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XXVII DOCTORAL PROGRAM

Prostate specific antigen (PSA): from the catalytic activity to the clinical value

L'antigene prostatico specifico (PSA): dall'attività catalitica all'uso clinico

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Chapter 1

Introduction and aims

1.1 – The prostate cancer

1.1.1. Anatomy of the prostate

The prostate, whose name derives from the greek *proseitos* (i.e. "set before", in relation to its position relative to the bladder), is an exocrine gland that surrounds the urethra. In adults prostate weighs about 20-25 g and histologically consists of glandular alveoli surrounded by a fibro-muscular matrix. The main function of the prostate is represented by the production of seminal liquid (Fig. 1).

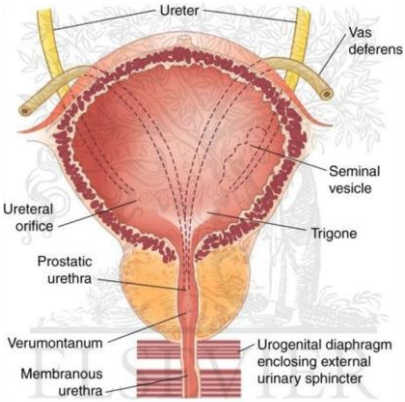

Figure 1. The male genitourinary system. Nicholson et al., 2007

Based on its pathophysiological and embryological characteristics, the prostate is divided into four zones (Fig. 2):

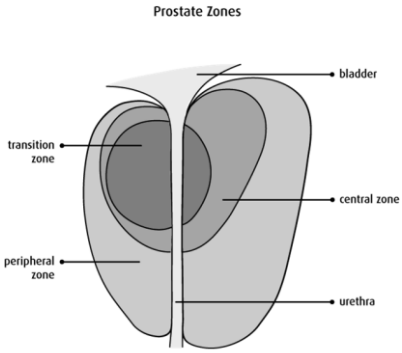

Figure 2. The prostatic zones. Nicholson et al., 2007

- *The transition zone*: it constitutes the 5% of the gland surrounding the urethra;
- *The central zone*: it accounts for about the 2% of the gland and it is placed under the proximal urethra;
- *The peripheral zone*: it comprises the 70-75% of the gland surrounding the central area and extending to the apex of the gland;
- *The fibromuscular stroma*: it is place before the other zones.

Most of the prostate cancers (PCa) originate in the peripheral zone, therefore, about the 70% of them are classified as adenocarcinoma [Schulz et al., 2003]. Less common is the possibility that neoplastic transformations could occur in the medial portion or in the transition zone of the gland (20%) that are typical sites of the benign prostatic hyperplasia (BPH). The central area, which constitutes the bulk of the prostate, rarely represent a tumor site (5%), but more often it is invaded by large tumors originated from the neighboring portions [Hising et al., 2006].

1.1.2. Epidemiology of the prostate cancer

PCa is the most common male cancer in Western populations and, after lung cancer and colon-rectum cancer, is the third leading cause of cancer related death [Siegel et al., 2013]. In the USA the highest incidence is found, with 217,730 new cases diagnosed in 2013, on a male population of 150 million people (0.14%) and 32,050 deaths (mortality = 0.025%) [Siegel et al., 2013]. In Europe, however, the incidence of PCa is similar, with 382,000 new diagnosed cases in 2012 on a male population of 360 million people (0.10%) and 87,400 deaths (mortality = 0.024%) [Ferlay et al., 2013]. On the other side, in some countries of Southeast Asia, from two to

ten times lower incidence rates were reported [Sankaranarayanan et al., 2011].
Over the last years, in many Western industrialized countries, an increase in PCa incidence occurred. This may reflect the introduction in the clinical practice of the determination of prostate specific antigen (PSA), in the form of opportunistic screening, with the consequent diagnosis of a higher number of asymptomatic or preclinical PCa forms [Croswell et al., 2011]. The use of this diagnostic tool, however, did not affect the mortality rate for this pathology [Jemal et al., 2010], probably because the majority of PCa identified by the PSA test is not intended to clinically manifest in the course of life even withot the screening [Guidelines AIOM 2009]. Tumors that exhibit this clinical course are called "latent cancers" and they are well documented also by post mortem autopsies. These analyses showed an incidence of PCa of 10-30% in men between 50 and 60 years old and of 50-70% in subjects between 70 and 80 years old [Haas et al., 2008].

1.1.3. Pathogenic mechanisms of PCa

As for the majority of solid tumors, the etiology of PCa is multifactorial as a result of a complex interaction between genetic factors (responsible for the familiar and racial incidence) and environmental factors (related to diet and lifestyle). This disease is steadily increasing and age is one of the most relevant risk factors; the PCa occurrence, in fact, is rare in men under 50 years old, but it increases dramatically after 65 years old, while the higher number is diagnosed between 70 and 74 years old [Carlsson et al., 2014].
Another factor that seems to be important in PCa development is the influence of male sex hormones. Since the prostate is an androgen-dependent gland it develops and maintains its tropism due to testosterone levels. However, there are no definitive data concerning the role of circulating androgens in PCa occurrence [Ismail et al., 2011]. On the other side, environment, lifestyle and diet are well documented risk factors for PCa. A particular work conducted on Asian immigrants moving to the USA, showed that the incidence of PCa increases in men starting from the second generation, thus emphasizing the importance of environmental factors in the development of this disease [Shimizu et al., 1991]. In other studies it was reported that the consumption of red meat in association with smoking, intake of alcohol and obesity, could play a significant role in higher the PCa risk [Meyerhardt et al., 2010]. The consumption of vegetables, however, seems to be important as a protective factor. The low incidence of this pathology in the Asian populations may therefore be related to the low consumption of red meat and the high consumption of vegetables, whose nutritional principles could play a protective role [Desgrandchamps et al., 2010].
From the molecular point of view, many are the mechanisms underlying the PCa onset and progression. In particular, it has been proposed as a recurring

or chronic inflammation may play a pivotal role in the neoplastic transformation [De Marzo et al., 2004]. During the inflammatory response, in fact, cells of the immune system synthesize numerous oxidizing agents capable to induce genetic damages to the resident epithelial cells [Sciarra et al., 2008]. One of the most interesting aspects, in that sense, was the finding of genetic alterations, characterizing the beginning stages of PCa, even in cells affected by inflammatory atrophic processes [Vecchione et al., 2007]. Moreover, at an inflammation level, epithelial cells often show signs of oxidative stress, such as increasing expression level of the glutathione-S-transferase (GSTP1).
On the other side, histological examination revealed the occurrence of specific lesions of the prostatic glandular tissue, defined as prostatic intraepithelial neoplasia (PIN). Such injuries are attributable to histopathological changes of low grade (LGPIN) or high grade (HGPIN) and, from many authors, they are considered direct precursors of PCa [Dickinson, 2010]. PIN lesions are frequently found in the peripheral zone of the prostate (where most of the PCa originate); the prostatic epithelial cells in those sites showed often the same chromosomal alterations detected in cancer cells. Moreover, cells of the PIN lesions show cell wall alterations similar to those observed in tumor cells; the thickening of the epithelium basal layer can be also be observed (Fig. 5). Changes in gene expression are also showed by the pre-neoplastic cells, as documented by the reduction of the cadherins and cytoskeleton components levels [Nelson et al., 2003].

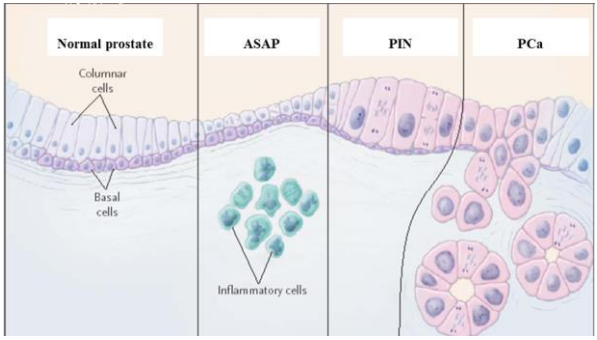

Figure 3. Pre-neoplastic and neoplastic forms of PCa. Nelson *et al*., 2003.

PIN lesions, on the other side, differ from PCa lesions for the presence of an intact basement membrane that does not allow the invasion of the glandular stroma. Furthermore, these lesions do not produce high levels of PSA and, therefore, they can be detected only by biopsy [Dickinson, 2010].
The international reference system used for classifying histologically a PCa is called Gleason system. This system consider the glandular differentiation

degree and the infiltration degree. Figure 4 shows the 5 pattern of increasing aggressiveness considered in the Gleason system. These patterns are classified as follows:

Gleason 1: Tumor composed by well defined, tight, uniform, single and not confluent glandular nodules.
Gleason 2: Tumor with a minimal extension of to the neoplastic glands toward the tumor lesion periphery. This lesion is localized in the context of normal tissue.
Gleason 3: Tumor invading the normal tissue; glands show considerable variability in shape and size.
Gleason 4: Glands with obvious neoplastic confluent alterations. Sometimes there are cribriform glands with irregular edges.
Gleason 5: Tumor without glandular differentiation, it is characterized by stretches of anaplastic cells and necrotic areas.

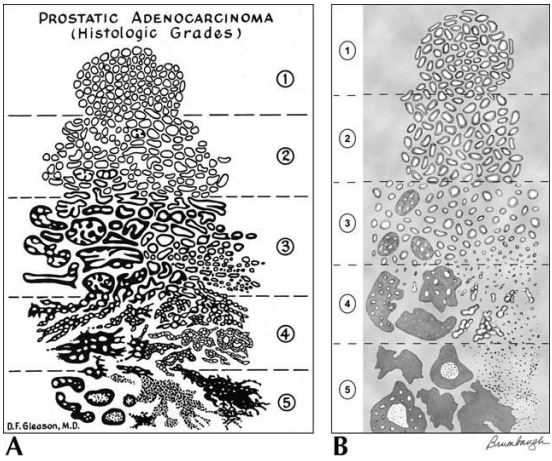

Figure 4 The Gleason system (Gs). Helpap et al., 2008

In the Gleason system the main framework (predominant) and the secondary framework (less represented) are considered and to both, a score between 1 and 5, is assigned. 1 indicates the most differentiated and 5 the less differentiated and most aggressive pattern. If a tumor shows a single histological framework, to the primary and secondary pattern the same score is assigned. The two scores are then combined in order to generate the so called Gleason score (Gs), whose value fluctuates from 2 (1 + 1) to 10 (5 + 5), that represent the highest degree of malignancy [Guidelines AIOM 2009].

