Overview
Infertility is defined as the inability to conceive after two years of unprotected intercourse and affects almost 15 percent of couples worldwide. Male and female factors contribute equally to this. Sperm cells undergo post-testicular maturation in the epididymis and reorganization during capacitation in the female reproductive tract; to bestow fertilizing ability on the sperm cell. Thus, sperm proteomics has gained much importance in the research community related to male fertility/infertility. SP is considered as a potential source of biomarkers for many disorders of the male reproductive system. SP proteins play critical roles in sperm maturation and are essentially required for sperm function mainly for their interactions with the environment of female reproductive tract and the oocyte. Some of them may even influence the relative intrinsic fertility of the male and may be sometimes the sole reason of infertility in a large population of infertile couples. Con A is a common lectin isolated from the jack bean, which has broad specificity, to isolate glycoproteins and Con A binding fraction of human SP shows decapacitating activity. PIP is a 17-kDa glycoprotein which is known to actively participate in fertility, immunoregulation, antimicrobial activity, apoptosis and tumour progression. PAP is most abundant phosphatase that is synthesized and secreted into seminal plasma under androgenic control. Many studies have reported increased expression of PAP in azoospermia and its levels are inversely correlated with sperm concentration.
2.1 Human seminal fluid

Human seminal fluid serves as a vehicle that helps to transport the spermatozoa out of urethra at the time of ejaculation and to guide their journey through the female reproductive tract. It is a mixture of various secretions produced by several glands of male reproductive tract and seminal vesicles contribute the majority of the ejaculate (Polakoski et al., 1976; Mann and Lutwak-Mann, 1981; Coffey, 1995). These secretions are incompletely mixed during ejaculation, due to which the initial ejaculate is not an entirely homogeneous mixture. The secretions of the organs contributing to the ejaculate differ in composition, and there has been a venerable interest in evaluating the composition of semen from a diagnostic point of view (Eliasson, 1982). It is a complex fluid composed of both inorganic and organic components and it functions to provide a nutritive and protective medium for the spermatozoa (Owen and Katz, 2005).

Seminal fluid is composed of mainly two components:

(i) **Cellular fraction** which consists of spermatozoa and the non-spermatozoan cellular elements including immature germ cells, leukocytes and epithelial cells.

(ii) **Seminal plasma** which consists of the fluid components derived from multiple sex accessory glands and includes prostasomes, membrane-bound vesicles of prostatic origin.

On release, the ejaculate is a thick fluid, but this typically liquefies within 15–30 minutes. The genetic material required for reproduction is contained within the motile spermatozoa and the SP provides nutrients and protects those spermatozoa (Pilch and Mann, 2006).

<table>
<thead>
<tr>
<th>Component</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>seminal vesicles</td>
<td>65%</td>
</tr>
<tr>
<td>prostate</td>
<td>25%</td>
</tr>
<tr>
<td>testes and epididymis</td>
<td>10%</td>
</tr>
<tr>
<td>periurethral glands</td>
<td>minimal</td>
</tr>
</tbody>
</table>

*(Source: Robert and Gagnon, 1994)*
In some species, SP may even represent up to 95–98% of total semen volume (Beyler and Zaneveld, 1982a and 1982b). It contains proteins, lipids, carbohydrates, enzymes, vitamins, hormones, and trace elements, and becomes a micro-environment for spermatozoa survival after ejaculation (Chiu and Chamley, 2003; Miro et al., 2005; Troedsson et al., 2005; Cardozo et al., 2006). SP has a very high buffering capacity, much higher than that of most other fluids in our body. It maintains pH near neutral in the acidic vaginal environment, providing the spermatozoa with the opportunity to enter the neutral pH cervical mucus. The acidic environment inside the vaginal canal is buffered by organic amines present in the seminal plasma including putrescine, spermine, spermidine, and cadaverine (Olle, 1986). Fructose serves as a major nutrient for the spermatozoa and its concentration is considered a measure of seminal vesicle function (Nun et al., 1972). A wide variation in fructose concentration had been reported among donors as its amount changes over time as a result of fructolysis (King and Mann, 1959; Mann and Lutwak-Mann, 1981). Duration of collection and the age of the donor are the major factors for this variation (Mauss et al., 1974; Kothari et al., 1977). Some studies have also reported that glucose might also serve as a likely source of energy to spermatozoa (Peterson and Freund, 1971; Martikainen et al., 1980).

Seminal plasma contains a fair amount of many ions, including calcium, magnesium, potassium, sodium and zinc. Calcium is considered the most vital due to its relation to sperm motility, metabolism, the acrosome reaction and overall fertilization (Sorensen et al., 1999). The concentrations of calcium, magnesium and zinc are highly correlated (Homonnai et al., 1978; Adamopulos and Deliyiannis, 1983). Magnesium and zinc have been reported to form complexes with proteins and bound to the surface of the sperm cells (Lindholmer and Eliasson, 1974; Mann and Lutwak-Mann, 1981; Hirsch et al., 1991). The presence of these ions is critical as they are essentially required for activity of various proteins/enzymes of SP having roles in fertility related biological processes.

