometry for absolute quantity (D).

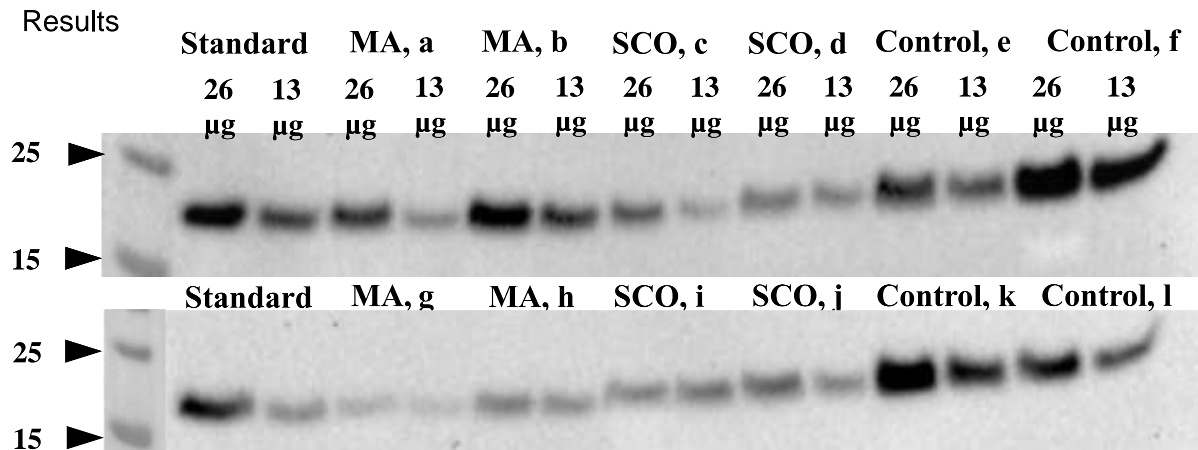


Figure 3-9: SOD1 Western blotting examples for different groups of patients and the group of control. 4 patients with mix testicular atrophy shown by MAa, MAb, Mag and MAh; 4 patients with Sertoli cell-only syndrome shown by SCOc, SCOd, SCOi and SCOj; 4 normal samples as controls shown by Controle, Controlf, Controlk and Controll. For each run of Western blot, the standard protein with two different concentration as following dilutions (26µg and 13µg) were loaded on to the gels. The same protein concentrations were loaded for all the patient and control samples. Later the min volume of following bands were calculated and normalized by min volume bands of standard protein in each gel. With this calculation, the min relative quantitative ratio was achieved and used for comparing among the patient and control groups.

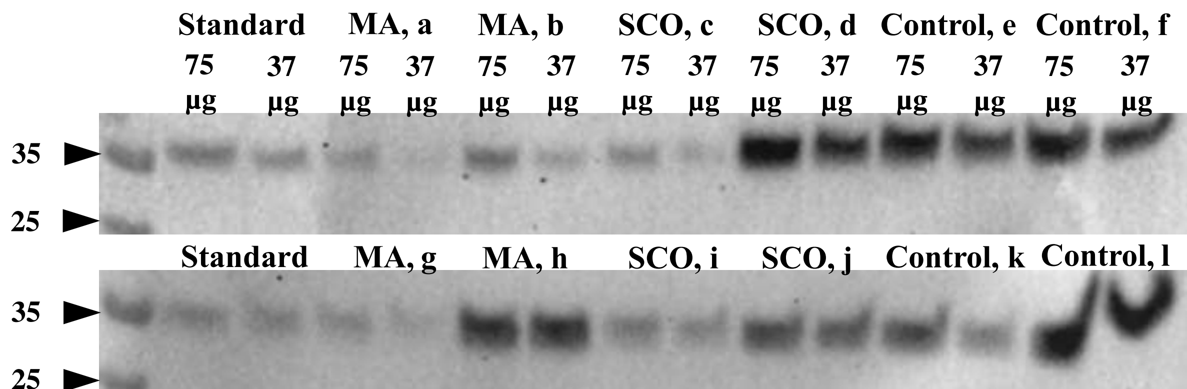


Figure 3-10: SOD3 Western blotting examples for different groups of patients and the group of control. 4 patients with mix testicular atrophy shown by MAa, MAb, Mag and MAh; 4 patients with Sertoli cell-only syndrome shown by SCOc, SCOd, SCOi and SCOj; 4 normal samples as controls shown by Controle, Controlf, Controlk and Controll. For each run of Western blot, the standard protein with two different concentration as following dilutions (75µg and 37µg) were loaded on to the gels. The same protein concentrations were loaded for all the patient and control samples. The calculation was similar to the explanation in the figure 3-9.

A B

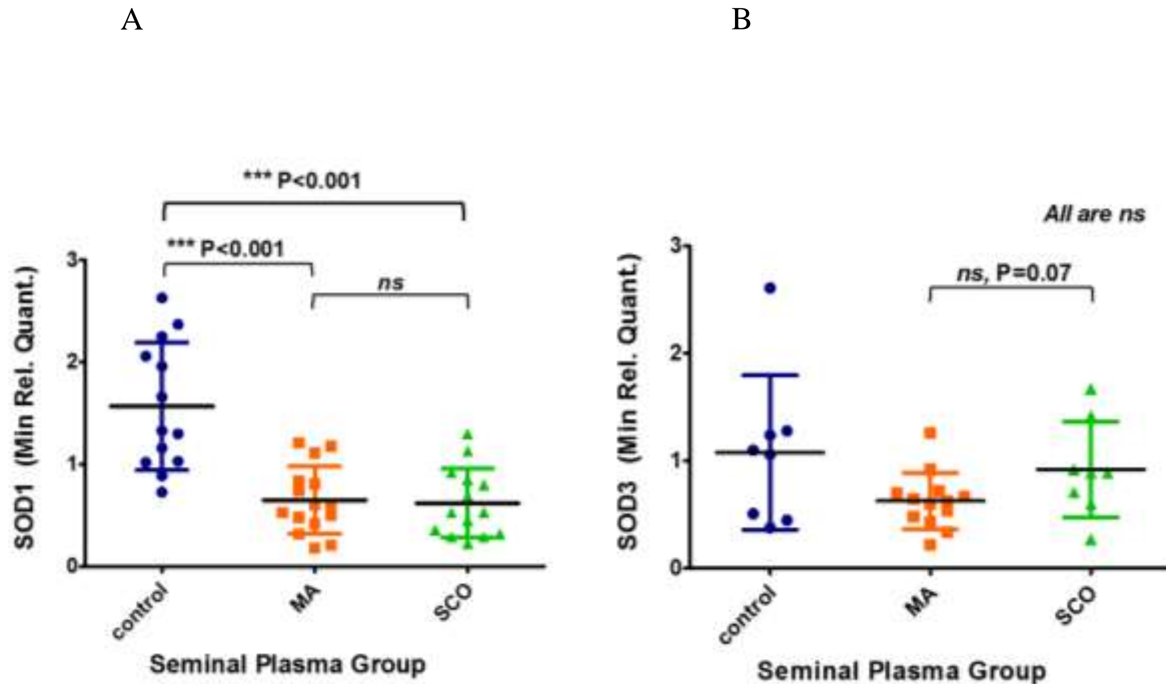


Figure 3-11: SOD1 and SOD3 protein concentrations of seminal plasma analysed by WB, was compared among various patient and control groups (mixed testicular atrophy, n=13; Sertoli cell-only syndrome, n=15; and normal-fertile groups, n=14). Comparisons of the SOD1 concentrations was significant between the azoospermic patient groups and normal-fertile group (p<0.01). However a significant difference was not found between mixed atrophy and SCO azoospermic groups.

SOD3 Protein concentrations of seminal plasma analysed by WB, was compared among various patient and control groups (mixed testicular atrophy, n=8; Sertoli cell-only syndrome, n=13; and normal-fertile groups, n=8). Comparisons of the SOD3 concentrations was not significant between the azoospermic patient groups and normal-fertile group. No significant difference also was found between mixed atrophy and SCO azoospermic groups. Nevertheless the pValue in comparing MA versus SCO was p=0.07, which is very close to the significant ratio.

The reason why SOD1 groups have more data points than SOD3 is that the quality of bands in SOD3 were not as sharp as bands in SOD1 plus in some gels there was a high background. All these reasons let to exclude some of samples from final calculations.

3.2. CRISP1

3.2.1. Two-dimensional gel electrophoresis

A comprehensive total of 84 different spots per gel were detected in comparison between groups (seminal plasma before vasectomy and after vasectomy). Proteins were considered to be differentially expressed when their mean spot intensity differed by over 1.5-fold between groups, with $P < 0.05$ (analysis of variance). The comparison between groups (seminal plasma before vasectomy and after vasectomy) revealed 27 significant proteins. Of those, none was testis-specific, while three epididymis-specific candidates were identified: CRISP1 (Cystein-Rich Secretory Protein 1), FAM12B (Human Epididymis-specific protein 3-beta) and LTF (Lactotransferrin). CRISP1 was shown to be more than 3 fold down-regulated in seminal plasma after vasectomy. As already mentioned the method of two-dimensional gel electrophoresis was done previously at the department of urology, pediatric urology and andrology, (Justus Liebig University, Giessen, Germany). The results were not published. I used those unpublished data as a source, for my project to select a protein candidate for further validation by another proteomics method means Western blotting. In addition, the validation occurred in my project for the group of azoospermic patients and not for the group of men requesting vasectomy (before and after vasectomy). It was because of the semen analysis in men after vasectomy has a mimic like azoospermia. Therefore in this project, the protein analysis for CRISP1 was performed in 5 subgroups of azoospermia including men with mixed testicular atrophy, Sertoli cell-only syndrome, spermatogenesis arrest, Klinefelter syndrome and obstructive azoospermia. The normal samples from the group of controls also were examined for more comparisons.

3.2.2. Demographic Data of samples

The average patient age in the group of mixed testicular atrophy was 36.9 ± 6.0 years old (Mean± SD), 33.9 ± 4.7 years old for the group of Sertoli cell-only syndrome, 31.3 ± 4.8 years old for the group of spermatogenesis arrest, 34.3 ± 5.2 years old for the group of Klinefelter syndrome and 37.0 ± 6.6 years old for the group of obstructive azoospermia. For fertile-normozoospermic men the average of age was 39.8 ± 5.5 years old (there was no significant difference in age between any groups). All the other parameters of semen analysis

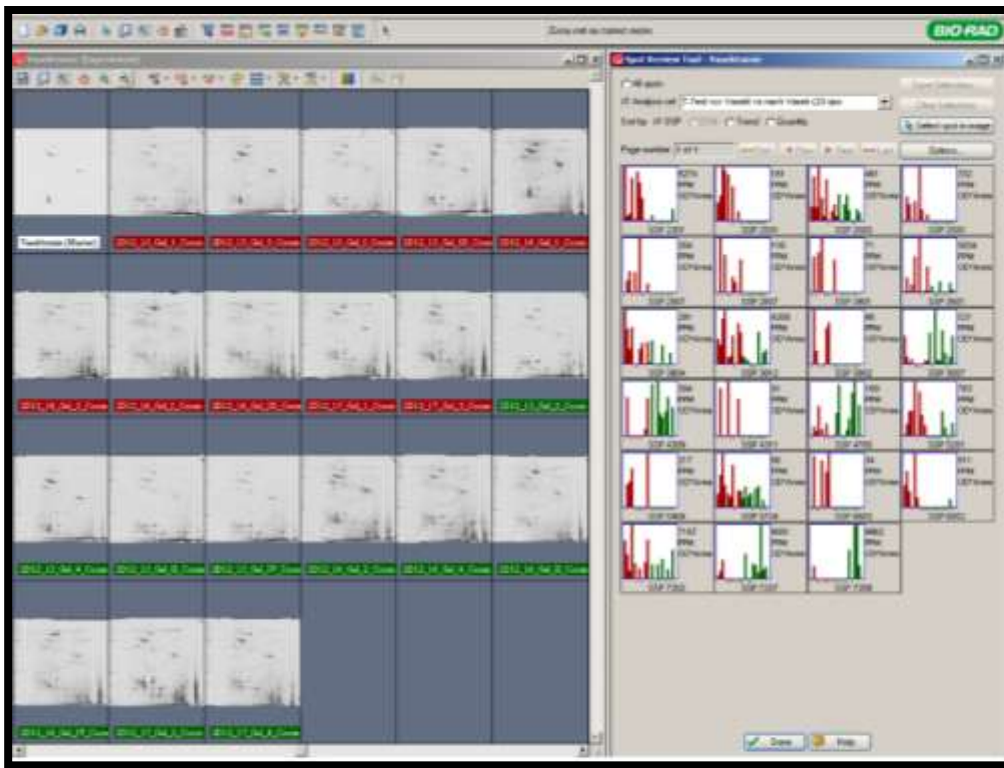


Figure 3-12: Comparison between groups (seminal plasma before vasectomy and after vasectomy), for characterization of the proteins analyzed by two dimensional gel electrophoresis. Gels described with red color belong to seminal plasma of patients before vasectomy. Gels described with green color belong to seminal plasma of patients after vasectomy.