The diagnosis of PCa is exclusively made by biopsy. The importance of the ultrasound-guided prostate biopsy is due not only to obtain a definitive diagnosis, but also some useful information for guiding the therapeutic strategy [Eichler et al., 2006]. This examination, however, is not diriment in case of a negative report; in fact the 10-30% of patients with a negative biopsy may have a PCa diagnosis in further biopsies [Djavan et al., 2005]. The detection rate for a PCa biopsy depends not only on the sampling technique, but also on the criteria used to perform eventual further biopsies [Eichler et al., 2006]. Moreover, a negative biopsy is usually associated with a risk reduction of finding a high degree PCa in a subsequent biopsy [Borden et al., 2007]. To date, in Italy, it is recommended to repeat a PCa biopsy only one or more of the following indications are satisfied [Guidelines AIOM 2009]:

- Inadequacy of the first biopsy (under 6 sampling, absence of prostate glands, too small fragments);
- Previous histological diagnosis of uncertain or suspicious pre-neoplastic lesions, such as HGPIN or atypical small acinar proliferation (ASAP);
- Progressively increasing PSA serum levels, or changes in digital rectal examination (DRE) results.

During a prostate biopsy is not uncommon to experience adverse events such as pain, hematuria, hematospermia and rectal bleeding; more serious adverse events, such as infections (1.8%) or considerable bleeding (0.6%), are instead infrequent. Complications related to the prostate biopsy brought the researchers to study new strategies to facilitate the PCa diagnosis in order to avoid this invasive clinical practice when it is not firmly recommended [Eichler et al., 2006].

The correct identification of the tumor differentiation state is important to determine the best therapeutic strategy and to obtain prognostic information. Despite considerable advances in imaging technology, it is not yet possible to get these information through such diagnostic tools. In fact, especially for the early stages, PCa can be diagnosed only with a biopsy [Verma et al., 2011].

In the TNM classification the local extension (T), the commitment of the lymph nodes (N) and the presence of distant metastasis (M) are considered. The study of pathological material analyzed after the radical prostatectomy (RP) provides information in the tumor stage definition according to the TNM system that involves the following indications [Schröder et al., 1992]:

Primary tumor (T)

pT2 Organ confined

pT2a Unilateral, involving one-half of 1 lobe or less

pT2b Unilateral, involving more than one-half of 1 lobe but not both lobes

pT2c Bilateral disease
pT3 Extraprostatic extension
pT3a Extraprostatic extension or microscopic invasion of the bladder neck
pT3b Seminal vesicle invasion
pT4 Invasion of the bladder and rectum

Regional lymph nodes (N)
pN0 No positive regional nodes
pN1 Metastases in regional nodes(s)

Distant metastasis (M)
M0 No distant metastasis
M1 Distant metastasis
M1a Non-regional lymph nodes(s)
M1b Bone(s)
M1c Other site(s) with or without bone disease

1.1.4. Laboratory medicine

Since it is not expected, at least in the short term, to reach a reduction in PCa incidence through an effective primary prevention, there is no doubt that secondary prevention remains the only available tool to influence the evolution of this disease and reduce, consequently, the PCa-related mortality. Therefore, the best way to obtain an early detection appears to be an individual or population opportunistic screening. The screening test that seems to be the more appropriate for this purpose, considering the cost, convenience, and diagnostic accuracy, is the PSA [Guidelines AIOM 2009]. On the other side, the role of PSA screening in reducing PCa-related mortality is still controversial. In fact, conflicting results have emerged from several observational studies [Crawford et al., 2011; Sciarra et al., 2011]; Therefore, the recommendations for PSA screening differ between the various organizations and scientific societies.
Although the PSA serum test increased the early diagnosis of PCa, a major disadvantage of this marker is its low specificity, which brings, every year, to the execution of a high percentage of negative biopsies (60-75%), especially in patients with PSA levels between 4 and 10 ng/ml, the so called gray zone [Hessels et al., 2009]. The PSA low specificity is due to the fact that its increase in serum is not an event that closely reflects the presence of a PCa, but it can also be found in patients with BPH and prostatitis. Consequently, although the normal cutoff value for PSA is 4 ng/ml, the probability to have a PCa exists even below this threshold, as well as values higher than 4 ng/ml do not necessarily indicate a PCa. Therefore, the strategy to perform a biopsy whenever serum PSA levels increase exposes

the male population to undergo a biopsy that is often useless and linked to several complications [Nogueira et al., 2010].
A great effort is therefore constantly turned to the research of new biomarkers, in order to improve the PCa diagnosis and/or the ability to detect the asymptomatic and most aggressive forms. Among the new identified biomarkers, the prostate cancer gene 3 (PCA3) seemed to have good diagnostic potential, giving conflicting results as concerns its prognostic value [Hessels et al., 2009].
The PCA3 gene (also known as DD3 or DD3PCA3) is located on chromosome 9 and is transcribed into a non-coding mRNA which is overexpressed in tumor cells, with a level from 60 to 100 times higher compared to normal cells [Nogueira et al., 2010]. Numerous studies have demonstrated the clinical utility of the PCA3 assay [Ankerst et al., 2008; Deras et al., 2008; Kirby et al., 2009; De la Taille et al., 2011], stressing that this tests could be useful in the following cases [Schilling et al., 2010]:

- Males with a high PSA serum levels who underwent one or more negative biopsies.
- Males with a normal PSA serum levels and a family history of PCa.
- Males with high PSA serum levels and a concomitant disease of the urinary tract.

Some preliminary studies also suggest the utility of the PCA3 assay in discriminating tumors of different aggressiveness [Haese et al., 2008; Nakanishi et al., 2008], even if the most promising are those in which it emerges how the PCA3 test is able to predict a prostate biopsy outcome after a previous negative biopsy [Marks et al., 2007] [Haese et al., 2008]. These studies also contributed to investigate another open question concerning PCA3 test, that is its optimal cutoff. Most of the published data indicated that a threshold of 35 (dimensionless, see paragraph 1.3.1) represents a point in which a better balance between sensitivity and specificity can be found for PCa diagnosis [Kouriefs et al., 2009].
The role of the laboratory medicine is therefore to validate such new biomarkers with the help of well conducted and independent prospective studies, in order to clarify the effective usefulness of their introduction in the clinical practice, in order to do not commit the same errors made with the old biomarkers. In this light, two more markers are currently under investigation in PCa early diagnosis: a particular truncated isoform of the PSA pro-enzyme, the [-2]proPSA (p2PSA) and an adhesion molecule that seems to be involved in PCa progression, the Galectin 3 (Gal3). Immunohistochemical studies showed that p2PSA is the most abundant form of truncated proPSA in tumor tissues [Mikolajczyk et al., 2000] and several studies were able to demonstrate the utility of the serum quantification of this biomarker in patients with serum PSA in the grey zone candidate to a further biopsy after at least previous negative biopsy [Mikolajczyk et al., 2003; Catalona et al., 2003; Sokoll et al., 2003; Sokoll

et al., 2008; Le et al., 2010; Catalona et al., 2011; Guazzoni et al., 2012; Lazzeri et al., 2012]. On the other side Gal3 expression has been reported to vary between healthy and tumor conditions [Takenaka et al., 2004; Balan et al., 2010; Newlaczyl et al., 2011; Wang et al., 2013]. A recent study demonstrated that the expression levels of Gal3 decrease in prostate tumor tissue when compared with normal tissue [Araújo-Filho et al., 2013], while another research found that, in patients with metastatic PCa, Gal3 serum levels were significantly higher than those observed in normal patients, opening, for the first time, to an hypothetical application of this marker in PCa diagnosis [Balan et al., 2013].
In this scenario it is clear that, to date, the possibility to early detect a PCa has increased, since beside the old diagnostic factors, such as the PSA serum levels, the DRE and the diagnostic imaging techniques, some interesting and innovative tests can be used giving a valid support.

1.2 – The prostate specific antigen (PSA)

1.2.1 – Enzymology of the PSA

The prostate specific antigen is a 30 kDa serine protease belonging to the kallikrein family and it's also known as the kallikrein related peptidase 3 (KLK3). PSA is produced almost exclusively by the prostate glandular cells and it is secreted as part of the seminal fluid in order to keep the semen fluidity after ejaculation.
Like all other members of the kallikrein family, PSA is synthesized in an inactive form as a zymogen which is composed of a pre-peptide (also known as signal peptide) and a pro-peptide (which maintains the enzyme in the latent form). Inside the epithelial cell, the 17 amino acid pre-sequence is first cleaved off by signal peptidases. Afterwards, in the extracellular environment, the additional 7 amino acid pro-sequence is removed by human kallikrein 2 (hK2) [Williams et al., 2007]. PSA shows a conserved position of the Asp102 / His57 / Ser195 catalytic triad [Watt et l., 1986], however, unlike most of kallikreins, which display a trypsin-like proteolytic specificity (i.e., they cleave on the carboxyl side of a positively charged amino acid residue, namely Arg and Lys), PSA shows instead a chymotrypsin-like substrate specificity (i.e., it cleaves on the carboxyl side of a hydrophobic amino acid residue, namely Tyr, Phe, Trp, and Leu). In addition, PSA is the only member of the kallikrein family that catalyzes the cleavage of substrates displaying the Gln residue at the P1 position [LeBeau et al., 2009].
PCa can increase the amount of PSA released into the bloodstream, even though serum PSA is kept inactive in a variety of different forms. As a matter of fact, serum PSA falls into two general categories: the free PSA

(fPSA), which includes all the unbound zymogen forms, and the complexed PSA, where also active forms are kept latent through the binding of serum protease inhibitors. Notably, PSA present in the extracellular fluid, surrounding prostate epithelial cells, has been reported to be enzymatically active, suggesting that its proteolytic activity plays a role in the PCa physiopathology [Denmeade et al., 2001].
The most important physiological substrates for PSA have been proposed to be semenogelin I (SgI) and semenogelin II (SgII). These proteins are synthesized and secreted by the seminal vesicles in spermatic fluid and are involved in the formation of a gel matrix that wraps around ejaculated spermatozoa, preventing their functionalization (mainly via inhibition of reactive oxygen species) [Malm et al., 2000]. The gel matrix breaks down under the PSA enzymatic action, facilitating the spermatozoa movements [Suzuki et al., 2007]. PSA cleaves preferentially the Tyr-Glu peptide bonds and generates multiple soluble fragments of SgI and SgII [Peter et al., 1998] that seem to be the main antibacterial components in human seminal plasma [Edström et al., 2008]. These findings, together with the ability of PSA to process a number of growth regulatory proteins that are important in cancer growth and survival (such as Insulin-like growth factor binding protein, Parathyroid hormone-related protein, latent Transforming growth factor-beta 2 as well as extracellular matrix components, like fibronectin and laminin) [Cohen et al., 1992; Iwamura et al., 1996; Lilja et al., 2000; Dallas et al., 2005], suggest that PSA can facilitate tumor growth and metastasis dissemination [Williams et al., 2007; Webber et al., 1995; Ishii et al., 2004]. On the other hand, PSA has been reported to slow down blood vessel formation, thus playing likely an important role in slowing the growth of prostate cancer [Mattsson et al., 2008]. PSA is synthesized to high levels by normal and malignant prostate epithelial cells and, under pathological conditions, it is abundantly secreted in the extracellular compartments. For this reason, it is the main biomarker currently used for early diagnosis of prostate cancer. Therefore, serum levels of PSA are also useful to detect eventual recurrent forms and to follow up treatment response in not operable and metastatic tumors [Ilic et al., 2013]. As a whole, although PSA is currently used as a PCa biomarker, its role in the PCa pathobiology remains obscure [Williams et al., 2007].