A large number of evidences favours that the SP exerts several effects on spermatozoa function and on the female genital tract that lead to successful fertilization (Pixton KL et al., 2004). Among the wide range of components of the SP, the peptides and proteins have a specific role in the regulation of fertilization process. Most of the
proteins found in the SP bind to plasma membrane of spermatozoon and coat its surface, preventing sperm agglutination, premature acrosome reaction and phagocytosis in the female reproductive tract (Jonakova and Ticha 2003). Usually, the proteins in SP are combined with carbohydrates, for example, polysaccharides or lipids to form glycoproteins or proteolipids, and play vital roles related to sperm functions (Mandal et al., 1989; Barrier-Battut et al., 2005; Topfer-Petersen et al., 2005).

2.2 Seminal plasma proteins

SP has the feature common to other body fluids, that it is characterized by a high dynamic range of protein abundance, making low-abundance components difficult to analyze. Attempts at identifying protein constituents of seminal plasma have a long history. The electrophoretic pattern of human seminal plasma was first reported by Gray and Huggins (1942) and Ross et al. (1942, 1943) in the 1940’s, and the major proteins, including phosphatases, aminopeptidases, glycosidases, hyaluronidase and mucin, have been known for almost half a century (Mann, 1964).

Initially, two-dimensional gel electrophoresis associated with immune-staining was used in combination with mass spectrometric (MS) identification of protein spots to visualize the whole seminal proteome. Edwards et al. (1981) mapped the proteins in human SP by high-resolution 2-dimensional electrophoresis from normal fertile males and revealed a pattern of over 200 proteins, ranging in mass from 10-100 kDa. Unfortunately, despite the large number of proteins resolved on the gels, protein spots were typically not identified in such studies. Subsequently, Utleg et al. (2003) identified 139 proteins of the prostasome proteome by employing a direct iterative approach using gas phase fractionation and microcapillary HPLC-tandem mass spectrometry. In the mean time, Fung et al. (2004) separated protein components of pooled human SP (n = 5) by gel electrophoresis (1-D and 2-D) and identified over 100 proteins by either matrix-assisted laser desorption ionization-time-of-flight-mass spectrometry or capillary liquid chromatography tandem mass spectrometry. The first and most extensive analysis of SP proteome was performed by Pilch and Mann (2006) who have identified 923 proteins in SP derived from a single person by LTQ-FT mass spectrometer using a bottom-up approach. Among the identified proteins, the most abundant were the so called gel-
forming proteins which are secreted by seminal vesicles: fibronectin, semenogelin I and semenogelin II. Cleaved by PSA (a kallikrein-like protease), they form a viscous gel entrapping spermatozoa immediately after ejaculation (Lilja et al., 1987). Another highly expressed seminal vesicle protein was LF, which have an antimicrobial role in seminal plasma. Serum albumin was another predominant element of human plasma, having a role as a sink for cholesterol, which is removed from the sperm membrane during capacitation (Cross, 1998).

**Figure 2.1:** A comparison between proteomic datasets of human seminal plasma generated by Utleg et al. (2003), Fung et al. (2004) and Pilch and Mann (2006).

Then, Drake et al. (2010) reported the identification of 916 unique proteins in 9 samples of expressed prostatic secretion by the MudPIT approach and by high-mass accuracy Orbitrap-MS. In a recent study, Batruch et al. (2011) have performed proteomic analysis using the offline MudPIT approach and high-resolution MS and identified 2022 proteins in pooled SP from 5 fertile volunteers; thus far this is the largest list of proteins identified in SP. Their study also revealed that highly abundant proteins of seminal plasma were semenogelins, fibronectin, PSA, albumin and LF. Other important proteins identified, include PAP, β-microseminoprotein, PIP and immunoglobulins. Miraldi et al. (2012) have reported very recently a panel of common seminal proteins in human seminal plasma by fertile men that might be involved in successful reproduction. They
identified 919 to 1,487 unique proteins per individual subject sample and among these, 83 proteins were expressed in all samples. Common list includes some proteins that might be critical for male fertility, such as semenogelin I, semenogelin II, olfactory receptor 5R1, LF, hCAP18, spindling and clusterin. They also performed Go annotations of common proteins to identify their molecular functions and their cellular distribution (Figures 2.2 and 2.3).

**Figure 2.2:** Biological process GO annotations of commonly expressed fertility related proteins in fertile donors. *(Source: Miraldi et al., 2012).*

**Figure 2.3:** Cellular distribution GO annotations of commonly expressed fertility related proteins in fertile donors. *(Source: Miraldi et al., 2012).*
A common feature of most of the seminal plasma proteins is their ability to interact with different types of inorganic and organic materials present in seminal fluid (Cameron et al., 2007; Russell et al., 1984). More extensively studied fractions of seminal plasma include Phosphorylcholine binding proteins, heparin binding proteins and zinc binding proteins. Phosphorylcholine binding proteins of seminal plasma are essentially required for the coating of the seminal plasma proteins on the sperm membrane (Varilova et al., 2006). In the female reproductive tract, seminal plasma proteins bound on the sperm surface most probably participate firstly in the formation of the oviductal sperm reservoir (Evans and Kopf 1998; Jansen et al., 2001), secondly the control of sperm capacitation by the intensive action of negative (decapacitation factors) and positive regulatory (capacitation stimulating) factors and finally in central fertilization events like sperm-zona pellucida interaction and sperm-egg fusion (Primakoff and Myles 2002; Yi et al., 2007). Seminal plasma proteins have also been shown to modulate the immune responses in the uterine environment. Thus, the combined effects of the seminal plasma components support the survival of the sperm within the female reproductive tract and ensure that functionally competent sperm meets the ovulated egg at the site of fertilization (Kanwar et al., 1979).