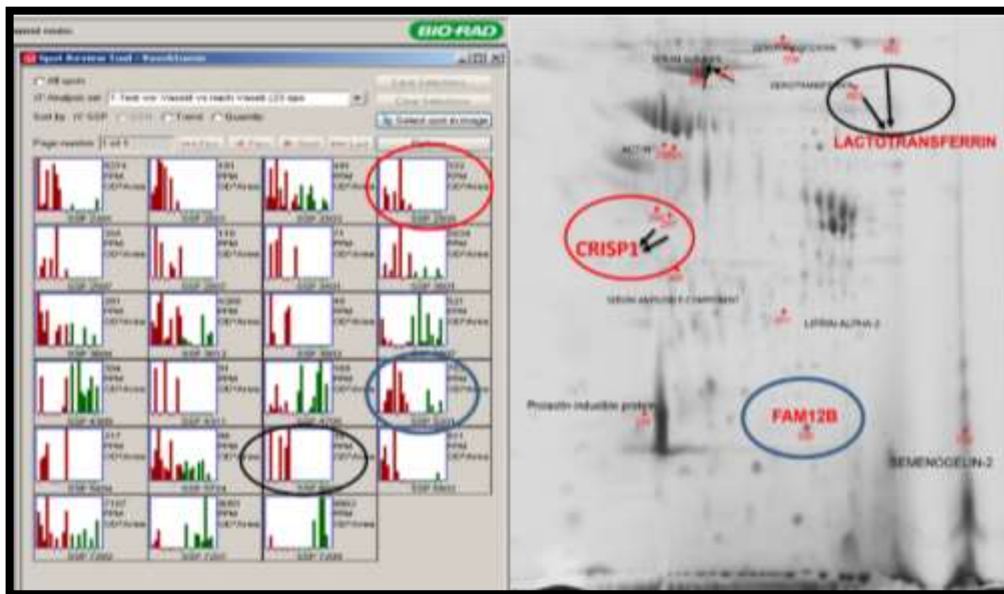


Figure 3-13: Two-dimensional map of proteins that are differentially regulated in seminal plasma before vasectomy and after vasectomy. Comparison between two groups revealed 27 significant proteins. Proteins were visualized by flamingo staining. Proteins were identified using the IPI database. Specifically indicated spots related to the significant proteins of CRISP1, FAM12B and LTF.

were in the standard range of WHO frame. Other parameters of semen analysis are presented in Table 3-4.

Table 3-4: Semen analysis parameters in the study groups (Mean± SD)

Groups / Parameters	Mixed testicular atrophy, n=10	Sertoli cell-only syndrome, n=12	spermatogenesis arrest, n=6	Klinefelter syndrome, n=14	Obstructive Azoospermia, n=8	Fertile-(Control), n=10	pValue
Volume of Semen (ml)	4.18±1.7	3.38±1.6	3.38±1.2	1.84±0.7	3.30±1.7	2.58±1.3	$p > 0.05$
pH of Semen	7.5±0.2	7.4±0.2	7.3±0.2	7.3±0.3	7.6±0.3	7.6±0.2	$p > 0.05$
Sperm Concentration (Mio/ml)	0.00	0.00	0.00	0.00	0.00	113.2±55.2	$p < 0.05$
Motility (a+b+c)	-	-	-	-	-	49.15±16.3	-
Sperm Vitality (%)	-	-	-	-	-	69.8±22.8	-
Sperm Normal Morphology	-	-	-	-	-	9.6±4.9	-

3.2.3. Immunohistochemistry

Immunohistochemistry was performed on normal testis and normal epididymis tissues with applying both anti CRISP1 antibodies (antibody A and antibody B). The purpose of performing the immunoreactivity in epididymis was to check the antibody performance and compare it with the references to know it has the same pattern or not (human protein atlas). The results showed the same stained patterns in epididymis with reacting to both antibodies (Figure 3-14, C, D, I and J). It was interesting also to see the reaction of antibody agains an epididmys specific protein in testis and therefore the performance of both antibodies in testis tissues was investigated. No reaction was seen with germ cells in testis and CRISP1 antibodies (Figure 3-14, A, B, G and H).

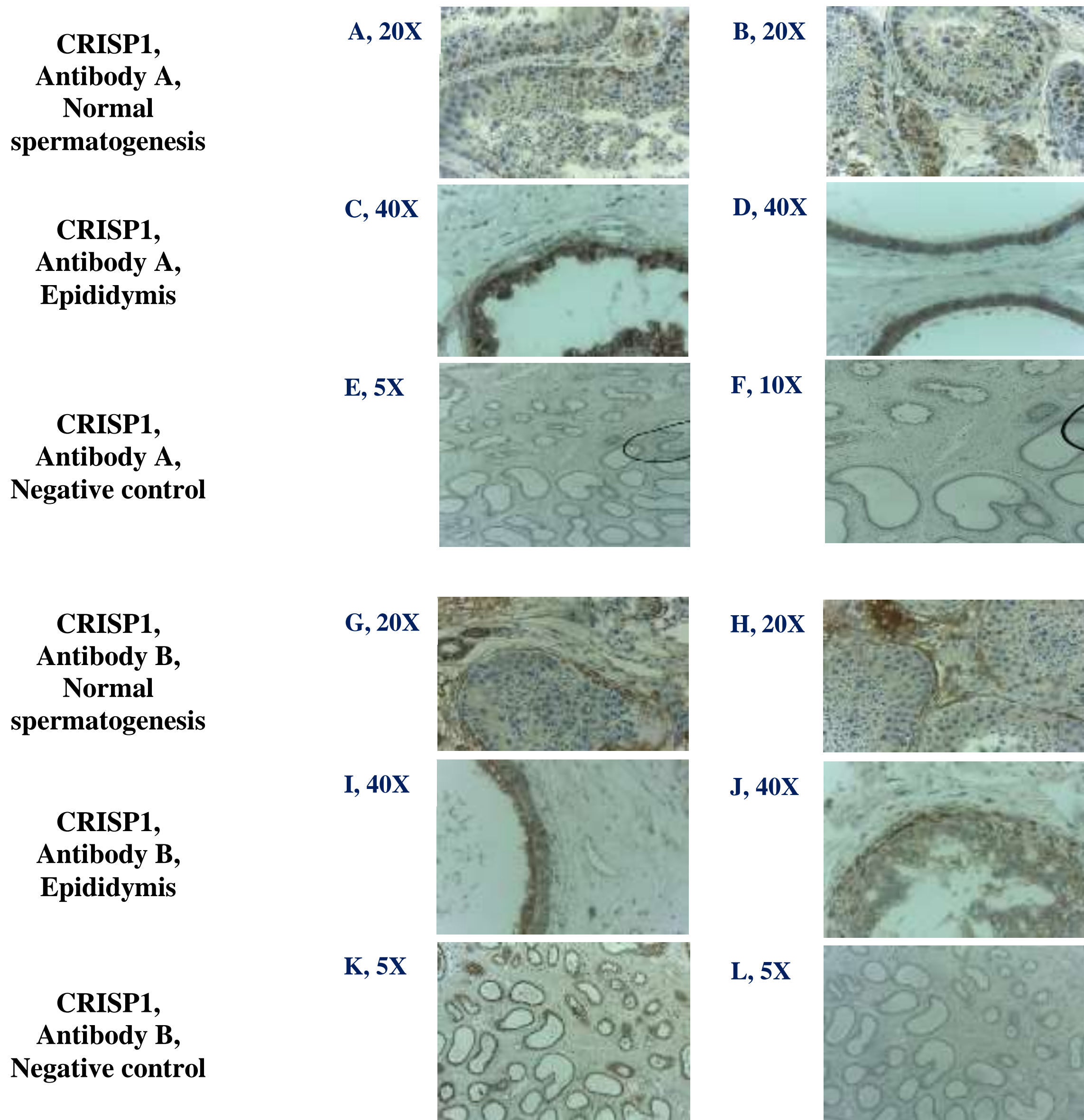


Figure 3-14: IHC staining for CRISP1 stained in the normal testis and epididymis. No reaction with germ cells in testis and CRISP1 antibody A and antibody B (A, B, G and H). Positive reaction were seen with epididym (efferent duct cells) and both CRISP1 antibodies A and B (C, D, L and J). Negative reaction with pre absorption test (IgG negative control) was used for both antibodies (E, F, K and L).

3.2.4. Protein Assay

Table 3-5: Seminal plasma protein concentrations for various groups in the study for CRISP1

	group mean (mg/ml)	Group SD	CV (%)
Mixed testicular atrophy (n=10)	32.60	7.30	22.41
Sertoli cell-only syndrome (n=12)	35.50	12.49	35.19
Spermatogenesis arrest (n=6)	37.63	8.34	22.19
Klinefelter syndrome (n=14)	34.93	9.80	28.06
Klinefelter syndrome, MA (n=7)*	39.43	9.84	24.97
Klinefelter syndrome, SCO (n=7)**	30.43	7.99	26.28
Obstructive Azoospermia (n=8)	35.88	13.35	37.20
Fertile-normozoospermic (n=10)	27.79	9.91	35.69

*and ** are two subgroups of patients with Klinefelter syndrome. Based on the testis tissue histology evaluation, half of the patients with Klinefelter syndrome showed histology as mixed testicular atrophy and the next half showed histology of Sertoli cell-only pattern.

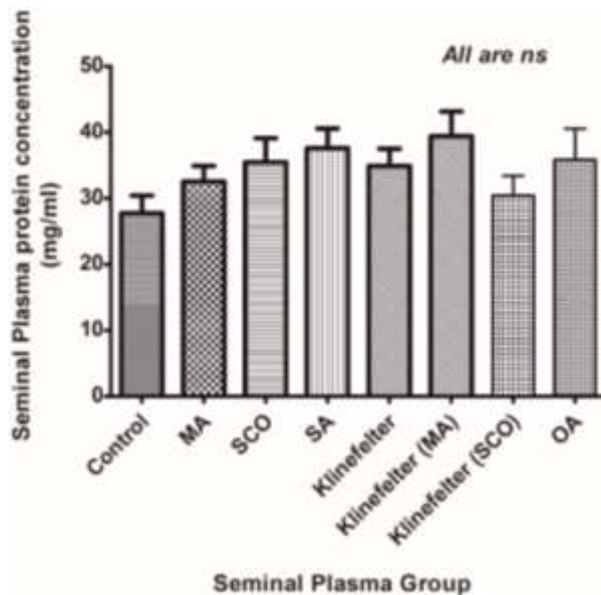


Figure 3-15: Seminal plasma protein concentration for various study groups involved to the study project of CRISP1. No significant relationship was seen among the groups. Control: Fertile-normozoospermic men, MA: Mixed testicular atrophy, SCO: Sertoli cell-only syndrome, SA: Spermatogenesis arrest, Klinefelter (MA): testis tissue histology looklikes mixed testicular atrophy, Klinefelter (SCO): testis tissue histology looklikes Sertoli cell-only syndrome, OA: Obstructive azoospermia.

3.2.5 Western Blot Analysis

The protein of CRISP1 has been relatively quantified by Western blotting with two different antibodies (antibody A and antibody B). Each antibody recognized the different epitope of the protein. The procedure of Western blotting setting up for CRISP1 (both antibodies) including the creation of a linear standard curve and choose the best following concentrations for using in the relative quantitation can showen in the figure of 3-15. The achieved results showed the same pattern of CRISP1 protein concentration for all different groups of patients (Figuer 3-16).

Patients after vasectomy in results of semen analysis are the same as obstructive azoospermic patients. CRISP1 was significant less in the seminal plasma after vasectomy, therefore in this study the CRISP1 protein concentration was compaired between obstructive and non-obstructive azoospermia (OA vs. NOA). The significant difference was found between obstructive and non-obstructive azoospermia (OA vs. NOA). This subject had been proved and reported already by Legare *et al.* 2013 and our results proved it again (Figuer 3-17). Afterwards the CRISP1concentration from the patients with Klinefelter syndrome was compared to the previous two groups. The significant difference was found only in results came from the CRISP1 antibody B (Figuer 3-17). The next statistical analysis was done among only non-obstructive azoospermia groups. Results came out similar with applying both antibodies except between MA and Klinefelter syndrome (Figuer 3-18). Means CRISP1 was seen significantly difference between MA and Klinefelter syndrome only with using antibody antibody B (Figuer 3-18). When the group of control was compared individually with one by one of all other groups, the results were more or less similar for both antibodies but not 100%. For example the first antibody showed significant difference between control and SA, but the second antibody did not show (Figuer 3-19).