1.2.2 – Clinical use of the PSA

The PSA can be found in the circulation in both free form (fPSA) and conjugated to inhibitors, such as α-1-antichymotrypsin (ACT) and the α-2-macroglobulin (αMG) (Fig. 5). The immunoassays commonly used in todays clinical practice are able to quantify both the fPSA fraction and the one linked to the ACT (tPSA), while they can not measure the PSA linked to the αMG [Shariat et al., 2011]. Although the PSA can be found in other

biological fluids (such as amniotic fluid, saliva and human milk), only the amount produced by the prostate can reach significant blood levels, so it can be considered a prostate specific marker [Kouriefs et al., 2009].
However it is important to remember that PSA serum levels can increase not only in course of a PCa, but also in many non-malignant diseases, such as BPH, infections and chronic inflammations [Pienta et al., 2009]. Generally PSA levels are considered pathological whenever they exceed 4 ng/ml in serum. However, a critical point is represented by the overlap between patients with organ-confined PCa and those with BPH, particularly for PSA values falling in the gray zone (i.e. 4-10 ng/ml) [Tamimi et al., 2010].

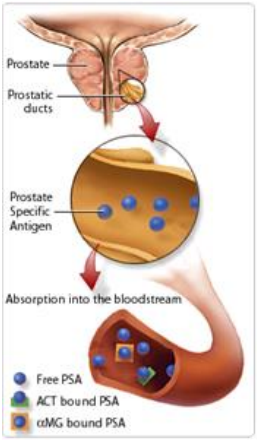

Figure 5. Different form of PSA in bloodstream. Kouriefs *et al*., 2009.

On the other side, it is important to observe that the 25-30% of patients with PCa show PSA values between 2.5 and 4 ng/ml [Hessels et al., 2009]. However, changing the PSA threshold is very risky, in fact, reducing the cutoff to 1.1 ng/ml, the 83.4% of PCa would be diagnosed, but the false positives would be the 61%. Conversely, with a threshold of 3.1 ng/ml, the test sensitivity and specificity would be 32% and 87%, respectively, while using a cutoff of 2.1 ng/ml they would be 53% and 73%, respectively. Today a threshold of 4 ng/ml should therefore be considered a conventional cutoff, characterized by a low predictive value, both negative and positive, no longer suitable for the decision to undergo a biopsy or not [Nogueira et al., 2010].
Attempting to improve the specificity of PSA test for the early diagnosis of PCa, some PSA-related parameters were used. The PSA velocity (Fig. 6): it is an index of the increasing rate of PSA over time and is obtained measuring the quantitative annual variation of PSA. This parameter is used to monitor patients with PSA levels in the gray zone. It was observed that in PCa the increase in PSA levels generally exceeds 0.75 ng/ml per year, or it undergoes an annual increase of 20% compared to baseline value. This parameter therefore represent an important diagnostic approach, but it requires careful standardization protocols before a possible routine use. To adopt this criterion, in fact, repeated PSA testing are necessary, for a minimum period of twelve months and preferably for several years. The inability to provide answers of clinical relevance in a short time is, therefore, the limit of this approach [Roobol et al., 2004].

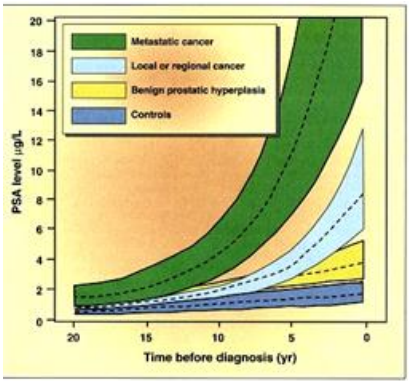

Figure 6. PSA velocity. Carter *et al.*, 2006.

The PSA ratio: it is a mathematical index calculated as fPSA/tPSA and it is also known as percentage of fPSA (%fPSA). For unknown reasons, patients with PCa tend to have a reduced amount of circulating fPSA compared to patients with a benign prostatic disease [Hoffman et al., 2000]. It was demonstrated that the PSA ratio reduces the number of unnecessary biopsies in subjects with tPSA between 4 and 10 ng/ml, but the optimal cutoff, even in this case, is not unanimously agreed [Pepe et al., 2010].
The introduction of PSA serum test in the clinical practice was an important step in the history of oncology; in fact before this test the two/third of the PCa were diagnosed only after metastasization. PSA mass screening improved early diagnosis of PCa, permitting more effective therapeutic interventions [Makarov et al., 2006].
In 2001, the American Cancer Society guidelines, suggested that men after 50 years old and with a normal risk of PCa should carry out an annual PSA and digital rectal testing, anticipating this timing in high-risk subjects. However in order to classify a screening procedure as acceptable it is necessary that its effectiveness, in terms of mortality reduction and cost/benefit, is confirmed by prospective and randomized studies. A large scale clinical trial questioning the real usefulness of PSA screening was conducted in Europe and produced, in 2009, some interesting data concerning the PSA impact on PCa-related mortality. The European Randomized Study of Screening for Prostate Cancer (ERSPC) started in the early 90s and enrolled, in seven European countries, a total of 182,000 individuals from 50 to 74 years old. This men underwent a PSA serum test every four years on average. After a mean follow-up of 9 years, the cumulative incidence of PCa was 8.2% for the PSA screened group and 4.8% in the control group, with a PCa-related mortality rate, between the first and the second group, of 0.80 ($p = 0.04$). The difference in the absolute death risk, instead, was found to be of 0.71 deaths per 1000 men, indicating

that 1408 people should be screened with PSA to prevent one case of PCa-related death. Authors concluded that beside a reduction of cancer-related mortality of about 20%, the PSA screening is associated to a high percentage of false positives [Schröder et al., 2009].
The potential benefits resulting from a screening program based on the determination of the serum PSA levels are, therefore, still unsure and not supported by clear evidence. The remarkable early diagnosis, the high number of false positives and the latent PCa treatment are, in fact, important negative effects of the PSA screening. Moreover this aspect should be taken together to the inability of this marker to discriminate between patients with aggressive PCa forms from those that are not intended to clinically manifest [Guidelines AIOM 2009].

1.3 – The new PSA-related biomarkers

1.3.1 – The prostate cancer gene 3 (PCA3)

In 1999, a gene specifically expressed in prostate cells was identified, using the differential display analysis, a technique that compares the expression profiles of mRNAs in the tumor tissue to the adjacent normal tissue [Bussemakers et al., 1999]. Using Northern blot analysis, the DD3 (differential display clone 3) was found to be significantly overexpressed in tumor tissue compared to normal tissue from the same patients. In particular, the median expression of this mRNA resulted to be 34 times higher in cancer cells than in normal cells [Hessels et al. , 2003]. According to the current nomenclature of the human genome, the gene was then renamed PCA3 (prostate cancer gene 3), in order to highlight its close relationship with PCa. Using a RT-PCR, it was shown that the PCA3 is a gene specifically expressed in the prostate resulting silent in the other human tissues, although, to date, it is not clear the role of this mRNA in prostate epithelial cells [Day et al., 2011]. PCA3 is a 25 kb gene located on chromosome 9q21-22 and it is composed by four exons. The molecular characterization of the PCA3 transcript revealed that alternative polyadenylation in three different positions of exon 4 could generate different transcripts. Furthermore, an alternative splicing event, may give a transcript in which exon 2 is totally deleted. The transcript that is found more frequently in prostate cells, however, contains exons 1, 3, 4a and 4b (Fig. 7).

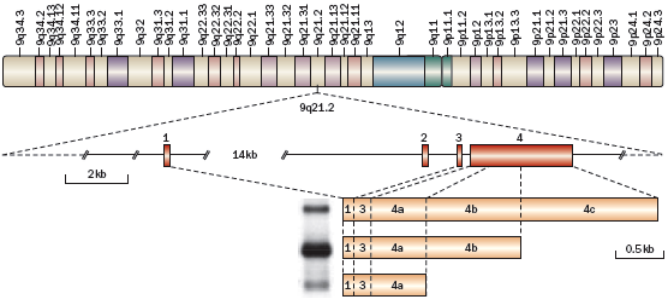

Figure 7. PCA3 gene and mRNAs. Bussemakers *et al*., 1999.

The absence of an Open Reading Frame (ORF) and the presence of stop codons which interrupt the protein structure, indicate that the PCA3 does not encode for a specific protein and that its transcript is not then translated [Bussemakers et al., 1999]. Even if the role of this gene is still unknown, it was proposed that its transcript could be implicated in gene expression or splicing regulation [Hessels et al., 2009]. Recently, it was demonstrated that the PCA3 gene is incorporated in the intron 6 of a second gene, BMCC1, implicated in the control of normal cells transformation into cancer cells [Clarke et al., 2009].