Heparin-binding proteins (HBPs) of SP regulate capacitation and acrosome reaction processes. Induction of sperm capacitation in the female reproductive tract is aided by HBPs, secreted by male accessory sex glands (Miller et al., 1990). HBPs are supposed to attach themselves to the sperm surface, especially lipids containing the phosphoryl-choline group, thus allowing heparin-like GAGs in the female reproductive tract to activate sperm capacitation (Miller et al., 1987). Kumar et al. (2009), using two-dimensional (2-D) electrophoresis and mass spectrometry (MS), performed proteomic analysis of human seminal plasma and identified approximately 60 heparin binding proteins. Heparin-binding proteins take part in capacitation and acrosome reaction. Major HBPs are Semenogelin I, semenogelin II, fibronectin, LF, and PSA.

SP zinc binding proteins regulate sperm chromatin condensation state and play active roles in sperm motility and acrosome reaction. Important zinc-binding proteins of human SP are semenogelins (Robert and Gagnon, 1999). They participate in coagulum formation, regulate DNA stability, inhibit sperm movement (Robert and Gagnon, 1999;
de Lamirande, 2007; Yoshida et al., 2008) and possess antibacterial activity (Edstrom et al., 2008). In addition, they hyperpolarize sperm plasma membranes (Yoshida et al., 2008) and prevent capacitation (de Lamirande et al., 2001). Zinc is secreted by human prostate gland in two forms: free and associated with high molecular weight protein complexes. Prostasomes are small lipoprotein vesicles containing zinc, which is secreted by the prostate to the human SP and acts as a regulator of physiological properties of sperm. Beside chromatin stabilization, prostasomes participate in acrosome reaction and sperm motility regulation. They are also reported to exhibit immunosuppressive, antioxidative, and antibacterial properties (Vivacqua et al., 2004).

**Figure 2.4:** A model of seminal plasma structures and functions. The model focuses on the main components of SP and their functions on spermatozoa. PM indicates plasma membrane; AST, aspartate amino transferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; LPO, lipid peroxidation; ROS, reactive oxygen species; ET, epithelium. *(Source: Juyena and Stelletta, 2012).*
2.3 Infertility

Parenthood is undeniably one of the most universally desired goals in adulthood, and most people have life plans that include children. However, not all couples who desire a pregnancy will achieve one spontaneously and a proportion of couples will need medical help to resolve underlying fertility problems. Infertility has been recognized as a public health issue worldwide by the World Health Organization (WHO). Infertility is generally viewed as a painful condition for the individuals and couples it affects however, it is usually seen as a private matter to be resolved. It is defined as the inability to conceive after two years of unprotected intercourse.

Although infertility is often attributed to female causes, fertility is a two person phenomenon. Successful conception depends on many complicated events, including satisfactory sexual and ejaculatory function, appropriate timing, and a complex set of interactions between the male and the female reproductive tracts. Male and female factors coexist in about one third of cases, while one third of cases are secondary to male factors only. Therefore, evaluation of both partners is critical, and the woman’s evaluation should proceed simultaneously with the man’s.

2.3.1 Statistics of infertility

For healthy young couples, the probability of achieving pregnancy per reproductive cycle is approximately 20 to 25%. The cumulative probabilities of conception are 60% within the first 6 months, 84% within the first year and 92% within the second year of fertility-focused sexual activity (Kamel, 2010). In the last decade, infertility rates have increased greatly in both men and women worldwide. The main cause of infertility is attributable to biological reasons along with psychological and emotional reasons.

The World Health Organization (WHO) estimated in 2004 that 60 to 80 million couples worldwide currently suffer from infertility. Boivin et al. (2007) estimated in their study that infertility prevalence rate ranges from 3.5% to 16.7% in more developed nations and from 6.9% to 9.3% in less-developed nations, with an estimated overall median prevalence of 9%. In general, when it comes to infertility, statistics suggest that 35 to 40 percent of the problems are caused by male conditions, another 35 to 40 percent by female conditions, and the last 20 to 30 percent a combination of the two.
2.3.2 Male infertility

Male infertility is one of the most rapidly growing fields in medicine, with dramatic advances in diagnosis and treatment. Infertility in men could be categorized as a reduced sperm count (oligospermia), reduced sperm motility (asthenospermia), abnormal sperm morphology (teratospermia) or with more severe cases, a complete lack of spermatozoa in semen, referred to as azoospermia.

Azoospermia accounts for 5−20% of infertility cases in men and could be further categorized as (a) pretesticular azoospermia, (b) testicular failure or non-obstructive azoospermia (NOA), and (c) obstructive azoospermia (OA) due to congenital bilateral absence or blockage of the vas deferens or epididymis (Jarow et al., 1989). Non-obstructive azoospermia can result from (a) reduced spermatogenesis, otherwise known as hypospermatogenesis, (b) maturation arrest at early or late stages of spermatogenesis, or (c) Sertoli-cell only syndrome resulting in complete lack of spermatogenesis (Sigman and Jarow, 2002).

In present work, oligospermia and azoospermia (two of the major causes of male infertility worldwide) were studied as infertile groups.