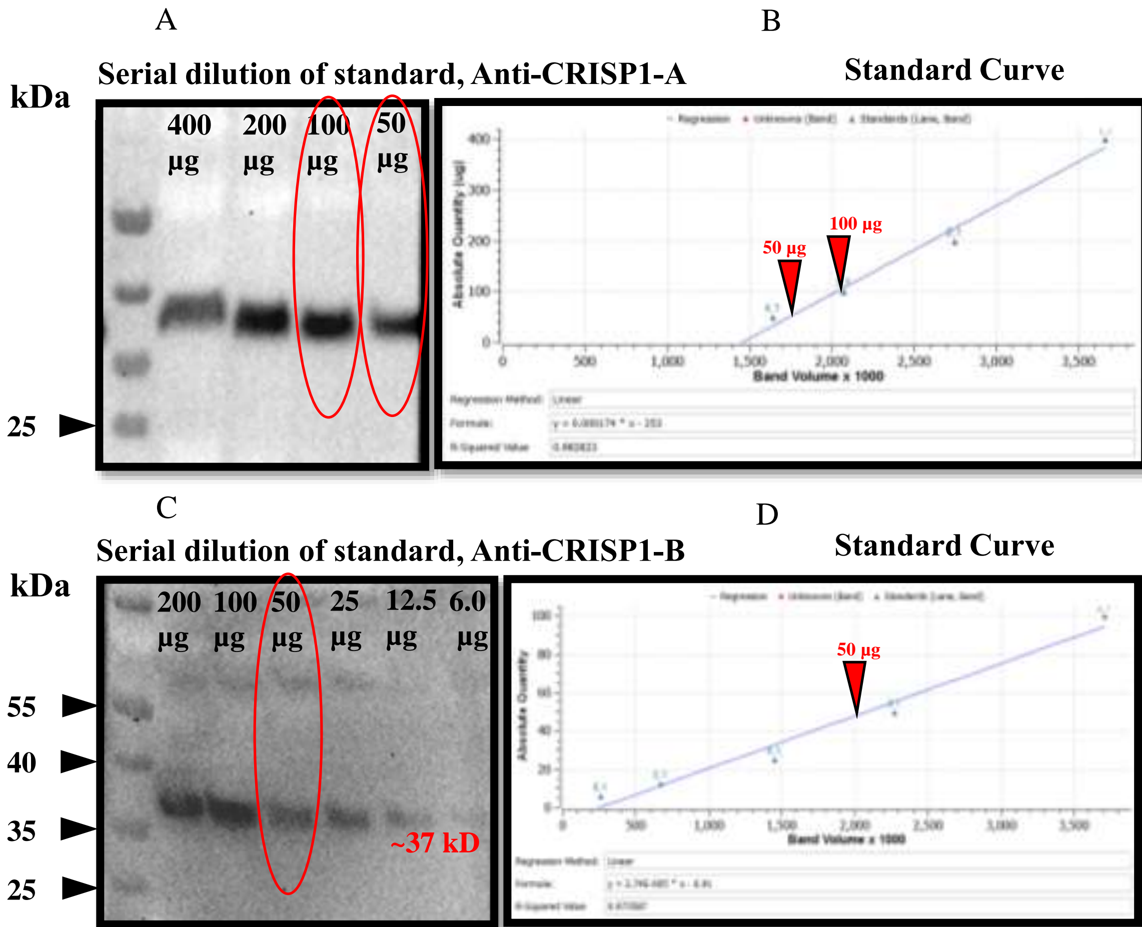


Figure 3-16: The flow of the Western blotting setup for the protein of CRISP1 with two different antibodies is summarized here.

Serial dilution of protein concentration for the protein of CRISP1 in the reaction with anti-CRISP1-A were including 400µg, 200µg, 100µg and 50µg (A). Serial dilution of protein concentration in the reaction with anti-CRISP1-B were including 200µg, 100µg, 50µg, 25µg, 12.5µg and 6µg (C). The standard curves were linear for serial dilutions in reaction with both antibodies (B and D). 100µg and 50µg as two following concentrations, selected as standard concentrations for applying in all Western blots to provide a basic standard for final quantitation analysis with anti-CRISP1-A (A and B). 50µg as the single concentration, selected as standard and union concentration for applying in all Western blots and therefore it was provided a basic standard for final quantitation analysis with anti-CRISP1-B (C and D). Anti CRISP1 showed a single band in 37kD in reaction with both antibodies (A and C). The location of standard proteins on the linear standard curve, based on their densitometry for absolute quantity (B and D).

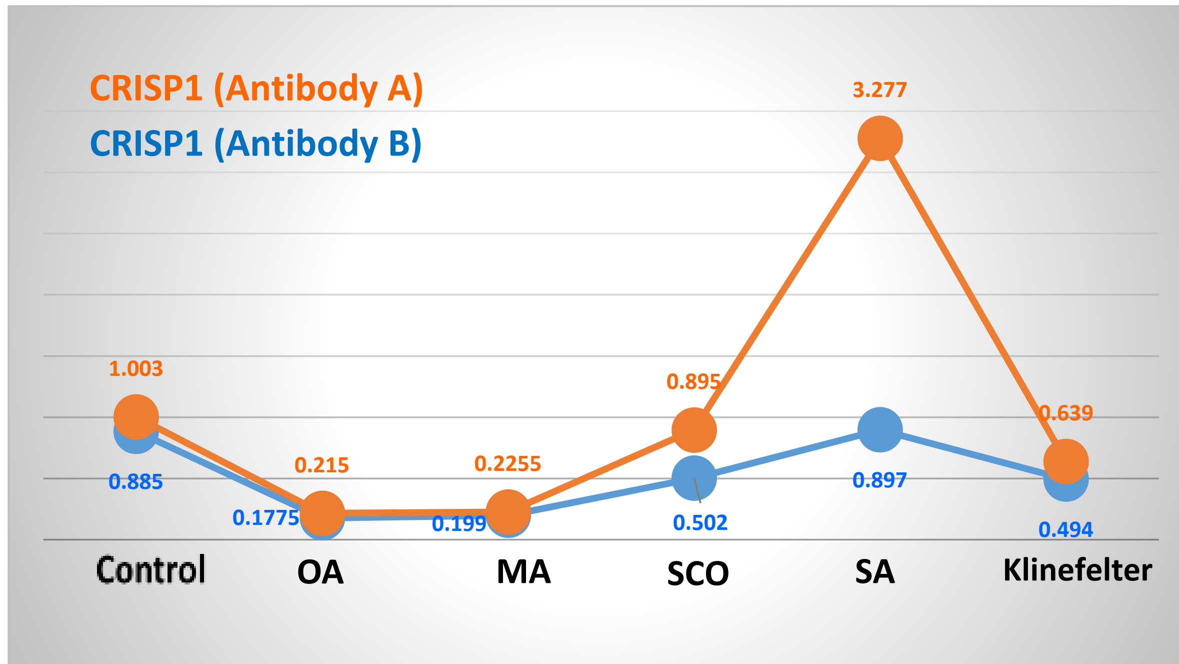
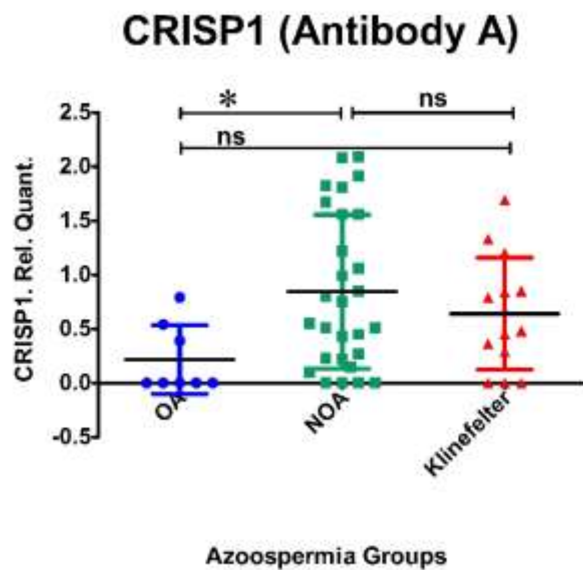


Figure 3-17: Comparison of CRISP1 protein concentration among the various azoospermic patients (and control group) by applying two different antibodies. There is a similar pattern for both antibodies (in this figure only the patterns in due to the reactions of CRISP1-antibody A and CRISP1-antibody B with study groups has been considered without consider the relationships among them). The numbers in the figure are indicating the mean protein concentreation of CRISP1 (mean, SD). The number of each study group was similar for both antibodies: Control (n=10), OA (n=8), MA (n=10), SCO (n=12), SA (n=6) and Klinefelter syndrome (n=14).

Control: Fertile-normozoospermic men, OA: Obstructive azoospermia, MA: Mixed testicular atrophy, SCO: Sertoli cell-only syndrome, SA: Spermatogenesis arrest, Klinefelter (Klinefelter syndrome).

A



B

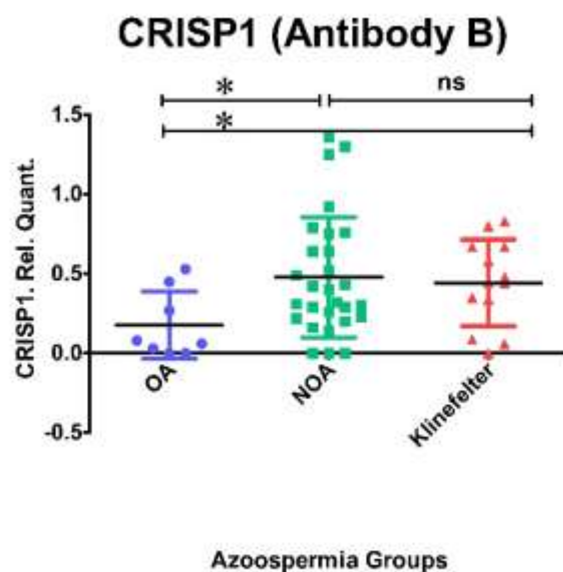


Figure 3-18: The Western blotting results for protein of CRISP1 in reaction with two differenet antibodies, antibody A and antibody B, which recognizing two different epitopes of the protein, among three major groups of OA, NOA and patients with Klinefelter syndrome.

The significant difference was found between obstructive (n=8) and non-obstructive azoospermia (n=28) with using both antibody A and antibody B (A, mean, SD, Mann Whitney test, *$P<0.05$; B, mean, SD, Mann Whitney test, *$P<0.05$). No significant difference between non-obstructive azoospermia (n=28) and patients with Klinefelter syndrome (n=14) and with using both antibodies (A, mean, SD, Mann Whitney test, $P>0.05$; B, mean, SD, Mann Whitney test, $P>0.05$). The significant difference between obstructive azoospermia (n=8) and Klinefelter syndrome (n=14) was found only in results of reaction with antibody B (A, mean, SD, Mann Whitney test, $P>0.05$; B, mean, SD, Mann Whitney test, *$P<0.05$).