The association between PCA3 increased expression levels and PCa highlighted the potential of its mRNA as oncologic marker [Deras et al., 2008]. In 2006 a commercial kit, approved by the Food and Drug Administration (FDA), was produced in order to quantify the number of PCA3-mRNA copies in urine samples. This test is based on the technology of the transcription-mediated amplification (TMA) and it was called PROGENSA PCA3 assay (Gen-Probe Inc., San Diego, CA, USA). This test allows the quantification of the number of PSA-mRNA copies too, in order to obtained the PCA3 score calculated as PCA3-mRNA copies per ml/ PSA-mRNA copies per ml x 1000 [Groskopf et al., 2006]. The number of mRNA-PSA copies is an index of the amount of nuclear material derived from prostate cells in the urine sample, so the PCA3 score gives the expression of the PCA3 gene corrected for the amount of prostate cells in the sample, estimated through the evaluation of the mRNA-PSA copies. The cutoff for this test was set at 35, a value that seemed to give the balance in terms of sensitivity and specificity [Kouriefs et al., 2009]. To date, many studies have been performed and most of them showed how the PCA3 test represented a useful tool to predict PCa, but questions about the optimal cutoff and the ability of PCA3 to predict tumor aggressiveness still remain highly controversial [Day et al., 2011; Luo et al., 2014].

Several studies suggested that the threshold of 35 proposed by Gen-Probe Inc., using the PROGENSA PCA3 assay, could be modified, getting lower

or even higher, in a way that is probably dependent on the population features. In this respect, the cutoff value of 20 seems to increase the PCA3 test sensitivity without affecting the specificity [Hessels et al., 2003; Van Gils et al., 2008; Haese et al., 2008; Bollito et al., 2012; Filella et al., 2013; Gittelman et al., 2013]. Some studies also demonstrated that PCA3 is effective only after the first negative biopsy, but a recently published meta-analysis showed that PCA3 can be used for repeat biopsy to improve accuracy of PCa detection, since a large number of unnecessary biopsies can be avoided by using a PCA3 score cutoff of 20 [Fall et al., 2007; Luo et al., 2014].

The second debated aspect in which scientists focused in the last period concerns the possible association between the PCA3 score and the tumor stage. The PCA3 score is strongly associated to the fraction of cancer cells in the urine sample as a result of the DRE. In this view, larger and more aggressive tumors could release more easily a wider number of neoplastic cells respect to smaller and less aggressive PCa forms, producing higher values of PCA3 score [Hessels et al., 2010]. Many authors attempted to validate this hypothesis by evaluating the association between PCA3 score and tumor volume, measured after radical prostatectomy (RP), and other clinical and pathological PCa features, often reporting conflicting results [Van Gils et al, 2008]. From this point of view it is well known that subjects with organ-confined PCa and Gs $\geq$ 7 have a worst prognosis than those with Gs $\leq$ 6, even following RP or radiation therapy [Heidenreich et al., 2011; Albertsen et al., 2011; van den Bergh et al., 2014]. To recognize a low grade from a more aggressive PCa is therefore essential for therapeutic purposes, but currently the only way to discriminate patients with low or high grade PCa is to perform a biopsy. The possibility of using the PCA3 test as a prognostic marker is desirable, but the possibility to evaluate tumor aggressiveness by the PCA3 test is openly debated [Auprich et al., 2011; Haese et al., 2008; Filella et al., 2013; van Poppel et al., 2012; Hessels et al., 2010; Durand et al., 2012; Liss et al., 2011; Auprich et al., 2011; Nakanishi et al., 2008]. Indeed, the wide range of results obtained in previous studies may be due to different experimental conditions and may reflect the selected cohort features. In fact, the use of urine sediments or whole urine samples, collected before or without a previous DRE, can give rise to different results that are not often comparable in judging the prognostic value capabilities of the PCA3 test. On the other hand, the characteristics of the screened population could be important too. In fact, the choice to enroll only patients with a certain risk for PCa, or depending on the number of previous biopsies, can drive data towards an easier or less easy association between the result of the PCA3 test and the tumor aggressiveness.

1.3.2 – The [-2]proPSA

PSA is normally secreted from the prostatic epithelial cells as proPSA, an inactive proenzyme containing 244 amino acids. Once released into the prostate lumen, the 7-amino acid peptide is eliminated extracellularly by human kallikrein enzymes hK-2 and hK-4, becoming the active or mature form of PSA with 237 amino acids. The forms with some part of the peptide yet bound to them remain as proPSA (Fig. 8) [Mikolajczyk et al., 2001].

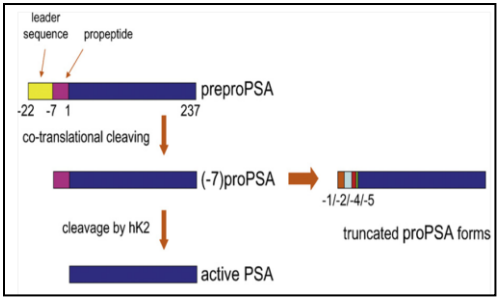

Figura 8. Activation of PSA and proPSA isoforms. Jansen et al., 2009

In proPSA, the smaller the part bound to the peptide in the leader region, the more difficult it is to activate. This makes the isoform of proPSA containing 2 residues in the leader region (the [-2]proPSA) the most stable component of proPSA in the serum. The [-2]proPSA (p2PSA) is produced much more in the periphery of the prostate, particularly under neoplastic conditions, so, although other proPSA isoforms maybe present in significant levels in serum samples, p2PSA appears to be more consistently correlated with PCa [Mikolajczyk et al., 2000]. In men with PSA levels between 6.0 and 24.0 ng/ml, the p2PSA fraction was found to be significantly higher in men with PCa and some prospective studies demonstrated that p2PSA better discriminated between PCa and benign disease compared to PSA and PSA ratio [Mikolajczyk et al., 2002].

Further studies evidenced that, among men with PSA levels between 2 and 10 ng/ml, a combination of p2PSA and fPSA, the so called percentage of p2PSA (%p2PSA = p2PSA/fPSA) was more cancer-specific than PSA and PSA ratio [Catalona et al., 2003]. Moreover, subsequent studies demonstrated a correlation between p2PSA levels and clinically significant cancer, including more advanced pathologic stage, higher tumor volume, and higher tumor grade [Catalona et al., 2004]. In addition, more recently Beckman Coulter Inc. has also developed a mathematical formula combining tPSA, fPSA and p2PSA: the Beckman Coulter prostate health index (PHI = (p2PSA/fPSA)× $tPSA^{1/2}$). This mathematical regression model was approved by the FDA in June 2012 and provided a better overall result for PCa discrimination in the tPSA range of 2-10 ng/ml [Le et al., 2010;

Guazzoni et al., 2011; Romero Otero et al., 2014]. In this light, the use of p2PSA, either incorporated in %p2PSA or PHI, provides superior discrimination between PCa and benign disease in men with tPSA levels of 2.5 to 10 ng/ml and negative DRE; however, confirmatory validation studies are needed to determine the optimal incorporation of this marker into clinical practice, as well as to definitively assess its ability in the identification of the most aggressive PCa forms.

1.3.3 – The Galectin 3

Galectin 3 (Gal3) is one of the proteins which can be cleaved by PSA. It is a unique chimera-type member of the galectin family, which contains a small N-terminal part, collagen-like sequence, and carbohydrate-binding domain similar to other galectins. Gal3 is the only member of the galectin family that can form oligomers through intermolecular interactions involving the collagen-like sequence [Hirabayashi et al., 1998; Barondes et al., 1994; Barondes et al., 1994]. The collagen-like sequence, rich in proline, tyrosine, and glycine residues contributes to self-aggregation [Lepur et al., 2012].

Until today, the three-dimensional structure of intact Gal3is unknown. However, the X-ray crystal structure of its carbohydrate recognition domain (CRD) was resolved, showing high similarity to the structure of CRD domains of other galectins [Seetharaman et al., 1998]. The unfolded structure of collagen-like sequence, which probably exhibits random-coil conformation, opens this sequence to different post-translational modifications, such as phosphorylation and cleavage by proteases, which in turn change the ability of Gal3 to create oligomers and change the localization in the cell.

Gal3 is mainly a cytosolic protein that often can be found in the nucleus and is secreted outside of the cell despite the fact that it lacks the classical leader signals at the N-terminal [Strik et al., 2001; Davidson et al., 2002; Yu et al., 2002]. Lack of Gal3 in knockout mice is associated with reduced mast cell function, reduced accumulation of asthma-associated leukocytes in airway inflammation and reduced peritoneal inflammatory responses. Endogenous Gal3 has also been shown to play a role in phagocytosis by macrophages and can mediate cytokine production by mast cells when functioning intracellularly [Cummings et al., 2009]. The fact that galectin-3 knockout mice do not show more drastic phenotypic changes leads to the assumption that other galectins can take over the role of Gal3. Most adult tissues without Gal3 do not show pathological changes; however, its role is more obvious in inflammatory responses, cell proliferation, motility, and apoptosis [Dumic et al., 2006].

This protein can be found in a wide variety of tissues as well as in blood. Experimental data available today demonstrate an association between Gal3 levels (in terms of up-regulation as well as down-regulation) and numerous

pathological conditions such as heart failure, infection with microorganisms, diabetes, and tumor progression [Chen et al., 2005; Takenaka et al., 2004; Shekhar et al., 2004; Puglisi et al., 2004; Califice et al., 2004; Balan et al., 2010; Newlaczyl et al., 2011]. Outside of the cell Gal3 is involved with a variety of extracellular functions such as cell adhesion, migration, invasion, angiogenesis, immune functions, apoptosis, and endocytosis [Ochieng et al., 2004; Nangia-Makker et al., 2008]. Experimental and clinical data demonstrate a correlation between galectin expression and tumor progression and metastasis, and therefore, galectins have the potential to serve as reliable tumor markers [Balan et al., 2010]. The expression of Gal3 in PCa is controversial. Previously published work demonstrated that expression of Gal3 was significantly decreased compared with normal and pre-malignant tissue [Araújo-Filho et al., 2013]. However, another study demonstrated an increased cleavage of Gal3 during the progression of PCa. This data implicate Gal3 in PCa progression and suggest that this protein may serve as both a diagnostic marker and a therapeutic target for future disease treatments [Wang et al., 2009]. To confirm this hypothesis, a recent work showed a significant increase of Gal3 serum levels in patients with metastatic PCa compared to normal patients [Balan et al., 2013]. It is therefore now essential to understand whether this marker can also discriminate PCa patients (even at an organ-confined tumor stage) and patients with benign prostatic pathologies, as well as it could work as a standalone marker or only in combination with the PSA, as the other previously screened biomarkers, resulting then only complementary and not substitutive of the PSA test.