2.3.3 Causes of male infertility

Any process that affects sperm production and quality is potentially harmful to male fertility. A variety of disorders ranging from hormonal disturbances to physical problems, to psychological problems can cause male infertility. In many instances, male infertility is caused by testicular damage resulting in an inability of the testicle to produce sperm. Once damaged, the testicle will not usually regain its sperm-making capabilities and cannot usually be treated. Besides testicular damage, the main causes of male infertility are low sperm production and poor sperm quality.

Other important causes include varicocele, genital tract obstruction, gonadotoxin exposure, genetic conditions, infections, hormonal dysfunction, immunological conditions (presence of anti-sperm antibodies), ejaculatory/sexual dysfunction, cancer and systemic diseases. Modern adult lifestyle, smoking, alcohol and food habits are also important factors which affect the fertility of men (Sharpe and Franks, 2002). Despite the advancement in the field of instrumentation and development of better evaluation methods, 30% to 50% cases of male infertility are idiopathic (Turek, 2005).
2.4 Evaluation of male infertility

Traditionally, the diagnosis of male infertility relies on microscopic assessment and biochemical assays to determine human semen quality. The conventional parameters have been given the most importance are sperm concentration, motility and morphology in the ejaculate. Some additional tests, including estimations of vitality, anti-sperm antibodies, contaminant cells, and total motile counts before and after sperm preparation for assisted conception; are also added to the list in the recent years (Lewis, 2007). The first person who suggested that semen analysis should be a routine investigation in the evaluation of every case of infertility was Edward Martin in 1902 (Jequier, 1991); however, one of the earliest published assessments of sperm concentration in human semen was by Macomber and Sanders (1929) who reported a median of ~100 million spermatozoa per millilitre, using blood pipettes and an unidentified counting chamber.

In order to establish uniformity in laboratory procedures, the WHO first published a laboratory manual for the examination of human semen in 1980. The manual also set out standards to exclude influences such as the health of patient over the previous spermatogenic cycle, length of sexual abstinence, time, and temperature from ejaculation to analysis. The manual has been regularly updated (1987, 1999, and 2008) with the latest version in 2010 with modifications to recommended tests for sperm morphology and motility (Cooper et al., 2010).

The main goals of evaluating the semen of infertile man are to identify correctable causes of infertility and to help him and his partner conceive by the most natural, least invasive means possible. The complete clinical evaluation of male infertility includes a detailed history, physical examination, laboratory tests of semen, endocrine evaluation and genetic tests (karyotyping).

The two main purposes of the evaluation are:

(1) To identify any modifiable factors that can improve the man’s fertility status.
(2) To identify any serious underlying conditions, such as testis cancer, osteoporosis, and endocrine or genetic problems that present first as infertility (Lipshultz et al., 2009).
2.4.1 Medical history

A detailed medical history should be obtained for any factor that may impact fertility potential. Information regarding the following areas should be collected (Esteves et al., 2011):

i. prior fertility, previous diseases during childhood and puberty such as viral orchitis and cryptorchidism;

ii. surgeries performed, especially those involving the pelvic and inguinal regions and genitalia;

iii. genital traumas;

iv. infections such as orchiepididymitis and urethritis;

**Figure 2.5:** Algorithm for the management of infertile patient. *(Source: Esteves et al., 2011)*.
v. physical and sexual development;
vi. social and sexual habits;
vii. exposure to gonadotoxic agents such as radiotherapy or chemotherapy, recent fevers or heat exposures;
viii. current or recent medications and
ix. family history of birth defects, mental retardation, reproductive failure or cystic fibrosis.

2.4.2 Physical Examination

Some of the essential physical examinations which are suggested by various studies to appropriately identify reasons of male infertility include,

i. Sexual development: In the presence of diminished body hair distribution, gynecomastia or eunuchoid proportions, androgen deficiency should be suspected.

ii. Genital examination: To reveal the presence of a hypospadiac urethral meatus, pathologic curvature of the phallus or an active sexually transmitted disease.

iii. Testicles: Normal adult testicles should measure about 4 cm (length) x 2.5 cm (width), resulting in a volume of approximately 20 mL. Testicles should present with a firm consistency. Approximately 85% of the testicular parenchyma is involved in spermatogenesis, but there is no lower limit for testicular volume to exclude the presence of spermatozoa. As such, testicle size cannot be relied on as a clinical marker to preclude a trial of sperm retrieval. Both primary and secondary testicular failure may lead to bilateral testicular hypotrophy. When serum testosterone is low, the volume of seminal fluid is often small. An endocrine workup helps to distinguish both conditions. High FSH levels accompanied by normal or low testosterone levels imply primary testicular failure.

iv. Epididymis: A healthy epididymis should be firm, but an obstructed epididymis is augmented and soft. The partial or complete absence regression of an epididymis may represent CBAVD. The vasa are easily palpable inside the posterior aspect of the spermatic cord as a distinct, firm, round, “spaghetti-like” structure. A unilateral or bilateral congenital absence of the vas results in oligospermia or azoospermia, respectively.