A B

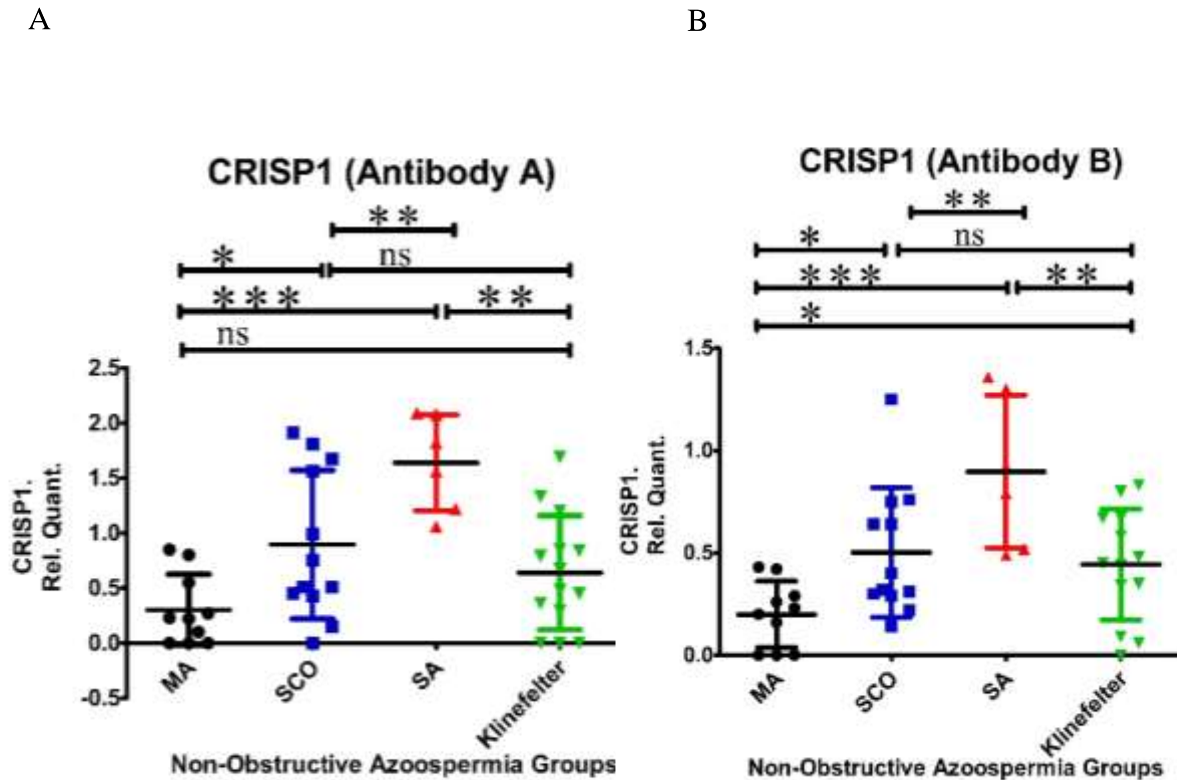
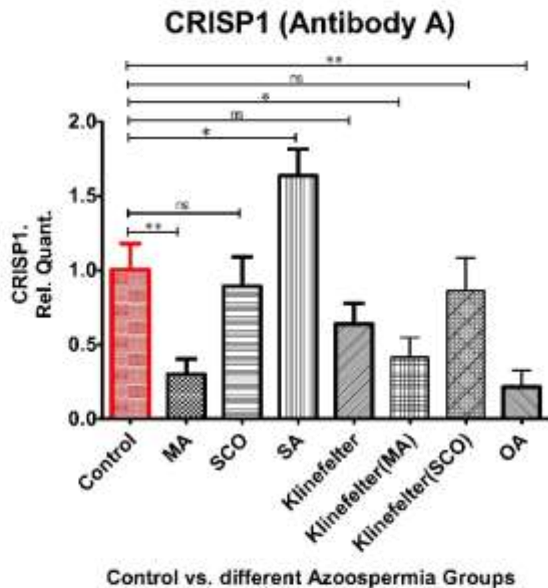


Figure 3-19: The Western blotting results with using two different antibodies for protein of CRISP1 among the various subgroups of non-obstructive azoospermia (NOA) including MA, SCO, SA and Klinefelter syndrome.

The significant difference was found between MA (n=10) and SCO (n=12) with using both antibodies A and antibody B (A, mean, SD, *P<0.05; B, mean, SD, *P<0.05). A high significant difference was found between MA (n=10) and SA (n=6) with using both antibodies A and antibody B (A, mean, SD, ***P<0.001; B, mean, SD, ***P<0.001). The significant difference was found between SCO (n=12) and SA (n=6) with using both antibody A and antibody B (A, mean, SD, **P<0.01; B, mean, SD, **P<0.01). No significant difference was seen between SCO (n=12) and Klinefelter syndrome (n=14) with using both antibodies A and antibody B (A, mean, SD, P>0.05; B, mean, SD, P>0.05). The significant difference was found between MA (n=10) and Klinefelter syndrome (n=14) with using only antibody B (B, mean, SD, *P<0.05). No significant difference was seen when the antibody A reacted with those both non-obstructive azoospermia groups, which means MA versus Klinefelter syndrome (A, mean, SD, P>0.05). All statistic analysis for providing the graphs of this figure was done by the method of One-way ANOVA test (Newman-Keuls Multiple Comparison Test).

A



B

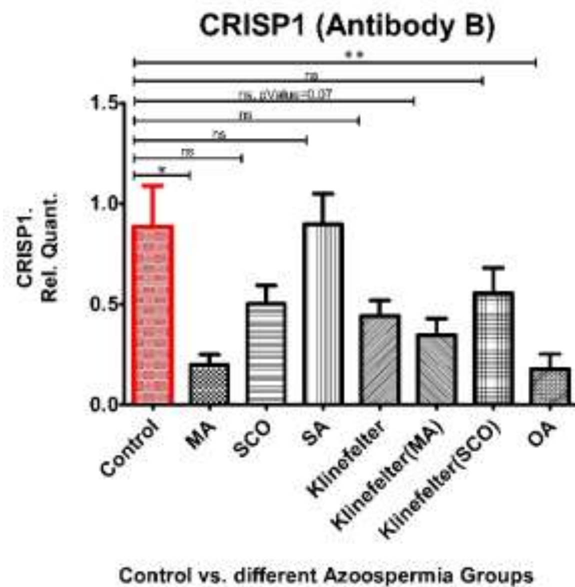


Figure 3-20: The Western blotting results with using two different antibodies for protein of CRISP1 between the control group (specifically) and each single of other non-obstructive azoospermic (NOA) groups including MA, SCO, SA and Klinefelter syndrome, plus the comparison between the control group and obstructive azoospermia (OA).

The significant difference was found between control (n=10) and MA (n=10) with using both antibody A and antibody B (A, mean, SD, **P<0.001; B, mean, SD, *P<0.05). No significant difference was seen between control (n=10) and SCO (n=12) with using both antibody A and antibody B (A, mean, SD, P>0.05; B, mean, SD, P>0.05). A significant difference was found between control (n=10) and SA (n=6) with using the only antibody A (A, mean, SD, *P<0.05) and this significant relationship was not found by applying the antibody B (B, mean, SD, P>0.05). No significant difference was seen between control (n=10) and Klinefelter syndrome (n=14) with using both antibody A and antibody B (A, mean, SD, P>0.05; B, mean, SD, P>0.05). The significant difference was found between control (n=10) and OA (n=8) with using both antibody A and antibody B (A, mean, SD, **P<0.001; B, mean, SD, **P<0.001).

All statistical analysis was done by the method of student t-test (Mann Whitney test).

3.3. LGALS3BP

3.3.1. Demographic data of samples

The average patient age in the group of mixed testicular atrophy was 36.7 ± 5.0 years old (Mean± SD), 33.1 ± 4.1 years old for the group of Sertoli cell-only syndrome, 31.7 ± 4.2 years old or the group of Klinefelter syndrome. For fertile-normozoospermic men the average of age was 38.7 ± 4.5 years old (there was no significant difference in age between any groups). All the other parameters of semen analysis were in the standard range of WHO frame. Other parameters of semen analysis are presented in Table 3-6.

Table 3-6: Semen analysis parameters in the study groups (Mean± SD) for LGALS3BP

Groups **Parameters**	**Mixed testicular atrophy, n=15**	**Sertoli cell-only syndrome, n=15**	**Klinefelter syndrome, n=14**	**Fertile (Control), n=15**	**pValue**
Volume of Semen (ml)	4.18±1.7	3.38±1.6	1.84±0.7	2.58±1.3	$p > 0.05$
pH of Semen	7.5±0.2	7.4±0.2	7.3±0.3	7.6±0.2	$p > 0.05$
Sperm Concentration (Mio/ml)	0.00	0.00	0.00	113.2±55.2	$p < 0.05$
Motility (a+b+c)	-	-	-	49.15±16.3	-
Sperm Vitality (%)	-	-	-	69.8±22.8	-
Sperm Normal Morphology	-	-	-	9.6±4.9	-

3.3.2. Comparison of seminal plasma protein content resulted from mass spectrometry between the group of mixed testicular atrophy and Sertoli cell-only syndrome

As already was mentioned in the section of 3.1.2. a total of 1354 proteins revealed by the method of label-free liquid chromatography-mass spectrometry in comparison of seminal plasma from two patient groups, namely mixed testicular atrophy and Sertoli cell-only syndrome. Differentially-expressed proteins were defined to have at least fold-change >2.0 with significance p<0.05 and therefore 61 proteins were identified. Among the significant proteins, a new candidate, LGALS3BP was selected for further validation by Western blotting. LGALS3BP was shown by MS to have a 3 fold down-regulation with a fold change of 0.39 and significance level of 0.02 (*P* Value =0.02) between the group of mixed testicular atrophy and Sertoli cell-only syndrome.

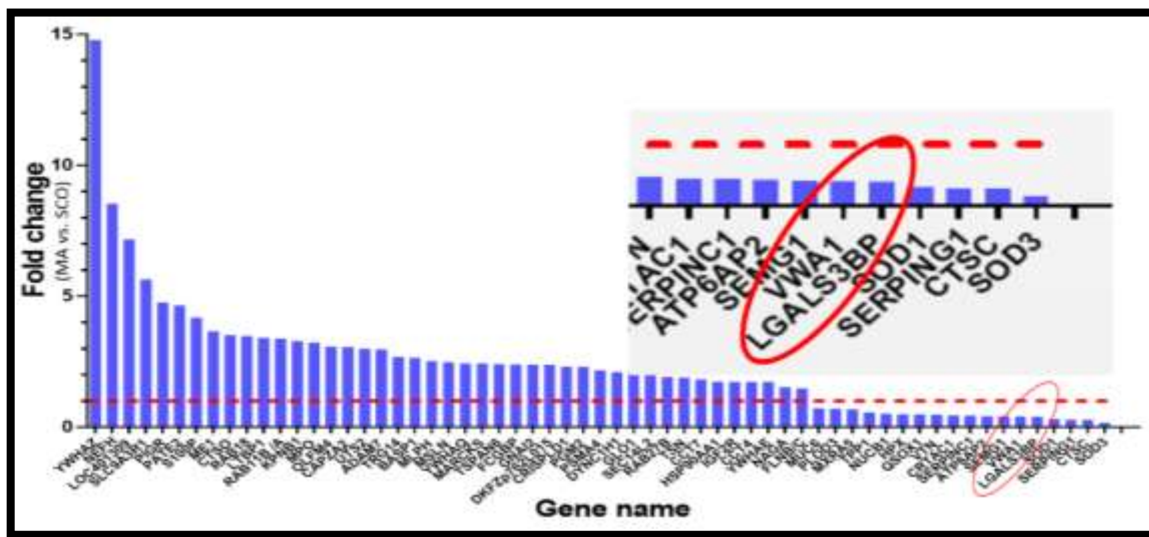


Figure 3-21: The location of LGALS3BP in the list of significant proteins came out from the method of MS. Among 61 proteins the third candidate for this study means the protein of LGALS3BP was selected for further validation. The location of LGALS3BP, has been magnified.

3.3.3 Western Blot Analysis

A

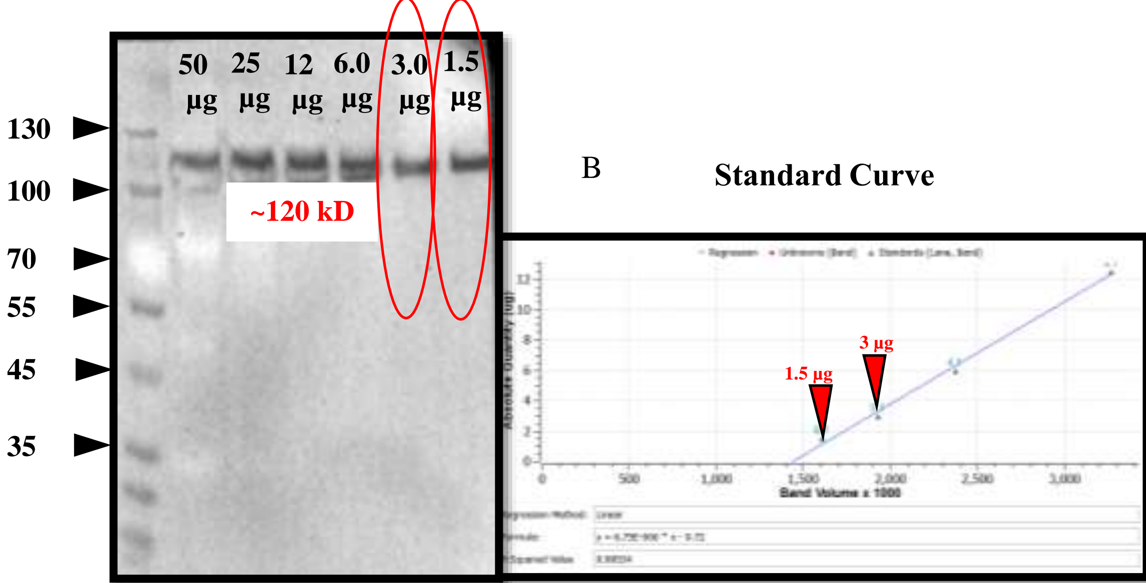


Figure 3-22: The flow of the Western blotting setup for the protein of LGALS3BP is summarized here.