1.4 – PCa treatment: the radical prostatectomy (RP)

Although some controversies remain over ideal diagnostic and treatment strategies for PCa, complete removal of the prostate remains the gold standard in the surgical management of localized disease. Hugh Hampton Young first described the perineal prostatectomy over 100 years ago in 1905 [Young, 1905]. Subsequently, the first retropubic radical prostatectomy (RRP) was performed by Millin in 1947 [Millin, 1947]. Anatomic studies in the 1970s and early 1980s led to improved appreciation of periprostatic features (dorsal venous complex, endopelvic fascia, autonomic innervation, and striated sphincter) to decrease morbidity of surgery and improve overall outcomes [Walsh, 1998; Bianco et al., 2005]. More recently, in 1997, Schuessler et al. described the first laparoscopic radical prostatectomy (LRP) reporting the feasibility of technique despite its association with long operative times [Schuessler et al., 1997]. Since that time, numerous European and US centers continued to improve and refine

technical aspects of the laparoscopic approach [Guillonneau et al., 1999; Touijer et al., 2005]. Several robotic systems were introduced around the turn of the century. The da Vinci system (Intuitive Surgical Inc, CA, USA) was first introduced in 1999. Following a merger with Computer Motion Inc. (AESOP and ZEUS systems) in 2003, Intuitive Surgical has become the sole producer of robotic surgical devices [Yates, et al., 2011]. After initially embarking into cardiothoracic surgery, the da Vinci robot found popularity within the urological community. From the initial descriptions of robot assisted laoaroscopic prostatectomy (RALP) in 2000 [Abbou et al., 2001; Binder et al., 2001], it has become widely adopted by urologists. By 2008, roughly 80% of RPs in the United States were performed robotically [Freire et al., 2010]. RALP has continued to evolve rapidly since that time with contributions including procedural step by steps, technical modifications, and outcomes data from various surgeons throughout the literature.

The surgical resection of a tumor in the absence of distal lymph node metastases is generally resolutive. However, some neoplasia, including PCa, are able to generate metastases after a few years, even if they are not detected at the moment of the surgery [Roato et al., 2008; Gupta et al., 2010; Pietras et al., 2010, Meyer et al., 2010; Roberts et al., 2013]. Accordingly, some investigations have suggested that a number of factors in the perioperative period could promote metastasization. These include the surgery approach and its associated stress response, the anaesthetic regimen, the acute pain and the administration of opioid analgesics, all of which could induce the liberation of angiogenic factors [Condon et al., 2004; Lee et al., 2009; Gottschalk et al., 2010; Mao et al., 2013]. One of the most affective factors seemed to be the anaesthetic regimen and this opened a wide debate regarding the use of a regional anaesthesia (RA) in place of the classic general anaesthesia (GA). In particular, RA, aside from reducing the amount of intra-operatively required GA and postoperative opioid consumption, has been consistently shown to attenuate the neuroendocrine response to surgery and, therefore, peri-operative immunosuppression [Melamed et al., 2003; Snyder et al., 2010; Mao et al., 2013]. Recent retrospective analyses indicate that RA for breast and prostate cancer surgery is associated with a markedly reduced risk of tumor recurrence and metastasization compared to systemic opioid administration [Exadaktylos et al., 2006; Biki et al., 2008]. This finding strengthens the hypothesis that paravertebral RA might reduce the incidence of metastases and recurrences compared to GA. Deepening these issues could be very important to understand if some anaesthetic regimens (e.g. GA and RA) or RP technique (e.g. LRP and RALP) could promote angiogenesis in vivo and if the administration of anti-angiogenic agents could be helpful in preventing the pro-metastasization events derived from the peri-operative manipulations.

1.5 - Aims of the thesis

Prostate cancer is the most common cancer in men of the Western countries [Siegel et al., 2013], but the molecular mechanisms of prostate cells neoplastic transformation are still unclear [Koul et al., 2010]. Despite the high incidence rate, an early diagnosis followed by surgery, radiotherapy or chemotherapy could be resolutive for this pathology [Roberts et al., 2013]. In this context, researches focused on the PCa pathogenic mechanisms, the evaluation of new PCa-related markers and less invasive PCa surgical methods, hoping to reach a positive impact on PCa-related recurrence and mortality [Kirby, 2014]. Early detection of PCa is currently based on the trans-rectal ultrasound scan, the digital rectal examination and the evaluation of the PSA serum levels [Romero et al., 2014]. PSA is a serine proteases that was found to be able to cleave a number of growth regulatory proteins that are important in cancer growth and survival, so it was related to tumor growth and metastasis dissemination [Ishii et al., 2004]. PSA serum level, on the other side, has been used for several decades as the only PCa-related marker, but in the last years clear limits for this test were demonstrated [Schröder et al., 2009; Crawford et al., 2011]. New PCa-related biomarkers have been intensively scrutinized over the last decade, but despite the new findings and the disclosure of some positive diagnostic performances, they are currently not uniformly accepted in clinical practice [Romero et al., 2014]. Even if the PSA serum test is no longer considered a helpful tool for PCa early diagnosis it is still a fundamental marker of PCa recurrence after surgical prostate removal [Carthon et al., 2013]. At the same time, a debate on whether some surgery approaches or anaesthetic drugs in PCa patient management could increase the risk of tumor spreading and metastasization was recently opened [Lee et al., 2009; Mao et al., 2013].

In light of these considerations, the present thesis aims to approach the PCa pathology from different points of view, trying to *i)* reveal new insights for the catalytic mechanism of PSA, *ii)* evaluate the PSA clinical utility as a diagnostic test, as well as the diagnostic and prognostic potential of new PSA-related biomarkers such as PCA3, p2PSA and Gal3, *iii)* to investigate changes in activation markers of the haemostatic system, endothelium and angiogenesis in patients with PCa undergoing different laparoscopic radical prostatectomy protocol and intra-operative anaesthetic regimens.

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Papers introduction

Prostate-specific antigen (PSA) is a serine protease belonging to the kallikrein family and it is also known as kallikrein-related peptidase 3 (KLK3) [Williams et al., 2007]. The major physiologic substrates for PSA appear to be the gel-forming proteins in freshly ejaculated semen, semenogelin I (SgI) and semenogelin II (SgII) which are synthesized and secreted by the seminal vesicles [Malm et al., 2000]. PSA can also cleave a number of growth regulatory proteins that are important in cancer development and survival as IGFBP, PTH-related protein, latent TGF-β2, and extracellular matrix components fibronectin and laminin [Webber et al., 1995]. Part of this thesis is dedicated to deepening the catalytic activity of PSA in order to clarify the hypothetical role of this enzyme in prostate cancer (PCa) progression and metastasization. In this light, the steady state and pre-steady state kinetics of the PSA-catalyzed hydrolysis of a fluorogenic substrate (Mu-His-Ser-Ser-Lys-Leu-Gln-AMC) has been determined between pH 6.5 and 9.0 at 37°C temperature. The pH-dependence of the enzymatic steps (i.e., acylation and deacylation) has been separately characterized, allowing the determination of pKa values. On this basis, possible residues which might regulate these steps, by interacting with the two portions of the substrate in PSA, were identified.

Consequently to a prostate tissue damage, large amount of PSA could be released in bloodstream and this explain the extensive clinical application of PSA in clinical practice as a PCa-related marker [Kouriefs et al., 2009]. Although the routine use of serum PSA testing has undoubtedly increased PCa detection, one of its main drawbacks is represented by its low specificity [Hessels et al., 2009]. High levels of PSA, in fact, can be found not only in malignant but also in benign prostatic pathologies, such as prostatitis and, first of all, in benign prostatic hyperplasia (BPH) [Tamimi et al., 2010]. This limitation contributes to the ongoing debate regarding the actual benefit of the PSA population-based screening for PCa detection [Schröder et al., 2009]. In this view several new PCa biomarkers have been intensively scrutinized over the last decade, but despite new findings and good performance characteristics, they are currently not uniformly accepted in clinical practice [Ilic et al., 2013]. In this thesis the diagnostic performances of one of this marker, the prostate cancer gene 3 (PCA3), are discussed in both term of its diagnostic and prognostic potential. A cohort of 407 high risk PCa men, with at least a previous negative biopsy and two or more risk factors for PCa, were tested for PSA serum test and PCA3 urine test before to undergo a further prostatic biopsy, to confirm or not the absence of a PCa. The 48% of patients (n=195) were found positive for PCa and were characterized for tumor aggressiveness with the evaluation of the Gleason score (Gs). Following the different distribution of PSA and PCA3

in patients with and without PCa, the diagnostic performances of these two tests were evaluated. In particular an association between the PCA3 score values and the probability to have a PCa was evaluated, as well as the ability of the PCA3 score to identify, between prostate cancers, the less significant forms that may directly enter the active surveillance protocols, lowering the economic effort for PCa diagnosis supported from public health.

Attempting to improve the specificity of the PSA serum test for the early diagnosis of PCa, the clinical utility of other PSA forms were evaluated [Pepe et al., 2010]. It is well known that PSA is normally secreted from the prostatic epithelial cells as proPSA, an inactive proenzyme containing 244 amino acids. However, different forms of proPSA exist, depending on the length of the leader region. The isoform containing two residues, the so called [-2]proPSA, is the most stable component of proPSA in the serum [Mikolajczyk et al., 2001]; moreover, the [-2]proPSA (p2PSA) is produced much more in the periphery of the prostate, particularly under neoplastic conditions, so, although other proPSA isoforms maybe present in significant levels in serum samples, p2PSA appears to be more consistently correlated with PCa [Mikolajczyk et al., 2003]. In this view, the present thesis aimed to assess the clinical utility of the p2PSA serum test, as well as the diagnostic and prognostic performances of another potential PCa-related serum biomarker, the Galectin 3 (Gal3). This is an extra- and intra-cellular β-galactoside-binding protein that has been found to be under-expressed in PCa tissue as well as at higher level in the serum of PCa patients [Balan et al., 2013]. Following the different distribution of PSA, p2PSA and Gal3 in patients with PCa and BPH, the diagnostic performances of these three tests were evaluated, both considered as stand-alone markers or after their combination to calculate indices such as the PSA ratio (fPSA/tPSA), the percentage of p2PSA (p2PSA/fPSA), the prostate health index ($p2PSA/fPSA*tPSA^{1/2}$) and a novel index of our creation, the Galphi (Gal3*tPSA/fPSA). Also in this case a possible association between the different parameters and tumor aggressiveness, expressed in terms of Gs, was determined.