2.4.3 Laboratory tests for semen analysis

Although semen analysis does not test sperm function, it is the basis of the initial laboratory evaluation which provides critical information and data from which a prognosis of fertility or the diagnosis of infertility can be deduced (Polansky and Lamb, 1988).

i. It is recommended that the semen should be collected in a specialized andrology laboratory and analyzed by well-trained technicians under rigorous quality control standards (Esteves, 2003).

ii. Results from at least two, but preferably three, separate seminal analyses must be obtained before a definitive conclusion can be drawn, as wide biological variability may exist within the very same individual.

iii. The interval between the analyses is arbitrary and is generally recommended to be 1-2 weeks. Ejaculatory abstinence should occur for a minimum of two days to a maximum of seven days, with two to three days being ideal (Cooper et al., 2010). Longer periods of abstinence may lead to higher ejaculatory volumes and increased spermatozoa quantity, but motility is usually negatively affected.

iv. The semen should be collected by masturbation and must be allowed to liquefy for 30 to 60 minutes before being analyzed.

Routine seminal analysis includes,

i. Physical characteristics of semen, including liquefaction, viscosity, pH, color and odour;

ii. Specimen volume;

iii. Sperm count;

iv. Sperm motility and progression;

v. Sperm morphology;

vi. Leukocyte quantification

vii. Fructose detection in cases where no spermatozoa are found, especially if the total volume of the sample is less than 1 mL.

The WHO criteria used to define normality have recently been updated (Cooper et al., 2010), as shown in Table 2.2. Apart from the total sperm number per ejaculate, the lower
limits of these distributions are lower than the previously presented normal or reference values (WHO manuals 1987, 1992, 1999) but are in agreement with recently published data from studying populations of fertile and infertile couples (Guzick et al., 2001; Gunalp et al., 2001; Menkveld et al., 2001).

**Table 2.2:** Reference values of semen variables.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>≥ 1.5 mL</td>
</tr>
<tr>
<td>pH</td>
<td>≥ 7.2</td>
</tr>
<tr>
<td>Sperm concentration</td>
<td>≥ 15x10^6 /mL</td>
</tr>
<tr>
<td>Total sperm number</td>
<td>≥ 39x10^6 /mL</td>
</tr>
<tr>
<td>Total Motility</td>
<td>≥ 40 %</td>
</tr>
<tr>
<td>Progressive motility</td>
<td>≥ 32 %</td>
</tr>
<tr>
<td>Normal morphology</td>
<td>&gt; 4 %</td>
</tr>
<tr>
<td>White blood cells</td>
<td>&lt; 1x10^6 /mL</td>
</tr>
<tr>
<td>Sperm vitality</td>
<td>≥ 58 % live</td>
</tr>
</tbody>
</table>

(Source: Cooper et al., 2010)

A morphometric description of spermatozoa using the strict criteria described by Kruger et al. (1988) was incorporated in the new WHO guidelines. Low values of 3–5% normal sperm morphology have been found by others to be optimal cut-off values between fertile and infertile men whose spermatozoa were used for in vitro fertilization (Coetzee et al., 1998), intrauterine insemination (Van Waart et al., 2001) and in spontaneous pregnancies (Van der Merwe et al., 2005). No single aspect of semen analysis was able to solely distinguish between fertile and infertile men, although morphology was suggested to be the most important. The coexistence of multiple altered seminal parameters increases the risk for infertility, but semen characteristics need to be interpreted in conjunction with the patient's clinical information. Abnormal white blood cell (leukocyte) counts are a frequent cause of male infertility. The incidence of leukocytospermia (>1 million leukocytes/mL of semen) in infertile men varies between 3
and 23% and has been correlated with clinical and subclinical genital infections, elevated levels of reactive oxygen species, elevated anti-sperm antibody levels and deficient sperm function (Bar-Chama and Fisch, 1993). Regarding sperm counts, azoospermic patients should have their diagnosis confirmed by verifying the lack of any spermatozoa in centrifuged seminal fluid on two separate occasions using a high-powered microscope. Azoospermia with low ejaculate volume (<1.0 mL) can be caused by ejaculatory dysfunction and the most common cause might be ejaculatory duct obstruction (EDO). When suspected, EDO can be confirmed by assessing the seminal pH and fructose levels, as normal semen is alkaline and contains fructose.

2.4.4 Endocrine evaluation

Endocrine evaluation is generally suggested in the following cases (Sokol and Swerdloff, 1997):

i. sperm concentration < 10 million/mL;
ii. erectile dysfunction;
iii. hypospermia (volume <1 mL);
iv. signs and symptoms of endocrinopathies or hypogonadism.

Measurements of hormone levels- Follicle-stimulating hormone, testosterone, LH, Gonadotropin, estradiol and prolactin: The minimal evaluation includes the assessment of serum follicle-stimulating hormone (FSH) and testosterone levels, which reflect germ cell epithelium and Leydig cell status, respectively. If the testosterone level is low, a second collection is recommended along with free testosterone, LH and prolactin measurements. Isolated FSH elevation is usually indicative of severe germ cell epithelium damage. Highly elevated FSH and LH levels, when associated with low-normal or below normal testosterone levels, suggest diffuse testicular failure and may have either a congenital (e.g., Klinefelter syndrome) or acquired cause. Concomitant low levels of FSH and LH may implicate hypogonadotropic hypogonadism. This condition may be congenital or secondary to a prolactin-producing pituitary tumour. In azoospermic men with a normal ejaculate volume, FSH serum level greater than two times the upper limit of the normal range is reliably diagnostic of dysfunctional spermatogenesis. Gonadotropin values within the normal range suggest an extraductal
obstruction in azoospermic subjects. However, azoospermic patients with testicular failure and testis histology exhibiting sperm maturation arrest and 10% of those diagnosed with Sertoli-cell-only syndrome may present with non-elevated FSH levels (Sokol and Swerdloff, 1997). Serum estradiol levels should be determined in patients presenting with gynecomastia. Infertile patients with a testosterone to estradiol ratio less than 10 can harbour significant but reversible seminal alterations (Raman and Schlegel, 2002). Vaucher et al. (2009) suggested that hyperestrogenism secondary to a higher conversion rate of testosterone into estradiol in Klinefelter syndrome patients inhibits testosterone production via a negative feedback pathway. Serum prolactin levels should be determined in infertile men with a complaint of concomitant sexual dysfunction or when there is clinical or laboratory evidence of pituitary disease; however, hyperprolactinemia is rarely a cause of infertility in healthy men (Sigman and Jarow, 1997). Although hormonal alterations may be present in approximately 10% of men who undergo assessment, clinically significant changes affect less than 3%.