Serial dilution of protein concentration for the protein of LGALS3BP including 50µg, 25µg, 12µg, 6µg, 3µg and 1.5µg. Anti LGALS3BP showed a single band in 120kD (A). The standard curve was linear for protein concentrations of serial dilutions (B). 3µg and 1.5µg as two following concentrations, selected as standard concentrations for applying in all Western blots to provide a basic standard for final quantitation analysis. The location of standard proteins on the linear standard curve, based on their densitometry for absolute quantity (B).

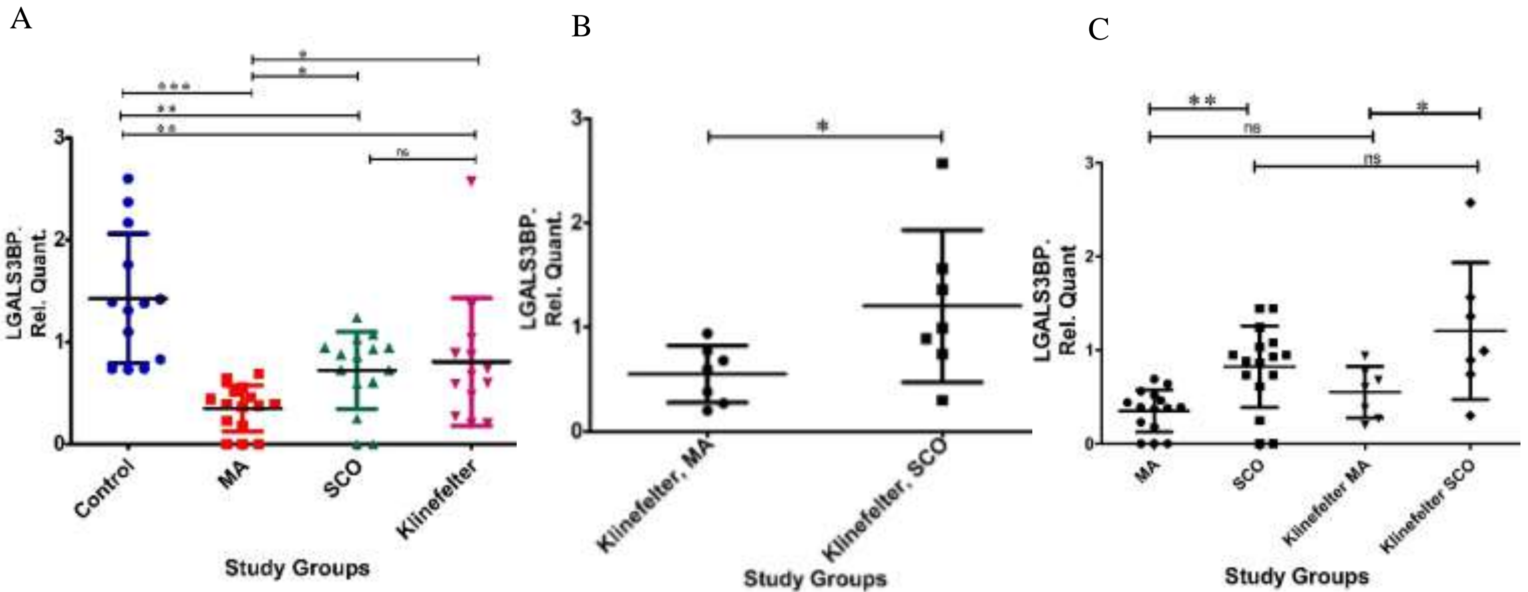


Figure 3-23: The Western blotting results for protein of LGALS3BP among the various subgroups of non-obstructive azoospermia (NOA) including MA, SCO and Klinefelter syndrome and also in comparison with normal-fertile men as group of control.

A: The significant difference was found between the group of controls and all other patient groups. Control (n=15) versus MA (n=15), mean, SD, ***P<0.001; Control (n=15) versus SCO (n=15), mean, SD, **P<0.01; Control (n=15) versus Klinefelter (n=14), mean, SD, **P<0.01. The patient group of SCO (n=15) in compare of patient group of MA (n=15) showed a significant relationship (mean, SD, *P<0.05). A significant difference was found between MA (n=15) and patient of Klinefelter syndrome (n=14) (mean, SD, *P<0.05). No significant difference was seen between SCO (n=15) and Klinefelter syndrome (n=14) (mean, SD, P>0.05). All statistic analysis for LGALS3BP was done by the method of One-way ANOVA test (Newman-Keuls Multiple Comparison Test).

B: The significant difference was found between two sub-groups of patients with Klinefelter syndrome, means Klinefelter, MA (n=7) and Klinefelter, SCO (n=7), mean, SD, *P<0.05 (Unpaired t test).

C: No significant difference was seen between MA patients (n=15) and Klinefelter-MA (n=7), mean, SD, P>0.05, as well as, no significant difference was seen between SCO patients (n=15) and Klinefelter-SCO (n=7), mean, SD, P>0.05. All statistical analysis for this graph was done by the method of One-way ANOVA test (Newman-Keuls Multiple Comparison Test).

4. DISCUSSION

4.1.SOD1 AND SOD3

Reproductive health problems affect 15 to 24% of worldwide couples, who are suffering from infertility (Agarwal *et al*., 2019). Azoospermic conditions are generally observed in 10 to 15% of infertile cases, however, in some references, it has been reported as high as 20% (Öztekin *et al*., 2019). Azoospermia is a pathological condition that is categorized under the umbrella of male fertility disorders. It is diagnosed by the absence of spermatozoa in the semen (Najari, 2019). Two different biological mechanisms are contributors to this condition. Therefore, azoospermia can be further categorized into non-obstructive azoospermia (NOA) or as obstructive azoospermia (OA). NOA occurs as a result of testicular dysfunction such as perturbations in sperm development which takes place in the seminiferous tubules. Additionally, genetic causes such as Klinefelter syndrome and hormone failures are some reasons for NOA, however, it still remains idiopathic.

On the other hand, OA is observed in males with an obstruction in the extra-testicular ducts resulting in azoospermia. OA has a prevalence of around 40% among the azoospermic patients (Blok *et al*., 2019). The remaining 60%, belongs to NOA displaying spermatogenic failure. NOA diagnosis can be further categorized based on various spermatogenic impairments (Table 1-1).

The significant differences between seminal proteome and transcriptome content in NOA and OA groups have been reported in many studies. Many efforts were made to find a non-invasive method to distinguish between NOA and OA groups. Thus, the use of protein or transcript biomarkers has been suggested. Azoospermic patients with various pathophysiological features are grouped as a single cohort in the previous studies, thus further studies to distinguish among the categories are required. The comparison of sub-groups within NOA is as important as distinguishing between NOA and OA groups. This enables physicians to make better clinical decisions. Currently, the only reliable approach to determine NOA diagnosis is the retrieval of surgical biopsies, an invasive method that would be preferred to be avoided if possible. Approximately half the NOA patients do not require surgery as spermatogenesis is absent. For the other half of NOA patients, this procedure

yields various success rates depending on the characteristics of azoospermia (Bromage *et al*., 2007).

Protein biomarkers may serve as a predictor of the spermatogenic status in patients suffering from azoospermia. Our study focuses on selected proteins expressed with a statistical significance from mass spectrometry screenings and also varies between the subgroups of NOA. The first part of the study was designed to compare the seminal proteomes of two different subgroups of NOA. Men with mixed-testicular atrophy (MA) with a positive sperm retrieval at microdissection TESE (M-TESE) and patients with Sertoli cell-only syndrome (SCO) with a negative sperm retrieval at M-TESE. Results were compared to a control group that consisted of fertile men with normozoospermic results of semen analysis. To the best of our knowledge, this is the first study to date to identify SOD family proteins in NOA subgroups patients. Two members of the SOD family, SOD1 and SOD3, were found significantly downregulated between MA and SCO patients in our proteomics data obtained from mass spectrometry studies (*P* Value<0.01).

To support our achieved data from the method of MS, we refer to the previous studies. In a study, the method of MS was used and both proteins of SOD1 and SOD3 were found in the list of proteins from seminal plasma for NOA patients (Batruch *et al*., 2012). However, in this study when the quantitation methods of spectral-counting and extracted ion current were used for determining the abundance ratios, only SOD1 was found significantly 0.6 folds increased in NOA in comparison to the control cohort (Batruch *et al*., 2012).

To validate this, further proteomics approaches such as WB, ELISA and IHC were performed. A literature search of the SOD family revealed these proteins are very interesting for the male reproductive scientists, as they have been studied widely among various infertile groups of men. Notably, the contribution of azoospermic patients was drastically less than other infertile groups such as teratozoospermia, oligozoospermia, asthenozoospermia and or patients suffering from varicocele (From 1983 to 2017, 17 papers have been published to report the SODs enzyme activity concerning the various groups of infertile men and among them only in two papers, the chosen study population consists of azoospermic men).

From another perspective and because the SOD family members have enzymatic functions, most of the previous studies have been tried to measure and compare their enzymatic

activities. Whereas there are lots of published papers indicating the results of SOD enzymatic activities, there are very few studies with the focus on the SODs protein concentration. From this point of view also our study considered as a novel study (Table 4-1).

Table 4-1: Presence of SOD1, SOD3, CRISP1 and LGALS3BP in the list of detected proteins found in the proteomics analysis of some high referral references

Reference	**Proteomics method**	**Study sample**	**Study group**	**SOD1**	**SOD 3**	**CRISP1**	**LGALS3BP**
Pilch and Mann, 2006	LC-MS/MS	SP	One normal man	Yes	-	Yes	Yes
Drabovich *et al.,* 2011	SRM	SP	Control/NOA	-	-	1,25 fold up in control	-
Batruch *et al.,* 2012	LC-MS/MS	SP	Control/NOA	0,6 fold up in NOA	-	-	-
Rolland *et al.,* 2013	MS	SP	Control/NOA	-	-	-	-
Freour *et al.,* 2013	ICPL	SP	*NOA+/*NOA-	-	-	0,89 fold up in NOA+	1,5 fold up in NOA+

*NOA+: Positive (successful) sperm retrieval

*NOA-: Negative (unsuccessful) sperm retrieval

First of all, it is necessary to discuss the reliability of the samples collected for this study. SOD1 and SOD3 are antioxidant enzymes and their substrate are reactive oxygen species. The relative amount of reactive oxygen species contained within body fluids (seminal plasma or blood serum) is highly variable. This variability is not limited to the individual physiology of the body, for example, the presence of leukocytes in semen, but also the influence by several environmental factors; smoking, alcohol consumption, air pollution and nutrition. Therefore it is important to choose the study population with equal conditions as much as possible. Leukocytes are one of the major producers of ROS in semen (Lobascio *et al.,* 2015). Measurement of leukocytes in the semen (, which are producing reactive oxygen species (ROS)) is a standard component of the semen analysis. All men involved in this study have a normal range of seminal leukocytes which has been defined by WHO (less than 1 million

leukocytes per milliliter of semen). Moreover, all patients in the sample and control groups have an equal rate of smoking and alcohol consumption.

SOD3 was found significantly different in seminal plasma from MA azoospermic patients in comparison to SCO azoospermic patients by two different proteomics-methods (mass spectrometry and ELISA). Thus it can be concluded SOD3 has the potential to predict the presence of sperm in the testis prior to biopsy. Western blotting results comparing MA versus SCO shows a statistical rate, which is very close to a significance (p=0.07) and with the current data in this thesis chapter it can be concluded as a potential trend. This subject indicates that with increasing the number of population to test by WB the significance result may be obtained.

The pattern of significant difference (for protein concentration of SOD3 in seminal plasma) among the three different groups of the study shows the highest protein concentration for the group of controls as expected.

However, between the two patient groups, the SOD3 concentration was significantly higher in the group of SCO in comparison to the MA cohort. This pattern (higher concentration of SOD3 in SCO patients) was seen in the results came out of three different proteomics methods (MS, ELISA and WB) and therefore it has been validated.

The molecular mechanisms and the identity of the molecules contributing to the increased SOD3 concentration in SCO patients in comparison to MA patients are yet to be elucidated. Even though the function of SOD3 as an antioxidant has been generally explained in the human body, however, it’s role in the male reproductive system is still unclear.