Once that a PCa is diagnosed and characterized in its histopathological features, a treatment approach must be planned. The first line treatment of organ-confined PCa involves, generally, a surgical resection of the prostate. The first described radical prostatectomy (RP) was performed over 100 years ago, in 1905, by Hugh Hampton Young and since that moment it remained the gold standard in the surgical management of localized PCa [Freire et al., 2010]. More recently, in 1997, Schuessler described the first laparoscopic radical prostatectomy (LRP) and since that time numerous European and US centers tried to improve and refine technical aspects of the laparoscopic approach, introducing, for example, several robotic systems such as the “da Vinci” system. After initially embarking into

cardiothoracic surgery, the “da Vinci” robot found popularity within the urological community and it is now used for robot assisted laoaroscopic prostatectomy (RALP) [Abbou et al., 2001]. In the last period, some investigations suggested that a number of factors in the perioperative period could promote metastasization. These include the surgery approach and its associated stress response, the anaesthetic regimen, the acute pain, and the administration of opioid analgesics [Mao et al., 2013]. The hypothesis, in fact, is that different anesthetic protocols and surgery techniques can differently activate the clotting system, or stimulate mononuclear cells, platelets and endothelial cells. The consequent formation of a fibrin matrix, together with cell activation, appear to promote tumor growth and neo-angiogenic processes [Falanga et al., 2013]. Part of this thesis is therefore dedicated to describe the effects on coagulation and platelet-activation markers of two established types of anaesthesia in 102 patients with primary PCa undergoing LRP or RALP. In particular, before the induction of anaesthesia (T0), 1 hr post-surgery (T1) and 24 hrs post-surgery (T2) plasma levels of fibrinogen, thrombin-antithrombin complex (TAT), prothrombin fragment 1+2 (PF12), factor VIII (FVIII), plasminogen-activator inhibitor (PAI-1), D-dimer (DD), p-selectin, anti-thrombin (AT), protein C (PC) and protein S (PS) were evaluated. In this light, perioperative variations of these parameters were followed in order to highlight the pro-thrombotic properties of different anesthetic protocols and surgery techniques during the treatment of PCa patients, trying to assess which manipulation could higher the risk of further complications.

Another pathway that could affect the tumor evolution is represented by the angiogenic process [Gupta et al., 2010]. This is a highly complex and dynamic event, regulated by a number of pro- and anti-angiogenic molecules. Surely one of the major pathways involved in this process is represented by the vascular endothelial growth factor (VEGF) family [Roberts et al., 2013]. At the same time, the von Willebrand factor (vWf), a pro-coagulant multimeric plasma protein synthesized by endothelial cells and megakaryocytes, was recently recognized as an anti-angiogenic and pro-apoptotic molecules, involved in the modulation of neo-vascularization processes and interacting with the same VEGF pathway [Franchini et al., 2013]. In the last period, some investigations have suggested that a number of factors in the perioperative period could promote metastasization. These include surgery and its associated stress response, anaesthesia, acute pain and opioid analgesics, all of which could induce the liberation of angiogenic factors [Mao et al., 2013]. Deepening these issues could be very important to understand if some anaesthetic drugs or surgey techniques could promote angiogenesis *in vivo*. In view of these opened questions, part of this thesis aims to investigate whether a cohort of PCa patients (n=87), undergoing conventional laparoscopic radical prostatectomy (LRP) or robot assisted laparoscopic prostatectomy (RALP), with two different intra-operative

anaesthetic regimens, total intravenous anesthesia with target-controlled infusion (TIVA-TCI) and balanced inhalation anaesthesia (BAL), showed different changes in plasma VEGF and plasma vWf antigen levels during the peri-operative period. In particular this evaluation aims to understand whether different anesthetic protocols and surgery techniques during the treatment of PCa patients can be associated to higher the tumor progression risk, as well as to reveal if VEGF and vWf showed synergic or opposite effects in the regulation of angiogenic processes.

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Papers

Characterization of the prostate-specific antigen (PSA) catalytic mechanism: a pre-steady-state and steady-state study

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Characterization of the Prostate-Specific Antigen (PSA) Catalytic Mechanism: A Pre-Steady-State and Steady-State Study

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Abstract

Prostate-specific antigen (PSA), an enzyme of 30 kDa grouped in the kallikrein family is synthesized to high levels by normal and malignant prostate epithelial cells. Therefore, it is the main biomarker currently used for early diagnosis of prostate cancer. Here, presteady-state and steady-state kinetics of the PSA-catalyzed hydrolysis of the fluorogenic substrate Mu-His-Ser-Ser-Lys-Leu-Gln-AMC (spanning from pH 6.5 to pH 9.0, at 37.0°C) are reported. Steady-state kinetics display at every pH value a peculiar feature, represented by an initial "burst" phase of the fluorescence signal before steady-state conditions are taking place. This behavior, which has been already observed in other members of the kallikrein family, suggests the occurrence of a proteolytic mechanism wherefore the acylation step is faster than the deacylation process. This feature allows to detect the acyl intermediate, where the newly formed *C*-terminal carboxylic acid of the cleaved substrate forms an ester bond with the -OH group of the Ser195 catalytic residue, whereas the AMC product has been already released. Therefore, the pH-dependence of the two enzymatic steps (*i.e.*, acylation and deacylation) has been separately characterized, allowing the determination of pK_a values. On this basis, possible residues are tentatively identified in PSA, which might regulate these two steps by interacting with the two portions of the substrate.

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Introduction

Prostate-specific antigen (PSA), an enzyme of 30 kDa grouped in the kallikrein family and also known as kallikrein-related peptidase 3 (KLK3) [1], is synthesized to high levels by normal and malignant prostate epithelial cells and, under pathological conditions, it is abundantly secreted in the extracellular compartments. For this reason, it is the main biomarker currently used for early diagnosis of prostate cancer. Therefore, serum levels of PSA are also useful to detect eventual recurrent forms and to follow up treatment response in not operable and metastatic tumors [2].

Like all other members of the kallikrein family, PSA is a serine protease that is synthesized in an inactive form as a zymogen which is composed of a pre-peptide (also known as signal peptide) and a pro-peptide (which maintains the enzyme in the latent form). Inside the epithelial cell, the 17 amino acid pre-sequence is first cleaved off by signal peptidases. Afterwards, in the extracellular environment, the additional 7 amino acid pro-sequence is removed by human kallikrein 2 (hK2) [3]. PSA shows a conserved position of the Asp102/His57/Ser195 catalytic triad [4] (see Fig. 1). However, unlike most of kallikreins, which display a trypsin-like proteolytic specificity (*i.e.*, they cleave on the carboxyl side of a positively charged amino acid residue, namely Arg and Lys), PSA shows instead a chymotrypsin-like substrate specificity (*i.e.*, it cleaves on the carboxyl side of a hydrophobic amino acid residue, namely Tyr, Phe, Trp, and Leu). In addition, PSA is the only member of the kallikrein family that catalyzes the cleavage of substrates displaying the Gln residue at the P_1 position [5].

Prostate cancer can increase the amount of PSA released into the blood stream, even though serum PSA is kept inactive in a variety of different forms. As a matter of fact, serum PSA falls into two general categories, namely: (*i*) free PSA, which includes all the unbound zymogen forms, and (*ii*) complexed PSA, where also active forms are kept latent through the binding of serum protease inhibitors. Notably, PSA present in the extracellular fluid, surrounding prostate epithelial cells, has been reported to be enzymatically active, suggesting that its proteolytic activity plays a role in the physiopathology of prostate cancer [6].

The most important physiological substrates for PSA have been proposed to be semenogelin I (SgI) and semenogelin II (SgII). These proteins are synthesized and secreted by the seminal vesicles in spermatic fluid and are involved in the formation of a gel matrix