2.4.5 Genetic evaluation

Male infertility can also be associated with various genetic factors, including chromosomal aberrations, genetic alterations and Y chromosome micro-deletions. Chromosomal aberrations are assessed through G-band karyotyping while genetic mutations and Y chromosome micro-deletions may be assessed by the analysis of the peripheral blood using molecular biology techniques as such, DNA is amplified by the polymerase chain reaction (PCR), analysed by agarose gel electrophoresis and sequenced (Esteves et al., 2011).

2.5 Searching male infertility marker proteins

Detailed proteomic analysis enhances our understanding related to various sub-processes and their regulation in a biological process. Advanced studies towards expression of proteins, their functions and interactomes give a clear picture of a disease mechanism. The simultaneous developments in the fields of proteomics and mass spectrometry have widely facilitated the systematic identification of proteins of very complex proteins mixtures from subcellular structures to tissues. These fields are integrated and considered as a powerful tool for searching new biomarkers for diagnosis.
and staging of a disease. Differential proteomics is commonly applied these days to identify variations in expressions of various proteins in normal and disease states. The improvements in mass-spectrometric instruments offer high sensitivity, mass accuracy and speed (Canas et al., 2006; Mann and Kelleher, 2008).

Studies towards identification of new and potential protein biomarkers for various diseases have shown an exponential growth in past decade and continuously growing in the present decade as a promising clinical research area in search for better therapeutic solutions for human kind. Thus, it is considered as the golden era of protein biomarkers research. The researchers, in the field of reproductive biology related to infertility, have also explored spermatozoa and SP proteomes for identifying novel infertility marker proteins for obvious reasons. The spermatozoa proteome is extensively studied in this regard, but similar studies in SP are premature. However, SP is a potential target for searching male infertility marker proteins as it is a rich source of proteins which are essentially required for development of sperm and successful fertilization.

### 2.5.1 Differentially expressed sperm proteins

The developments in proteomic technology and availability of bioinformatics tools/software for data analysis have enabled the researchers to generate dataset of differentially expressed proteins of spermatozoa in various functional states (normal vs. defective, motile vs. non-motile, capacitated vs. non-capacitated etc.). These studies can efficiently list the proteins which are functionally significant.

Zhao et al. (2007) did differential proteomic studies and compared 8 asthenozoospermia samples to normal controls. They visualized 17 differentially expressed proteins and 10 of them were identified. The proteins identified in this study are enzymes associated with sperm energy metabolism involved in the control of the human sperm motility (isocitrate dehydrogenase subunit, glutamate oxaloacetate transaminase-1, carbonic anhydrase II, semenogelin-1 precursor, glutamine synthetase and 26S protease regulatory subunit 7), glycolytic enzymes (phosphoglycerate mutase 2 and triosephosphate isomerase) and proteins associated with sperm structure (Rho GDP dissociation inhibitor and outer dense fiber protein).

Martínez-Heredia et al. (2008) measured the amount of the major sperm proteins in asthenozoospermic and normospermic control sperm donor samples and reported 17
differentially expressed proteins in the asthenozoospermic group. These proteins are cytochrome-C oxidase subunit-6B, dihydrioloipoamide dehydrogenase precursor, fumarate hydratase precursor and dienoyl-CoA isomerase precursor (energy production); cytoskeletal actin-B, histone H2A, prolactin-inducible protein, prolactin-inducible protein precursor and semenogelin 1 precursor (structure and movement); annexin-A5, calcium binding protein-S100A9 and inositol-1 monophosphatase (cell signalling and regulation); clusterin precursor (associated with apoptosis and fertility), heat shock protein-HSPA2 (spermatid development), proteasome subunit-PSMB3 (potential regulatory effect) and testis expressed sequence 12, 3-mercapto-pyruvate sulfurtransferase.

Siva et al. (2010) carried out proteome analysis of asthenozoospermic and normospermic sperms to understand the molecular basis of sperm motility. Analysis revealed eight proteins with altered intensity and these proteins are crucial for sperm structural organization and motility. These proteins are categorized into three main functional groups namely: proteins involved in energy and metabolism (triose phosphate isomerase, glycerol kinase 2, testis specific isoform and succinyl-CoA: 3-ketoacid coenzyme A transferase 1, mitochondrial precursor), sperm movement and structural organization (tubulin beta 2C and tektin 1) and protein folding and stress response (proteasome alpha 3 subunit and heat shock-related 70 kDa protein 2).