As a first question, it should be asked which sources secret SOD3 into the semen. It is known that the original source of SOD1 and SOD3 in the semen (except the portion, which is producing continuously by spermatozoa and released into the semen), originates from the prostate glands. However, prostate glands are not the sole producers of SOD proteins (Aumuller and Seitz, 1990; Peeker *et al*., 1997). For example, SOD proteins were identified and listed among the major human neosynthesized and secreted epididymal proteins (Dacheux *et al*., 2006). In this mass spectrometry-based research, SOD proteins were identified as a total union protein and the SOD members were not distinguished individually

(only one spot was seen in the two-dimensional gel electrophoresis). For this reason, it is unclear that exactly which SOD protein has more contribution to the secretions from the epididymis. Moreover, there is no substantial evidence to show the origination of SOD proteins from various germ cell types or somatic cells of seminiferous tubules located within the testis. Therefore, I performed IHC stainings for SOD3 in the testis. Immunoreactivity between the SOD3 proteins and antibody was seen in the Sertoli cells of the seminiferous epithelium in normal testis tissues. To date, no previous study has documented the testicular SOD3 protein expression utilizing IHC. To support this result, it was referred to the IHC staining of testis tissue available on the website of: “the human protein atlas”. Consistent with the results of this study, IHC staining of testis tissues in this website also shows an immunoreactivity of anti SOD3 protein in the Sertoli cells and Leydig cells (https://www.proteinatlas.org/ENSG00000109610-SOD3/tissue/testis#img).

This finding although it is very primitive and incomplete and needs more experiments to make a final conclusion. However, this may explain why the concentration of SOD3 was increased in the patients with SCO than MA and this is due to MA patients have many defective Sertoli cells. While SCO patients have intact and functional Sertoli cells. Additionally, it is also proposed that the SOD3 can be secreted by Sertoli cells into the seminal plasma. Therefore it is plausible to assume the protein concentration is higher in the seminal plasma of SCO patients. More investigations such as RT-PCR, qRT-PCR and protein quantitation in the testis biopsies are required to further validate this assumption. Via this method, both particular cells from seminiferous tubules, which have been shown a positive immunoreactivity with SOD3 antibody or have been shown negative staining, take apart from the tissue (with viewing with the microscope) and then undergo for mRNA or protein evaluation (Pleuger *et al*., 2016).

Another explanation for the reduced SOD3 in patients with MA compared to patients with SCO can be due to the presence of high ROS levels in their testicles. There are three different reasons for MA patients to have high levels of ROS. Firstly, sperm cells produce ROS and men with MA have a slight rate of spermatogenesis (according to the positive sperm retrieval at M-TESE), thus naturally there is an increased level of ROS present compared to SCO patients. Secondly, it has been reported that defected sperm cells produce ROS more than

normal sperms. On the other hand, also this fact has been shown although there is low spermatogenesis in the testis of MA patients, actually, most of the produced sperms are considered as abnormal sperms (because of their abnormal morphology or motility) which may contribute to the increase in the number of ROS in MA patients. Finally, the third reason refers to the defective Sertoli cells in the seminiferous epithelium of MA patients. There is a reference to indicate that reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is also responsible for the generation of endogenous ROS. These ROS are indispensable as signaling molecules regulating sperm capacitation and are considered another source of oxidative damage in spermatozoa (Donà *et al.*, 2011). Defective Sertoli cells located in the seminiferous epithelium of MA patients have an improper process of cytoplasmic extrusion during spermatogenesis. This can lead to an increased cellular level of glucose 6-phosphate dehydrogenase, a rate-limiting enzyme for nicotinamide adenine dinucleotide phosphate (NADP) reduction, thus leading to the increase in the amount of NADPH oxidase substrates and indirectly inducing oxidative stress via the generation many ROS (Rengan *et al.*, 2012). It seems because of many ROS components existing during the spermatogenesis procedure of MA patients, plentiful of antioxidants including the SOD3 enzyme involved to the oxidoreductase reaction and occupied by ROS components and therefore the pure concentration of SOD3 (in seminal plasma) reduces significantly. Moreover, the inefficiency in the antioxidant mechanisms in azoospermic patients is disrupted as the production and replacement rates of the antioxidant enzymes are not responsive for the large antioxidants consumption. Therefore this may contribute to the high SOD enzymatic activity reported in azoospermic groups in comparison to the controls.

Already in the above, we explained the presence of damaged germ cells and Sertoli cells in the testis of patients with MA. On the other hand, we explained there is an excessive ROS in MA patients. To combine these two subjects, there is a published reference that proves that excessive ROS is associated with induced damage to the germline (Agarwal and Said, 2005b). It seems severe ROS imbalance leads to necrosis of germ-cell precursors that may lead to tubular atrophy and abnormal differentiation of spermatids. A detailed explanation of how sensitivities to ROS vary between germ cells is yet to be determined.

However, the theory of having more ROS in MA patients has some opponents. In one study it was reported that testicular tissue for ROS concentrations was not statistically different in patients having normal spermatogenesis, with respect to Sertoli cell-only syndrome, maturation arrest and hypospermatogenesis, respectively (Yaman *et al*., 1999). In another study, a significant elevation in testicular ROS levels was observed in the SCO patients compared to hypospermatogenesis (Koksal *et al*., 2003).

The data obtained in our study, we argue in contrary to those mentioned papers in the above (Yaman *et al*., 1999; Koksal *et al*., 2003) as the methodology used in these studies measured only one kind of ROS means malondialdehyde (MDA) and not the whole content of testicular ROS. Although the effects of ROS have been extensively studied in mammals not much is known about its direct impact on human male germ cells. Further studies are also necessary to show differences in ROS among the NOA subgroups. For example, it could be interesting to know the superoxide anion as an important ROS component is produced mostly in which kind of NOA.

In this study, a significant concentration of SOD1 in patients with SCO was seen in comparison to MA patients (according to MS data reported in the chapter of results). With the methods of ELISA and WB, the results did not show the significant difference of SOD1 concentration between MA and SCO patients. The same outcomes apply to SOD3, still, this is yet to be documented whether SOD1 has some origins among the seminiferous tubules or not. Even the source of the SOD1 enzyme from other parts of the male reproductive system has not been completely discovered. To identify the localization of SOD1, it was performed IHC staining on human testis material which represented an intact spermatogenesis. Immunoreactivity between the SOD1 proteins and antibody was seen in the spermatogonia. The same results have been reported in one study (Celino *et al*., 2011) and also on the website of: “the human protein atlas” (https://www.proteinatlas.org/ENSG00000142168-SOD1/tissue/testis#img). Both references reported the strong expression for SOD1 in spermatogonia utilizing IHC. This can give an explanation that why there is a high concentration of SOD1 in patients with MA in comparison to SCO. Seminiferous tubules from testis tissue in patients with MA (despite the severe atrophy) contains a few intact spermatogonia germ cells. Those spermatogonia cells secret the SOD1 protein into the

lumen. More studies are required to know the contribution of other cells belongs to the seminiferous tubules regarding the secretion of SOD1.

The important question, which can be asked throughout this study and especially regarding the result of SOD1 is that why variable results were seen during the various proteomics methods? The answer is that undoubtedly, a high technology method like mass spectrometry is more sensitive (more reliable!) than others (e.g. ELISA or WB). On the other hand, in this study, two different antibodies were used for quantitation of protein (in both cases of SOD1 and SOD3) by Western-blotting compared to ELISA which may explain the variation in results. Moreover, the selected antibodies may not target the same sequence region that was detected by mass spectrometry.

In conclusion and sum up all results came out of this study, SOD3 can be accepted as a protein with having high potential to consider as a protein biomarker regarding distinguish the azoospermic patients with MA from azoospermic patients with SCO prior the surgical biopsy. It means, there is a chance to predict the presence of sperm in the biopsied tissue before the sperm retrieval surgery with protein concentration measurement of SOD3.

4.2.CRISP1

Initial studies reported that the protein of CRISP1 has an epididymal origin. When the presence of the protein of CRISP1 was investigated in the seminal plasma of three groups of men: normal men, men after vasectomy and men after vasovasostomy, it was seen that CRISP1 exists in the seminal plasma of normal and men after vasovasostomy but not in the seminal plasma of men after vasectomy (Légaré *et al.*, 2010). In this study, it was tried to expand the investigation on the whole proteome of seminal plasma from men before and after vasectomy by the method of two-dimensional gel electrophoresis. After comparison, CRISP1 was found among the significant proteins, which was significantly lower in the seminal plasma of men after vasectomy. In spite of the previous study, which had reported the completely missing of CRISP1 in seminal plasma of men after vasectomy, in this study it was seen that CRISP1 exists and its concentration is more than 3 fold down-regulated in compare of seminal plasma of men before vasectomy.

On the other hand, men with OA have the same mimic of men after vasectomy regarding their semen behavior. Therefore, the protein of CRISP1 was selected to do further proteomics investigation in men with OA. When the protein concentration of CRISP1 was measured in the seminal plasma of men with OA and then compared with seminal plasma of normal-fertile men, it was seen there is significantly lower concentration of this protein in the seminal plasma of men with OA (similar to the result of CRISP1 comparison in seminal plasma of men before and after vasectomy).

After applying two-dimensional gel electrophoresis, selecting the candidate protein (CRISP1) and measure its concentration in patients with OA, it was planned to do the analysis of this protein in various subgroups of patients with NOA. For further proteomics investigation, the method of Western blotting was used but with two different antibodies.

Here and in this part of the discussion, it is important to note that why in this study two different antibodies, which recognize two different epitopes have been used. The answer is because to reach more accurate results and having suitable tools for a better comparison. The behavior of proteins and the model of their formation (their folding structure) are different in different body liquids and this is maybe influenced by the affinity of the antibody. Epitope specificities are important for both scientific investigation and clinical translation, especially

for personalized medicine. Therefore the different epitopes of a protein targeted by different antibodies, if ended to similar results provide an accurate conclusion and if it ended to the different results then many challenges will start to find the reasons (Hua *et al*., 2017). Unknown protein modifications and protein residues remain from some procedures like apoptosis can provide a sequence of amino acids which are maybe exactly matching with the target of antibody and therefore come up with a wrong protein concentration measurement.

Regarding the accepted knowledge about the epididymal origin of CRISP1, a previous study tried to find the relationship of CRISP1 concentration between men with OA and men with NOA. It was shown that there is a significant difference in the protein of CRISP1 between those two groups (Légaré *et al*., 2013). In the present study, also the protein concentration of CRISP1was compared between OA and NOA (combine all the NOA patients as a union group). The results showed the seminal plasma of men with OA contains significantly less amount of protein of CRISP1 compare to the seminal plasma of men with NOA. Therefore the previous results came from Légaré et al., was proved by this study.

In this study beside the comparison between the groups of OA and NOA, a third group of azoospermic men means men with Klinefelter syndrome also was compared with those two previous groups. Men with Klinefelter syndrome are azoospermic and the same time are different from the other groups of OA and NOA. The reason refers to their abnormal chromosome numbers and having one extra chromosome of X (instead of the typical XY chromosomes in men, they have XXY). No significant relationship was found between the groups of Klinefelter syndrome and NOA. Pathohistological patterns of testis in men with Klinefelter syndrome are close to men with NOA. For example in their testis histology is seen, as the testis histology of NOA, two different patterns of mixed testicular atrophy and Sertoli cell-only syndrome. As already mentioned a significant relationship was found between OA and NOA for protein concentration of CRISP1 and it was assumed to find the same relationship between the OA and men with Klinefelter syndrome. After statistical analysis, this significant relationship was found but only with one of the antibodies. Here also CRISP1 concentration in seminal plasma was significantly lower in OA in compare to Klinefelter syndrome ($P<0.05$).

This study aimed to find a protein biomarker(s) that has the potential to distinguish among different types of non-obstructive azoospermia (NOA). The histology of various types of non-obstructive azoospermia (NOA) mainly defines with impairments in their testis. CRISP1 is known as an epididymal protein and therefore how it can be a protein biomarker candidate among the various types of NOA? The rational approach to consider CRISP1 as a testis biomarker refers to the new report of a human gene database (GeneCards), which showed the fold change of mRNA of CRISP1 is overexpressed in testis and it is 47.7 folds more compared to average of all tissues (GeneCards, Human Gene Database: http://www.genecards.org/cgi-bin/carddisp.pl?gene=CRISP1 and GTEx Portal: https://www.gtexportal.org/home/gene/CRISP1). This subject opens the door to look at the testis origin of CRISP1. More details are required to know about the situation of CRISP1 in the testicular epithelium. The patients with NOA (because of their problem with testis and seminiferous tubules failure) are a good target to investigate their seminal plasma for the protein of CRISP1. No study has been published to show the transcriptome or proteome condition of NOA's subgroups for CRISP1. Therefore the present study shows the results for the first time.

Four different subgroups of NOA have been quantified for the protein concentration of CRISP1. It was provided the evidence supporting that CRISP1 plays the prognostic role for the distinction among three different subgroups of NOA, namely spermatogenesis arrest (SA), mixed testicular atrophy (MA) and Sertoli cell-only syndrome (SCO). Statistical analysis did not support CRISP1 as a distinguished biomarker for differentiation of the group of Klinefelter syndrome among the other subgroups of NOA.