A

```
PSA       ----------------IVGGWECEKHSQPWQVLVA---SRGRAVCGGVLVHPQWVLTAAHC  42
KLK1      ----------------IVGGWECEQHSQPWQAALY---HFSTFQCGGILVHRQWVLTAAHC  42
KLK2      ----------------IVGGWECEKHSQPWQVAVY---SHGWAHCGGVLVHPQWVLTAAHC  42
KLK4      ----------------IINGEDCSPHSQPWQAALV---MENELFCSGVLVHPQWVLSAAHC  42
KLK6      ----------------LVHGGPCDKTSHPYQAALY---TSGHLLCGGVLIHPLWVLTAAHC  42
KLK7      ----------------IIDGAPCARGSHPWQVALL---SGNQLHCGGVLVNERWVLTAAHC  42
HPK       ----------------IVGGTNSSWGEWPWQVSLQVKLTAQRHLCGGSLIGHQWVLTAAHC  45
                             :: *  .   . *:*. :             *.* *:   ***:****
BCTRP     CGVPAIQPVLSGLARIVNGEDAVPGSWPWQVSLQ--DSTGFHFCGGSLISEDWVVTAAHC  58

PSA       IRN-----KSV-ILLGRHSLFHPED-TGQVFQVSHSFPHPLYDMSLLKNRFLRPGDDSSH  95
KLK1      ISD-----NYQ-LWLGRHNLFDDEN-TAQFVHVSESFPHPGFNMSLLENHTRQADEDYSH  95
KLK2      LKK-----NSQ-VWLGRHNLFEPED-TGQRVPVSHSFPHPLYNMSLLKHQSLRPDEDSSH  95
KLK4      FQN-----SYT-IGLGLHSLEADQEPGSQMVEASLSVRHPEYNRP----------LLAN  85
KLK6      KKP-----NLQ-VFLGKHNLRQRES-SQEQSSVVRAVIHPDYDAA----------SHDQ  84
KLK7      KWN-----EYT-VHLGSDTLGDR---RAQRIKASKSFRHPGYSTQ----------THVN  82
HPK       FDGLPLQDVWR-IYSGILNLSDITK-DTPFSQIKEIIIHQNYKVS----------EGNH  92
                                 * .*          * :.
BCTRP     GVT-----TSDVVVAGEFDQGLET-EDTQVLKIGKVFKNPKFSIL----------TVRN  101

PSA       LMLLRLSEPAE-LTDAVKVMDLPTQE--PALGTTCYASGWGSIEPEEFLTPKKLQCVDL  152
KLK1      LMLLRLTEPADTITDAVKVVELPTQE--PEVGSTCLASGWGSIEPENFSFPDDLQCVDL  153
KLK2      LMLLRLSEPAK-ITDVVKVLGLPTQE--PALGTTCYASGWGSIEPEEFLRPRSLQCVSL  152
KLK4      LMLIKLDESVS-ESDTIRSISIASQC--PTAGNSCLVSGWGLLAN--GRMPTVLQCVNV  140
KLK6      IMLLRLARPAK-LSELIQPLPLERDC--SANTTSCHILGWGKTADGD--FPDTIQCAYI  139
KLK7      LMLVKLNSQAR-LSSMVKKVRLPSRC--EPPGTTCIVSGWGTTTSPDVTFPSDLMCVDV  139
HPK       IALIKLQAPLN-YTEFQKPICLPSKGDTSTIYTNCWVTGWGFSKE-KGEIQNILQKVNI  150
          *. *:.*          :.  : : :  :          .*  ***             :  :
BCTRP     ITLLKLATPAQ-FSETVSAVCLPSADEDFPAGMLCATTGWGKTKYNALKTPDKLQQATL  160

PSA       HVISNDVCAQVHPQ-KVTKFMLCAGRWTGGKSTCSGDSGGPLVCNG----VLQGITSWGS  207
KLK1      KILPNDECKKVHVQ-KVTDFMLCVGHLEGGKDTCVGDSGGPLMCDG----VLQGVTSWGY  208
KLK2      HLLSNDMCARAYSE-KVTEFMLCAGLWTGGKDTCGGDSGGPLVCNG----VLQGITSWGP  207
KLK4      SVVSEEVCSKLYDP-LYHPSMFCAGGGQDQKDSCNGDSGGPLICNG----YLQGLVSFGK  195
KLK6      HLVSREECEHAYPG-QITQNMLCAGDEKYGKDSCQGDSGGPLVCGD----HLRGLVSWGN  194
KLK7      KLISPQDCTKVYKD-LLENSMLCAGIPDSKRNACNGDSGGPLVCRG----TLQGLVSWGT  194
HPK       FLVTNEECQKRYQDYKITQNMVCAGYKEGGKDACKGDSGGPLVCKNNGMWRLVGITSWGE  210
          :: : * : :          *.*.*    *.:* *******:*     * *:.*:*
BCTRP     PIVSNTDCRKYWGS-RVTDVMICAGA--SGVSSCMGDSGGPLVCQKNGAWTLAGIVSWGS  217

PSA       EPCALPERPSLYTKVVHYRKWIKDTIVANP--------  237
KLK1      VPCGTPNKPSVAVRVLSYVKWIEDTIAENS--------  238
KLK2      EPCALPEKPAVYTKVVHYRKWIKDTIAANP--------  237
KLK4      APCGQVGVPGVYINLCKFIEWIEKTVQAS--------  224
KLK6      IPCGSKEKPGVYTNVCRYTNWIQKTIQAK--------  223
KLK7      FPCGQPNDPGVYTQVCKFTNWINDTMKKHR--------  224
HPK       -GCARREQPGVYTKVAEYMDWILEKTQSSDGKAQMQSPA  248
            *.   *.: ..:  : .** ..
BCTRP     STCST-STPAVYARVTALMPWVQETLAAN--------  245
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B

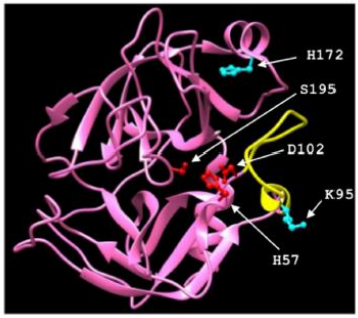

Figure 1. Sequence alignment of human kallikreins (panel A) and three-dimensional structure of PSA (panel B). Sequence alignment (panel A) is built with those human kallikreins for which the three-dimensional structure is available at the Protein Data Bank. The protein sequences were obtained from the NCBI database (http://www.ncbi.nlm-nih.gov). The progressive multiple alignment of PSA (also named kallikrein 3; NCBI entry number: CAD30845.1), kallikrein 1 (also named tissue kallikrein; KLK1; NCBI entry number: AAH05313.1), kallikrein 2 (KLK2; NCBI entry number: AAF08276.1), kallikrein 4 (KLK4; NCBI entry number: AAD38019.1), kallikrein 6 (KLK6; NCBI entry number: AAP35498.1), kallikrein 7 (KLK7; NCBI entry number: NP_644806.1), and human plasma kallikrein (HPK; NCBI entry number: AAF79940.1) was performed by the Clustal-Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo). Only the trypsin-like serine protease domain of HPK has been aligned. The "*" symbol means that the residues are identical in all the aligned sequences; the ":" symbol indicate conserved substitutions, and the "." symbol means semi-conserved substitutions. The amino acid sequence of bovine chymotrypsinogen (BCTRP; NCBI entry number: 681083A) has been reported as the template. Three-dimensional structure of PSA (panel B). In both panels, the image was produced using UCSF Chimera molecular graphics package [26]. The "kallikrein loop" is in yellow [24,27,28], amino acid residues forming the catalytic triad are in red, and amino acid residues affecting the pH dependence of the catalytic parameters are in cyan.
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that wraps around ejaculated spermatozoa, preventing their functionalization (mainly via inhibition of reactive oxygen species) [7]. The gel matrix breaks down under the PSA enzymatic action, facilitating the spermatozoa movements [8]. PSA cleaves preferentially the Tyr-Glu peptide bonds and generates multiple soluble fragments of SgI and SgII [9] that seem to be the main antibacterial components in human seminal plasma [10]. These findings, together with the ability of PSA to process a number of growth regulatory proteins that are important in cancer growth and survival (such as Insulin-like growth factor binding protein, Parathyroid hormone-related protein, latent Transforming growth factor-beta 2 as well as extracellular matrix components, like fibronectin and laminin) [11-14], indeed suggest that PSA can facilitate tumor growth and metastasis dissemination [3,15,16]. On the other hand, PSA has been reported to slow down blood vessel formation, thus playing likely an important role in slowing the growth of prostate cancer [17]. As a whole, although currently PSA is a biomarker, its role in the pathobiology of prostate cancer remains obscure [3].

In view of the PSA importance both from the physiological and the pathological viewpoints, the present study is focused on insights into the catalytic mechanism of PSA. In particular, it has been investigated the PSA-catalyzed hydrolysis of the fluorogenic substrate Mu-His-Ser-Ser-Lys-Leu-Gln-AMC (Mu-HSSKLQ-AMC), a PSA-specific substrate designed on the basis of a PSA cleavage map for SgI and SgII [18]. Under pre-steady-state and steady-state conditions, the release of the Mu-HSSKLQ product (*i.e.*, the deacylation process) is the rate-limiting step of catalysis. The independent analysis of the pH dependence of the acylation and deacylation steps allows to determine the pK_a values of residues involved in the modulation of the proteolytic activity.