Liao et al. (2009) have investigated the differences in protein expression between human round-headed spermatozoa (with an absent acrosome, an aberrant nuclear membrane and midpiece defects) and normal spermatozoa by two-dimensional fluorescence difference gel electrophoresis (DIGE) coupled with mass spectrometry. This particular study revealed that 9 proteins were upregulated and 26 proteins were downregulated in round-headed spermatozoa when compared with normal spermatozoa. The identified proteins have been suggested to be associated with a variety of cellular processes and structures, including spermatogenesis, cell skeleton, metabolism and spermatozoa motility.

Secciani et al. (2009) have studied protein profile of capacitated versus ejaculated human sperm. Comparative analysis of 2DE gels of freshly ejaculated sperm and capacitated sperm revealed significant quantitative and qualitative variations. The proteins with a significant decrease after capacitation were found to be involved in
protein fate, metabolism, and flagellar organization while increasing proteins were found to be related to cellular stress.

Baker et al. (2010) have also reported the analysis of proteomic changes associated with sperm capacitation. They have reported 210 significant peptide changes and identified 52 proteins that are altered during capacitation. Most of the proteins identified in this study are first time reported to be associated with the process of sperm capacitation. A large fraction of proteins is associated with mitochondrial energy metabolism and glycolysis; clearly emphasizing the importance of energy metabolism in allowing spermatozoa to achieve a capacitated state.

2.5.2 Differentially expressed proteins of seminal plasma

It is well known that seminal plasma proteins have an ample panorama of actions in reproductive physiological processes for establishing fertility. SP proteins play critical roles in sperm maturation and are essentially required for sperm function mainly for their interactions with the various environments of the tubular genital tract and the oocyte (Rodríguez-Martínez et al., 2011). Some of them may even influence the relative intrinsic fertility of the male and may be sometimes the sole reason of infertility in a large population of infertile couples. Even with normal semen parameters, the interruption in the normal expression of these proteins may lead to male infertility. Thus, identification of differentially expressed proteins in fertile and infertile seminal plasma states may provide us evidence about the fraction of proteome which is critical for fertility/infertility issues. The proteomic strategies are extensively applied to identify the constituents of seminal plasma; however, there have been very few studies designed to identify male infertility marker proteins in seminal plasma. Few but important studies performed in this direction are performed by Yamakawa et al. (2007); Bai et al. (2007); Wang et al. (2009); Bai et al. (2010) and Davalieva et al. (2012).

Yamakawa et al. (2007) performed comparative analysis of inter-individual variations in the seminal plasma proteome and identified potential markers of azoospermia. They have identified 4 possible candidate markers for non-obstructive azoospermia, namely stabilin 2, 125 kDa centrosomal protein, guanine nucleotide–releasing protein and prolactin-inducible protein.
Wang et al. (2009) did proteomic analysis of seminal plasma from asthenozoospermia patients. They reported the identification analysis of 741 proteins in the seminal plasma by LC-MS/MS and found that 45 proteins were upregulated and 56 proteins were downregulated in the asthenozoospermic group when compared with the normal. Bai et al. (2007) did comparative analysis of proteins in seminal plasma of non-obstructive azoospermia patients and healthy fertile males and identified 28 differentially expressed proteins. In another study, they have also identified asthenozoospermia-associated proteins in human seminal plasma by shotgun proteomic strategy (Bai et al., 2010). Overall, 172 unique proteins were identified, including 89 from the asthenozoospermia and 83 from normospermia.

In an important study, Ding et al. (2007) have identified human alpha-1-antitrypsin and zinc-alpha-2-glycoprotein (ZAG) as spermatozoa forward motility-related proteins in human seminal plasma using two-dimensional (2D)-gel electrophoresis and mass spectrometry analysis. These proteins may play important roles during maturation of spermatozoa, from the epididymis through fertilization in the female reproductive tract.

In a most recent study, Davalieva et al. (2012) compared the protein expression profiles of seminal plasma from four different groups of men: normospermic, asthenozoospermic, oligospermic and azoospermic groups, using two-dimensional differential gel electrophoresis and found eight proteins with statistically significant increased expression in azoospermia compared with at least one of the other studied groups. The differentially expressed proteins were fibronectin, PAP, proteasome subunit alpha type-3, beta-2-microglobulin, galectin-3-binding protein, PIP and cytosolic nonspecific dipeptidase.

2.6 Proteins of human seminal plasma, in context to this thesis

The present study includes the analysis of Concanavalin A binding proteins of human seminal plasma, PIP and PAP in view of male infertility.

2.6.1 Concanavalin A binding proteins

The studies related to expression of proteins, their functions and interactions are essential for understanding the various disease mechanisms. The developments in the
field of clinical proteomics have extensively facilitated the systematic identification of proteins of clinical importance including biomarker discovery. The discovery of proteins with post-translational modifications has become an important frontier of proteomics studies. Glycosylation, one of the most common post-translational modifications, plays essential roles in controlling various biological processes in immunology, cancer, protein folding, host-pathogen interactions, human diseases and signal transduction etc. In recent times, it has become an imperative target for proteomic research and has great potential for clinical applications. Comparative glycoproteomics is also emerged as a powerful tool in a wide variety of research, including biomarker discovery, disease diagnosis and glycosylation profiling (Wei and Li, 2009). Lectin affinity chromatography allows the isolation and enrichment of glycoproteins and glycopeptides.