CRISP1 was found significantly in higher quantitation in men with SA in comparison with SCO and MA subgroups. CRISP1 also was significantly more in SCO in compare to MA. Why seminal CRISP1concentration is in the group of SA more than SCO and SCO more than MA? There is not enough evidence to help for explaining the reason for this variate range of significant quantitations among the different groups of NOA. Other investigations are required to test the CRISP1, both in the mRNA level and the protein level, in the whole testis tissue plus separately in each various parts of that. For example to test the CRISP1 only in Sertoli cells, Leydig cells or germ cells. In this study, immunoreactivity of anti CRISP1

was seen with Sertoli cells through one of the selected antibodies. The other anti CRISP1 antibody showed immunoreactivity with the Leydig cells.

The condition of the epididymis is also unknown in the various subgroups of NOA. Epididymis has been investigated very well in men with OA, anatomically, histologically and physiologically. But the single reference related to the epididymis and NOA only was mentioned that the epididymis is flat (Cocuzza *et al*., 2013). No details regarding the structure, anatomy and physiology of different parts of epididymis like initial segment, caput, corpus and cauda in various subgroups of NOA or even NOA as a single union group.

In conclusion and according to the results of this study, CRISP1 may consider as a protein biomarker with having high capability to consider as a biomarker regarding specify the three various non-obstructive azoospermic groups, MA, SCO and SA from each other. The high capability is due to check the protein quantitation of seminal plasma by two different antibodies that have an affinity to two different parts of protein and reach to the same significant results.

Increase the number of study populations in all groups will help to find a cut off concentration range for CRISP1 to do a prediction of the specific subgroup in which the NOA patient belongs to that. Therefore before an invasive TESE, there is a chance to predict the presence of sperm.

4.3.LGALS3BP

In this study, LGALS3BP was identified as a potential predictive biomarker in NOA patients regarding successful or unsuccessful TESE outcomes. Briefly, a comparative proteomics-based approach was used to identify seminal biomarkers of residual spermatogenesis in patients suffering from NOA. The strategy of the study was the identifying proteins with differential expression patterns in seminal plasma of azoospermic men with histology of MA as NOA+ (positive means successful sperm retrieval) versus azoospermic men with histology of SCO as NOA- (negative means unsuccessful sperm retrieval). After test the seminal plasma samples of azoospermic men with the proteomics method of MS, a list of significant proteins (MA versus SCO) including 61 different proteins were obtained. LGALS3BP was one of the proteins in the list and it was significantly less in patients with MA (as NOA+) in comparison of patients with SCO (as NOA-)(Figure 3-21). Afterward, it was tried to prove the results of MS with other proteomics method means Western blotting. It was found again that LGALS3BP seminal expression was significantly lower in MA patients (as NOA+) than that in SCO patients (as NOA-)(Figure 3-23). With this achievement, the result from the method of MS was confirmed. In both analyses, the protein concentration of LGALS3BP was significantly less in the group of MA (as NOA+) in comparison to patients with SCO (as NOA-).

In the next step, it was planned to do a comparison for seminal LGALS3BP protein concentration not only between two groups of patients (MA versus SCO) but also among four different groups, Normal-fertile men, men with MA, men with SCO and finally men with Klinefelter syndrome. All the relationships were significant except for the protein concentration's difference between the group of MA and Klinefelter syndrome (Figure 3-23). In this study, for the first time, the protein concentration of LGALS3BP for men with Klinefelter syndrome was evaluated. It had a reason to include the patients with Klinefelter syndrome to the analysis. First of all these patients were azoospermic because of a known and clear reason, which was a chromosomal disorder (to have one extra chromosome of X, means XXY). It was interesting to know how the protein concentration of LGALS3BP in this group is. The second, from the viewpoint of histology, the testis biopsy of men with Klinefelter syndrome shows two different patterns. Some of them are the same as MA and the next group is as SCO. When the seminal plasma protein concentration of LGALS3BP

was compared between those two different categories of Klinefelter syndrome patients (Klinefelter syndrome-MA versus Klinefelter syndrome-SCO), it was seen, again, the protein concentration of LGALS3BP is significantly lower in the group of Klinefelter syndrome-MA. Thus, the less quantitation of LGALS3BP in the group of MA (NOA+) not only proved by two different proteomics methods (MS and Western blotting) but also showed the same results in two separate populations of azoospermia. 1: idiopathic azoospermia with histology of MA and SCO, 2: non-idiopathic azoospermia, means Klinefelter syndrome with again histology of MA and SCO (Figure 3-23).

In a previous study, it was reported that LGALS3BP seminal expression is significantly higher in NOA+ patients in comparison to patients with NOA- (Freour *et al*., 2013). The results of the present study is in the opposite of results came from the study of Freour et al. Regarding the defense of results in this study and argue with the opposite results, it is indicated some other studies, which their results are in the same line with the present study. In a research, it was found that the protein concentration of LGALS3BP increased in azoospermic samples in compare of oligozoospermic samples. It shows that when sperm cells exist in the semen, the protein concentration of LGALS3BP decreases (Davalieva *et al*., 2012). In another study, the seminal fluid proteome was analyzed and potential associations between proteins and fertility scores were reported. In this study, LGALS3BP showed the highest negative correlations with fertility ranking (Viana *et al*., 2018). Even though this study was animal-based research (Bos Taurus) and not human-based research, it still indicates that when the protein amount of LGALS3BP increases in the seminal plasma the fertility significantly decreases.

In conclusion, advances in technologies, such as mass spectrometry hold great promise for the identification of novel prognostic biomarkers for sperm retrieval operations. Seminal LGALS3BP protein concentration has potential as a novel noninvasive biomarker for the detection and prognosis of non-obstructive azoospermia (NOA+) from non-obstructive azoospermia (NOA-). Finally, novel biomarkers may provide important clues to our understanding of azoospermia, and ultimately lead to better treatment strategies. Simultaneous advancement in these many medical disciplines will hopefully initiate change in the poor prognosis of azoospermic patients.

5. SUMMARY

Men with non-obstructive azoospermia (NOA) have zero sperm in the ejaculate. Testicular sperm suitable for assisted reproductive technology (ART) could be present in NOA patients. Sperm is collected by micro-testicular sperm extraction (M-TESE). However, no diagnostic test exists to predict the presence of sperm in the testis biopsy before surgery. About 50% of azoospermic men are unable to have sperm retrieved at M-TESE and therefore have surgery unnecessarily. The ability to predict the presence of sperm in the testis prior to TESE would improve sperm retrieval rates and avoid unnecessary surgery. Identification of specific proteins related to spermatogenesis in seminal plasma (SP) could provide a novel proteomics assay for sperm presence prior to biopsy. SP is a rich, easily-accessible and promising source of spermatogenesis biomarkers. This study was designed to compare the SP proteomes of two different groups of NOA. 1) Men with mixed-testicular atrophy (MA) with a positive sperm retrieval at M-TESE. 2) Sertoli cell-only syndrome (SCO) patients with a negative sperm retrieval at M-TESE.

Results also compared with the control-group. In a part of study, SP was collected from MA and SCO patients. All samples were compared by label-free Liquid chromatography-mass spectrometry/MS proteomics. Differentially expressed proteins were defined as those with a fold-change >2.0 and a significant difference between the groups ($p<0.05$). In another part of the study, a comparative proteome analysis of seminal plasma was performed in men requesting vasectomy. Sampling was done before vasectomy and six weeks after vasectomy. 2D electrophoresis was performed separately with 100 µl seminal plasma for each sample. Gel images were acquired with a typhoon 9200 laser scanner and analysed with PdQuest software. Comparison between groups (before vs. after) revealed 84 significant different spots. Protein identification revealed 27 different proteins.

Among the significant proteins identified by mass spectrometry SOD1, SOD3 and LGALS3BP were selected for further validation by 3 different methods: Western blotting, immunohistochemistry and ELISA (n=15/group). Also, to compare with seminal plasma, the protein concentrations of SOD3 and SOD1 were measured in the blood-serum of participants. SOD3 immunoreactivity (ELISA) in SP from SCO men was 2.3 fold lower than controls

($p<0.05$), and SP from MA men was 4.6 fold lower than controls ($p<0.0001$). The SOD3 MA/SCO protein ratio was 0.49 ($p<0.05$) by ELISA and 0.16 ($p<0.01$) by mass spectrometry. SOD3 gave the expected band of 30 kDa by Western blot of SP samples, but no significant difference between MA and SCO groups was observed. In normal human testis, SOD3 staining was predominantly observed in Sertoli cells, while in the testis of azoospermic patients staining was more variable.

SOD1 immunoreactivity (ELISA) in SP from SCO and MA men was decreased significantly compared with controls ($p<0.05$). The MA/SCO ratio of SOD1 was significantly different by mass spectrometry but not by ELISA or quantitative Western blot. In normal human testis, SOD1 was predominantly localized in spermatogonia, while in the testis of azoospermic patients variable SOD1 staining was noted. There were no significant differences in the levels of SOD1 and SOD3 in the blood-serum of MA compared to SCO patients. Two different antibodies were used for quantitation of protein (in both cases of SOD1 and SOD3) by Western-blotting compared to ELISA which may explain the variation in results. Moreover, the selected antibodies may not target the same sequence region that was detected by the mass spectrometry. SOD3 was significantly different in SP from MA versus SCO azoospermic patients by at least 2 different proteomics-methods (mass spectrometry and ELISA), and thus has the potential to predict the presence of sperm in the testis prior to biopsy. LGALS3BP in SP from SCO and MA men was significantly lower than controls ($p<0.001$) by Western blot. Also, a significant difference for the LGALS3BP MA/SCO protein ratio was observed ($p<0.05$). Among the significant proteins identified by 2D electrophoresis, an epididymis specific candidates, namely, CRISP1 (Cystein-Rich Secretory Protein 1) were selected for further validation by Western blotting and using two different antibodies, which had affinity for two different epitopes of protein. The CRISP1 MA/SCO protein ratio was significantly different ($p<0.05$) by applying the both antibodies.

SOD3 (Superoxide dismutase 3), LGALS3BP and CRISP1 are proteins in seminal plasma which have potential to predict the success of micro-dissection sperm retrieval in non-obstructive azoospermia men (NOA).

6. ZUSAMMENFASSUNG

Das Ejakulat von Männern mit nicht-obstruktiver Azoospermie (NOA) enthält keine Spermienzellen. Wenn das Krankheitsbild der „Mixed Atrophie“ (MA) hingegen vorliegt, verbleiben im Hodengewebe fokale Bereiche mit Spermienbildung. In diesem Fall können aus dem Hodengewebe Spermien mit dem Potential einer Befruchtung durch assistierte Reproduktionstechniken (ART) gewonnen werden. Die Isolation erfolgt durch mikro-testikuläre Spermienextraktion (M-TESE). Vor Durchführung dieses invasiven Verfahrens gibt es bisher keine klinisch etablierten Tests zur Identifikation von MA Patienten. Da sich etwa 50% der Männer beim M-TESE Eingriff als vollständig azoosperm (Sertoli-Cell-Only Syndrom: SCO) herausstellen, können keine Spermien isoliert werden und der Eingriff ist folglich vergeblich. Eine Methode zur Vorhersage fokaler Spermienpopulationen im Hoden hätte das Potential einer effizienteren M-TESE mit Vermeidung unnötiger Eingriffe. Seminalplasma (SP) ist eine proteinreiche, leicht verfügbare Flüssigbiopsie und stellt eine vielversprechende Quelle von Spermatogenese-Biomarkern dar. In dieser Studie wurde die Proteomik von SP untersucht auf das Potential zur Diskrimierung von NOA Patienten in (1) Sertoli-Cell-Only (SCO) Patienten und (2) Mixed-Atrophie (MA) Patienten mit dem Potential der Spermienisolation durch M-TESE. Dabei erfolgte der Vergleich mit einer (3) gesunden Kontrollgruppe.

Im ersten Teil der Studie wurde SP von MA und SCO Patienten gesammelt und mittels einer Kombination aus ungelabelter Flüssigchromatographie und Massenspektrometrie (LC-MS) charakterisiert. Differentiell exprimierte Proteine wurden anhand eines Cutoff von >2.0 facher Regulation ($p<0.05$) identifiziert. In einem zweiten Teil der Studie wurde SP von Vasektomie-Kandidaten direkt vor und 6 Wochen nach dem Eingriff isoliert und eine 2D Gelelektrophorese durchgeführt. Die Gele wurden mit einem Typhoon 9200 Laserscanner aufgenommen und der Software PdQuest ausgewertet. Der Vergleich der Gruppen führte zur Identifikation von 84 Spots. Dies führte im Anschluss zur massenspektrometrischen (MALDI-TOF) Identifikation von 27 differentiell exprimierten Proteinen.

Unter den durch MS identifizierten Proteinen wurden die Kandidaten SOD1, SOD3 und LGALS3BP mit 3 weiteren Methoden validiert (n=15 pro Gruppe): Western Blotting (WB), Immunhisochemie (IHC) und enzym-gekoppelten Immunosorbant Assay (ELISA).

Zusätzlich wurden SOD1 und SOD3 Blutserum-Konzentrationen der Studienteilnehmer untersucht und mit den SP-Konzentrationen verglichen.

Bei Vergleich mit der gesunden Kontrollgruppe zeigten SP Proben von SCO Patienten eine 2.3fach ($p<0.05$) verringerte, und MA Patienten eine 4.6fach ($p<0.0001$) verringerte SOD3 Immunreaktivität (ELISA). Das SOD3 Protein-Verhältnis von MA/SCO Proben wurde mit ELISA und MS ermittelt und betrug 0.49 ($p<0.05$; ELISA) bzw. 0.16 ($p<0.01$, MS). WB Detektion von SOD3 führte zur erwarteten 30 kDa Bande, jedoch konnte kein signifikanter Unterschied zwischen den MA und SCO Gruppen beobachtet werden. In normalem humanem Testesgewebe konnte SOD3 durch IHC vorwiegend in Sertoli-Zellen detektiert werden, wobei im Gewebe der azoospermen Patienten eine hohe Variabilität beobachtet werden konnte.

SP von SCO Patienten und MA Patienten zeigte bei Vergleich mit der gesunden Kontrollgruppe zudem eine signifikant (jeweils $p<0.05$) verringerte SOD1 Immunreaktivität (ELISA). Obwohl die vorausgehende MS Quantifizierung signifikante Unterschiede zwischen MA/SCO Patienten zeigte, konnte dies mit ELISA oder WB nicht verifiziert werden. Durch IHC Experimente konnte SOD1 vorwiegend in Spermatogonien lokalisiert werden, in den Proben der MA/SCO Patienten war abermals eine hohe Variablität zu verzeichnen.

Die SOD1 und SOD3 Konzentrationen im Blutserum von MA und SCO Patienten unterschieden sich nicht signifikant voneinander. Dies könnte daran liegen, dass die Antikörper der verwendeten ELISA Kits anderer Herkunft waren als jene zur WB Quantifizierung. Darüber hinaus könnte es sein, dass die verwendeten Antikörper eine andere Proteinregion detektieren als jene, die zur MS Quantifizierung verwendet wurde.

Zwei unabhängige proteomische Methoden (Massenspektrometrie/ELISA) erlaubten eine Differenzierung von SCO und MA Patienten anhand der SP Level von SOD3, somit hat dieses Protein womöglich das Potential zukünftig M-TESE Erfolgsaussichten vorauszusagen. Bei WB Quantifizierung von LGALS3BP in SP Proben

konnte gezeigt werden, dass SCO oder MA Patienten im Vergleich zu der gesunden Kontrollgruppe signifikant verringerte Proteinlevel ($p<0.0001$) haben. Darüber hinaus waren

LGALS3BP Proteinkonzentrationen in SP von MA und SCO Patienten signifikant unterschiedlich.

Unter den durch 2D Gelelektrophorese und Massenspektrometrie (2D-GE/MS) identifizierten Proteinen wurde das Epididymis-spezifisch exprimierte Cystein-Rich Secretory Protein 1 (CRISP1) für eine Validierung mittels WB ausgewählt, wobei zwei Antikörper mit unterschiedlicher Epitopen-Spezifizität verwendet wurden. Mit beiden Antikörpern wurde ein signifikanter Unterschied (jeweils $p<0.05$) zwischen MA und SCO Patienten beobachtet.

Superoxid-Dismutase 3 (SOD3), LGALS3BP und CRISP1 handeln sich folglich um SP Proteine mit dem Potential, M-TESE Erfolgsaussichten in Männern mit nicht-obstruktiver Azoospermie (NOA) vorauszusagen.

7. List of abbreviations

ART	Assisted reproductive technology
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BS	Blood serum
cAMP	Cyclic adenosine monophosphate
C. elegans	Caenorhabditis elegans
CID	Collision-induced dissociation
CFD	Complement factor D
CRD	Carbohydrate-recognition-binding domain
CRD	Cysteine-rich domain
CRISP1	Cysteine-rich secretory protein 1
CRISP2	Cysteine-rich secretory protein 2
CRISP3	Cysteine-rich secretory protein 3
CRISP4	Cysteine-rich secretory protein 4
CV	Coefficient of variation
DAB	3, 3′-Diaminobenzidine tetra hydrochloride
ELISA	Enzyme-linked immunosorbent assay
FASP	Filter aided sample preparation
FDR	False discovery rate
gamma-TuRC	gamma-tubulin ring complex
GPX5	Glutathione peroxidase 5
HIV	Human immunodeficiency virus

HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HS	Hypospermatogenesis
ICPL	Isotope-coded protein label
ICSI	Intracytoplasmic sperm injection
IgG	Immunglobulin Gamma
IHC	Immunohistochemistry
IPI	International protein index
IUI	Intrauterine insemination
IVF	In vitro fertilization
LAK	Lymphokine-activated killer
LGALS1	Galectin-1
LGALS3	Galectin-3
LGALS3BP	Galectin-3-binding protein
LPO	Lipid peroxidation
MA	Mixed testicular atrophy
MDA	Malondialdehyde
Mg/ml	Milligrams per milliliters
MMP	Mitochondrial membrane potential
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry (mass spectrometry/mass spectrometry)
M-TESE	Microdissection TESE
Ng/mg	Nanogram per milligram

NK	Natural killer
NOA	Non-Obstructive Azoospermia
NS	Not significant
OA	Obstructive Azoospermia
PBS	Phosphate-buffered saline
PSM	Peptide-to-spectrum match
PVDF	Polyvinylidene difluoride
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SA	Spermatogenesis arrest
SCO	Sertoli cell-only syndrome
SD	Standard deviation
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
SOD3	Superoxide dismutase 3
SP	Seminal plasma
SRM	Selected reaction monitoring
TAC	Total antioxidant capacity
TBS	Tris-buffered saline
TBST	Tris-buffered saline+Tween20
TCEP	Tris-(2-carboxyethyl) phosphine
TESE	Testicular sperm extraction
UHR-QqTOF	Ultra-High Resolution Qq-Time-Of-Flight

WB	Western blotting
WHO	World health organization
2-DE	Two-dimensional gel electrophoresis

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11. LIST OF PUBLICATIONS

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Conference Papers

1. 2019 Savadi-Shiraz E., Pilatz A., Weidner W., Diemer T*., Stanton PG*. CRISP1 (Cysteine-rich secretory protein-1) as an epididymis-specific protein in seminal plasma has a prognostic role for the distinction among 3 different subgroups of non-obstructive azoospermia (mixed testicular atrophy (MA), Sertoli-cells-only syndrome (SCO) and spermatogenesis arrest (SA). 35th European Society of Human Reproduction and Embryology (ESHRE 2019), Vienna, Austria.

2. 2018 Savadi-Shiraz E., Pilatz A., Weidner W., Fietz D., O'Donnell L., Bergmann M., Diemer T*., Stanton PG*. Ability of SOD1 and SOD3 in seminal plasma to distinguish between two different azoospermic subgroups (mixed testicular atrophy and Sertoli cell-only syndrome). 34th European Society of Human Reproduction and Embryology (ESHRE 2018), Barcelona, Spain.

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12. DECLARATION OF HONOR

"I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation."

Elham Savadi Shiraz

Giessen 04.12.2019

Elham Savadi Shiraz

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14. APPENDIX

Table 14.1: Label-free liquid chromatography-mass spectrometry revealed 61 proteins that were identified significantly different in the seminal plasma from patients with mixed atrophy azoospermia versus Sertoli cell-only syndrome azoospermia

No	Elements included exclusively in seminal plasma: MA vs. SCO	Gene Names		Fold change
1	K7DDM2	YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	14.789
2	P12036	NEFH	neurofilament heavy [Homo sapiens	8.537
3	A4D110	LOC401309	uncharacterized protein	7.179
4	O14745	SLC9A3R1	SLC9A3 regulator 1	5.647
5	P01833	PIGR	polymeric immunoglobulin receptor	4.769
6	Q6UY27	PATE2	prostate and testis expressed 2	4.648
7	P25815	S100P (MIG9)	S100 calcium binding protein P (	4.188
8	P48163	ME1	malic enzyme 1	3.673
9	P43234	CTSO	cathepsin O	3.521
10	Q9NP72	RAB18	RAB18, member RAS oncogene family	3.475
11	C9JD84	LTBP1	latent transforming growth factor beta binding protein 1	3.409
12	Q15907	RAB11B; RAB11A	RAB11B, member RAS oncogene family	3.396
13	Q14974	KPNB1	karyopherin subunit beta 1	3.292
14	P05164	MPO	myeloperoxidase	3.238
15	Q6UX06	OLFM4	olfactomedin 4	3.082
16	Q53GE2	CAPZA2	capping actin protein of muscle Z-line alpha subunit 2	3.064
17	A0AVP6	UTS2	urotensin 2	3.002

18	C9JK28	ADAM7	a disintegrin and metallopeptidase domain 7	2.967
19	B3KU62	TRG14; NDRG1; NDRG1-ERG fusion	N-myc downstream regulated 1	2.681
20	P80723	BASP1	brain abundant membrane attached signal protein 1	2.653
21	H7C2D8	MLPH; UNQ8200	melanophilin	2.523
22	Q13421	MSLN	mesothelin	2.486
23	P27348	YWHAQ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta	2.436
24	P29966	MARCKS	myristoylated alanine rich protein kinase C substrate	2.432
25	O43657	TSPAN6	tetraspanin 6	2.414
26	Q9Y6R7	FCGBP	Fc fragment of IgG binding protein	2.392
27	P04899	GNAI2; WUGSC:H_LUCA16.1; WUGSC:H_LUCA15.1	G protein subunit alpha i2	2.385
28	Q658J0	DKFZp762L015; NIF3L1	NGG1 interacting factor 3 like 1	2.380
29	Q9H336	CRISPLD1	cysteine rich secretory protein LCCL domain containing 1	2.320
30	Q96G03	PGM2; FLJ10983	phosphoglucomutase 2	2.306
31	P25789	PSMA4	proteasome subunit alpha 4	2.180
32	Q14204	DYNC1H1; Dnchc1	dynein cytoplasmic 1 heavy chain 1	2.108
33	Q04760	GLO1	glyoxalase I	2.094
34	O76054	SEC14L2; SEC14L3	SEC14 like lipid binding 2	2.002
35	O00194	RAB27B	RAB27B, member RAS oncogene family	1.914
36	E9PGT1	TSN	translin	1.902
37	Q53HV2	CCT7	chaperonin containing TCP1 subunit 7	1.828

38	K9JA46	EL52; HSP90AA1; HSP90Af; HSP90AA2; HSP90AA5P	heat shock protein 90 alpha family class A member 1	1.735
39	P11717	IGF2R	insulin like growth factor 2 receptor	1.730
40	B7Z9L0	CCT4	chaperonin containing TCP1 subunit 4	1.730
41	P62258	YWHAE; YWHAE/FAM22 B fusion; YWHAE/FAM22 A fusion	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon	1.727
42	P17050	NAGA	alpha-N-acetylgalactosaminidase	1.540
43	O75369	FLNB; DKFZp686A166 8; FLNC	filamin B	1.481
44	Q6W4X9	MUC6	mucin 6	0.728
45	O60568	PLOD3; DKFZp564O182 2	procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	0.708
46	Q9NR99	MXRA5	matrix remodeling associated 5	0.689
47	O14773	TPP1	tripeptidyl peptidase 1	0.573
48	Q53GX6	NUCB1	nucleobindin 1	0.520
49	P02790	HPX	hemopexin	0.494
50	O00391	QSOX1; BPGF-1	quiescin sulfhydryl oxidase 1	0.492
51	P04004	VTN	vitronectin	0.478
52	Q9NQ79	CRTAC1	cartilage acidic protein 1	0.452
53	P01008	SERPINC1; AT3	serpin family C member 1	0.444
54	H7C3E1	ATP6AP2	ATPase H+ transporting accessory protein 2	0.425
55	P04279	SEMG1	semenogelin I	0.419
56	Q6PCB0	VWA1	von Willebrand factor A domain containing 1	0.407
57	Q08380	LGALS3BP	galectin 3 binding protein	0.396
58	P00441	SOD1	superoxide dismutase 1	0.305
59	B4E1H2	SERPING1	serpin family G member 1	0.294
60	B4DJQ8	CTSC	cathepsin C	0.292
61	P08294	SOD3	superoxide dismutase 3	0.161