Figure 2.6: Three dimensional structure of Concanavalin A (PDB ID: 1CVN). (Source: Naismith and Field, 1998).
Figure 2.7: Sugar (alpha-D-Mannose, shown as white surface) binding sites of Concanavalin A (circles highlight binding site in each chain, A-D). Con A is a metalloprotein containing manganese (purple) and calcium (green) in a bimetallic metal-binding site which facilitate its binding to saccharides. (Source: Naismith and Field, 1998).

Concanavalin A (Con A) is a common lectin isolated from the jack bean, which has broad specificity, to isolate glycoproteins rather than some specific structural types (Novotny and Mechref, 2005). Con A is a homo tetramer, 104 kDa lectin (having four 26 kDa monomer units), derived from the jack bean (Canavalia ensiformis). It binds to alpha-D-glucose and alpha-D-mannose containing saccharides and requires both Mn2+ and Ca2+ ions as a co-factor for binding (Naismith and Field, 1998).

Many of the previous studies have successfully applied Con A lectin affinity chromatography to enrich and isolate glycoproteins (Ogata et al., 1975; Brewer and
Bhattacharyya, 1986; Spiro, 2000; Owen et al., 2009). More captivatingly, Con A-interacting fraction of human seminal plasma shows decapacitating activity (Marquínez et al., 2003). Thus, the study towards isolation and identification of various proteins of this fraction is of vast importance and may help us in better understanding of fertility related processes.

2.6.2 Prolactin inducible protein

PIP is a ~17 kDa glycoprotein present in various human body fluids. In seminal fluid, it is secreted from prostate gland of testis (Autiero et al., 1991). Alternative names of PIP are gross cystic disease fluid protein 15, secretory actin-binding protein and gp17 (Hassan et al., 2009). It is known to play vital roles in immunoregulation, antimicrobial activity, apoptosis and tumour progression. PIP interacts with many proteins, such as fibrinogen, actin, keratin, myosin, tropomyosin (Schenkels et al., 1994), immunoglobulin G (Chiu and Chamley, 2003), zinc-alpha-2 glycoprotein (Hassan et al., 2008) and human serum albumin (Kumar et al., 2012).

![Figure 2.8: Three dimensional structure of prolactin inducible protein. Structure coordinates for PIP were retrieved from ZAG-PIP complex (PDB ID: 3ES6). (Source: Hassan et al., 2008).](image)
Due to large protein-protein interactome, various roles of PIP have been suggested in diverse biological processes including male fertility/infertility; however exact physiological function remains unclear till date. The role of PIP in cancer proliferation is extensively studied. The mitogenic activity assays on breast cancer cell lines suggested active role of PIP in tumor proliferation (Mazoujian et al., 1983; Cassoni et al., 1995). Its expression analysis in breast and prostate cancers identified it as a marker of these subtypes of cancer (Hassan et al., 2009). It had been found in various studies that PIP is differentially expressed in infertile and fertile sperm / seminal plasma, but most of these studies were performed at preliminary stage and detailed relative expression quantification was not performed.

Human PIP is synthesized as a single chain immature pre-protein that consists of 146 residues and Schaller et al. (1991) demonstrated for the first time the full amino acid sequence of mature PIP by using peptide cleavage and amino acid sequencing. The structure of PIP consists of seven anti-parallel β-strands organized in the form of two β-sheets and arranged in such a way that they form a sandwiched β-sheet structure with a cluster of hydrophobic residues, Ile9, Leu28, Val30, Thr42, Thr58, Ala60, Lys68, Phe70, Trp72, Val86, Phe109, and Thr111, filling the space between the two sheets (Hassan et al., 2008).

2.6.3 Prostatic acid phosphatase

PAP is a non-specific phosphomonoesterase that is synthesized and secreted into seminal plasma under androgenic control (Ostrowski and Kuciel, 1994). It belongs to the family of high-molecular mass phosphatases and is classified as an acid phosphatase due to its optimum pH range of 4–7. Mature PAP is secreted as a glycosylated homo-dimer consisting of two 50 kDa subunits. PAP is secreted by epithelial cells of the prostate (Vihko, 1978) and its concentrations in seminal fluid is about 1 mg/mL. Secreted PAP is consisting of multiple post-translationally modified isoforms, which differ in glycosylation and hydrophobicity (Taga et al., 1983). It was formerly used as a marker for diagnosis and therapy control of prostate cancer; however, its role as a prostate cancer marker has now been taken over by prostate-specific antigen. PAP is the most abundant phosphatase in human prostate tissue and has been studied extensively, primary due to its clinical relevance in male infertility and prostate carcinoma (Azumi et al., 1991).
Increased PAP activity in patients with azoospermia compared to normal controls had been reported in various studies and enzyme levels were found inversely correlated with sperm concentration (Vaubourdolle et al., 1985; Dave and Rindani, 1988; Ziyyat et al., 2008). Patients with severe oligospermia were also shown to have increased seminal PAP levels (Singh et al., 1996). Significantly increased levels of PAP in azoospermia in comparison to other conditions such as normospermia, oligospermia and asthenozoospermia are also reported in a very recent study (Davalieva et al., 2012).

**Figure 2.9:** Three dimensional structure of prostatic acid phosphatase (PDB ID: 1CVI). *(Source: Jacob et al., 2000)*.