Materials and Methods

PSA (pure grade >96%), obtained from seminal fluid, was purchased by SunnyLab (SCIPAC Ltd, Sittingbourne, UK). The highly-specific PSA fluorogenic substrate Mu-HSSKLQ-AMC (purity >97%) was purchased from Sigma-Aldrich (Buchs, Switzerland).

The PSA-catalyzed hydrolysis of Mu-HSSKLQ-AMC was monitored spectrofluorimetrically at 460 nm with a Cary Eclipe spectrofluorimeter (Varian, Palo Alto, Ca, USA). The excitation wavelength was 380 nm with a slit bandwidth of 5 nm. The Mu-HSSKLQ-AMC concentration ranged between 5 and 70 μM, whereas the PSA concentration was 50 nM for all determinations. The PSA-catalyzed hydrolysis of Mu-HSSKLQ-AMC was investigated between pH 6.5 and 9.0 using the following buffers: 25 mM bis-tris-HCl and 25 mM tris-HCl, in the presence of 100 mM NaCl, 10 mM $CaCl_2$, and 0.05% Brij (a nonionic detergent). All measurements were performed at 37.0°C.

Determination of kinetic parameters

The pre steady-state and steady-state parameters for the PSA-catalyzed hydrolysis of Mu-HSSKLQ-AMC were analyzed within the framework of the minimum three-step mechanism depicted by Figure 1: where E is the enzyme (*i.e.*, PSA), S is the fluorogenic peptide substrate (*i.e.*, Mu-HSSKLQ-AMC), ES is the enzyme-substrate complex, EP is the acyl intermediate, P_1 is AMC, P_2 is Mu-HSSKLQ, K_s is the fast pre-equilibrium constant (reflecting

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E + P_2 + P_1$$

Figure 2. Minimum three-step mechanism underlying the pre steady-state and steady-state parameters for the PSA-catalyzed hydrolysis of Mu-HSSKLQ-AMC.
doi:10.1371/journal.pone.0102470.g002

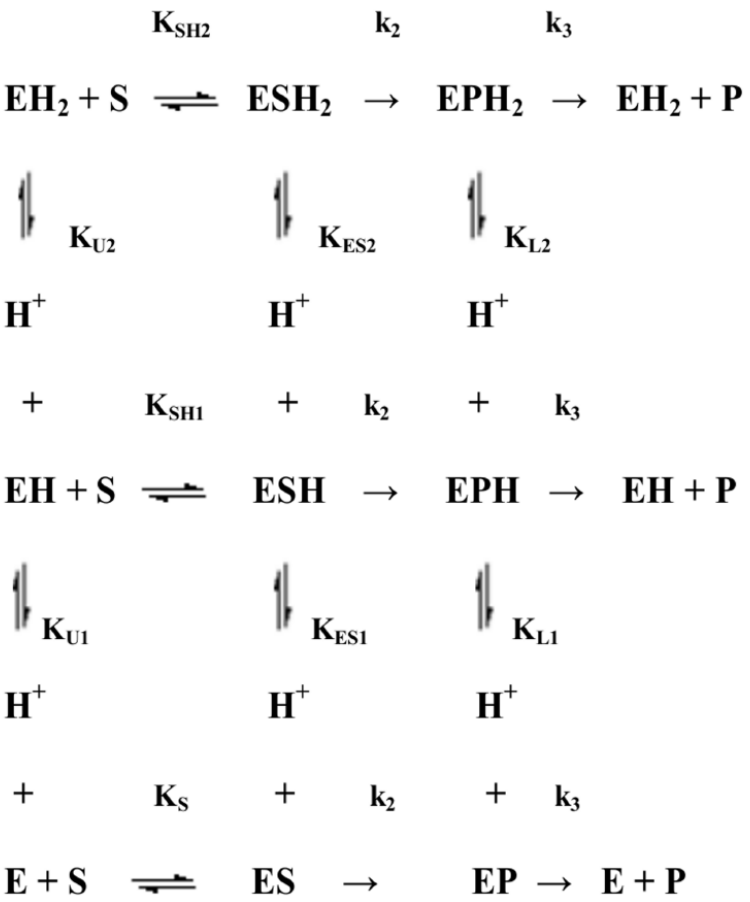

Figure 3. Minimum reaction mechanism for the pH dependence of pre-steady-state and steady-state parameters.
doi:10.1371/journal.pone.0102470.g003

the actual substrate affinity for the enzyme), k_2 is the acylation rate constant, and k_3 is the deacylation rate constant [19].

Since the fluorescence spectroscopic change is associated to the P_i release, the enzymatic mechanism described in Figure 2 results in a biphasic kinetic pattern whenever $k_3 < k_2$ [19]. Therefore, P_i release has been analyzed according to Eqn 1

$$[P_i] = \pi_0 \cdot (1 - e^{-k \cdot t}) + v \cdot t \quad (1)$$

where π_0 is the amplitude of the initial fast pre-steady-state phase

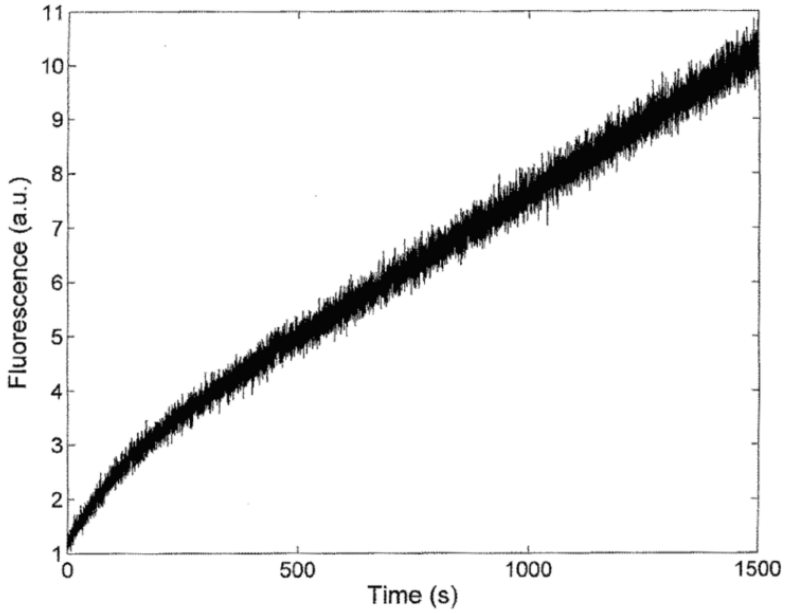

Figure 4. Time course of the PSA-catalyzed hydrolysis of Mu-HSSKLQ-AMC. Observation wavelength = 460 nm, pH = 7.5 and temperature = 37.0°C. The concentration of PSA was 50 nM. The concentration of Mu-HSSKLQ-AMC was 5 μM.
doi:10.1371/journal.pone.0102470.g004

(also known as the "burst"), k is the apparent rate constant of the initial fast pre-steady-state phase, v indicates the subsequent slow steady-state process, and t is the time.

The initial fast pre-steady-state kinetics (see Eqn. 1) was analyzed according to Eqns 2 and 3 [20]:

$$\pi = [E]\cdot\left\{\frac{k_2\cdot[S]}{(k_2+k_3)\cdot(K_m+[S])}\right\}^2 \quad (2)$$

and

$$k = \frac{k_2\cdot[S]}{K_s+[S]} + k_3 \quad (3)$$

The analysis of kinetics according to Eqns. (2) and (3) allowed to determine the actual concentration of active PSA (*i.e.*, [E]) and values of K_s, k_2, and k_3.

The subsequent slow steady-state kinetics (see Eqn. 1) was analyzed according to Eqn. 4:

$$v = \frac{k_{cat}\cdot[E]\cdot[S]}{K_m+[S]} \quad (4)$$

where k_{cat} is the catalytic constant (corresponding to the rate-limiting step), K_m is the Michaelis constant, and [E] and [S] are the enzyme and substrate concentrations, respectively.

Of note, the steady-state parameters k_{cat} and K_m are related to the pre-steady-state parameters K_s, k_2, and k_3 according to Eqns 5 and 6:

$$k_{cat} = \frac{k_2\cdot k_3}{k_2+k_3} \quad (5)$$

and

$$K_m = \frac{K_s\cdot k_3}{k_2+k_3} \quad (6)$$

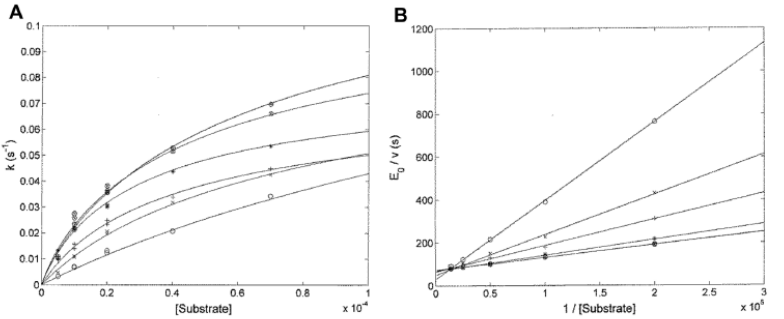

Figure 5. Dependence of *k* (panel A) and *v* (panel B) on the substrate concentration for the PSA-catalyzed hydrolysis of Mu-HSSKLQ-AMC. The continuous lines fitting the data reported in panels A and B were obtained according to Eqns. 3 and 4, respectively, with values of k_2, k_3, and K_s (panel A), and of k_{cat} and K_m (panel B) reported in Table 1. Values of pre-steady-state and steady-state parameters were obtained at pH 6.5 (o), pH 7.0 (x), pH 7.5 (+), pH 8.0 (*), pH 8.5 (▵), and pH 9.0 (⊕) at a temperature of 37.0°C.
doi:10.1371/journal.pone.0102470.g005

The pH dependence of pre-steady-state and steady-state parameters was analyzed in the framework of the minimum reaction mechanism depicted in Figure 3 [21,22], where two protonating residues are involved, according to Eqns. 7-12:

$$^{obs}k_{cat} = {}^{0}k_{cat}\cdot\frac{1}{P_L} + {}^{1}k_{cat}\cdot\frac{K_{L1}\cdot[H^+]}{P_L} + {}^{2}k_{cat}\cdot\frac{K_{L1}\cdot K_{L2}\cdot[H^+]^2}{P_L} \quad (7)$$

$$^{obs}k_2 = {}^{0}k_2\cdot\frac{1}{P_{ES}} + {}^{1}k_2\cdot\frac{K_{ES1}\cdot[H^+]}{P_{ES}} + {}^{2}k_2\cdot\frac{K_{ES1}\cdot K_{ES2}\cdot[H^+]^2}{P_{ES}} \quad (8)$$

$$^{obs}k_3 = {}^{0}k_3\cdot\frac{1}{P_L} + {}^{1}k_3\cdot\frac{K_{L1}\cdot[H^+]}{P_L} + {}^{2}k_3\cdot\frac{K_{L1}\cdot K_{L2}\cdot[H^+]^2}{P_L} \quad (9)$$

$$^{obs}K_m = {}^{0}K_m\cdot\frac{1+K_{U1}\cdot[H^+]+K_{U1}\cdot K_{U2}\cdot[H^+]^2}{1+K_{L1}\cdot[H^+]+K_{L1}\cdot K_{L2}\cdot[H^+]^2} \quad (10)$$

$$^{obs}K_s = {}^{0}K_s\cdot\frac{1+K_{U1}\cdot[H^+]+K_{U1}\cdot K_{U2}\cdot[H^+]^2}{1+K_{ES1}\cdot[H^+]+K_{ES1}\cdot K_{ES2}\cdot[H^+]^2} \quad (11)$$

$$^{obs}(k_{cat}/K_m) = {}^{0}(k_{cat}/K_m)\cdot\frac{1}{P_U} + {}^{1}(k_{cat}/K_m)\cdot\frac{K_{U1}\cdot[H^+]}{P_U} + {}^{2}(k_{cat}/K_m)\cdot\frac{K_{U1}\cdot K_{U2}\cdot[H^+]^2}{P_U} \quad (12)$$

where

Table 1. Different parameters at various pH values, as obtained from the analysis of steady-state kinetics according to Eq. (1c) and of pre-steady-state kinetics according to Eq. (1d).

pH	k_{cat} (s^{-1})	K_m (M)	k_2 (s^{-1})	k_3 (s^{-1})	K_s (M)
6.5	$3.4(\pm0.5)\times10^{-2}$	$1.3(\pm0.3)\times10^{-4}$	$1.3(\pm0.3)\times10^{-1}$	$4.7(\pm0.6)\times10^{-2}$	$4.9(\pm0.6)\times10^{-4}$
7.0	$2.0(\pm0.3)\times10^{-2}$	$3.8(\pm0.5)\times10^{-5}$	$6.6(\pm0.9)\times10^{-2}$	$2.9(\pm0.5)\times10^{-2}$	$1.2(\pm0.3)\times10^{-4}$
7.5	$1.5(\pm0.3)\times10^{-2}$	$1.9(\pm0.3)\times10^{-5}$	$5.1(\pm0.7)\times10^{-2}$	$2.2(\pm0.4)\times10^{-2}$	$6.2(\pm0.8)\times10^{-5}$
8.0	$1.4(\pm0.3)\times10^{-2}$	$1.1(\pm0.2)\times10^{-5}$	$5.9(\pm0.9)\times10^{-2}$	$1.9(\pm0.3)\times10^{-2}$	$4.2(\pm0.7)\times10^{-5}$
8.5	$1.4(\pm0.3)\times10^{-2}$	$8.4(\pm1.1)\times10^{-6}$	$9.1(\pm1.7)\times10^{-2}$	$1.6(\pm0.3)\times10^{-2}$	$5.5(\pm0.9)\times10^{-5}$
9.0	$1.4(\pm0.2)\times10^{-2}$	$8.3(\pm1.0)\times10^{-6}$	$1.1(\pm0.2)\times10^{-1}$	$1.6(\pm0.3)\times10^{-2}$	$7.5(\pm1.0)\times10^{-5}$

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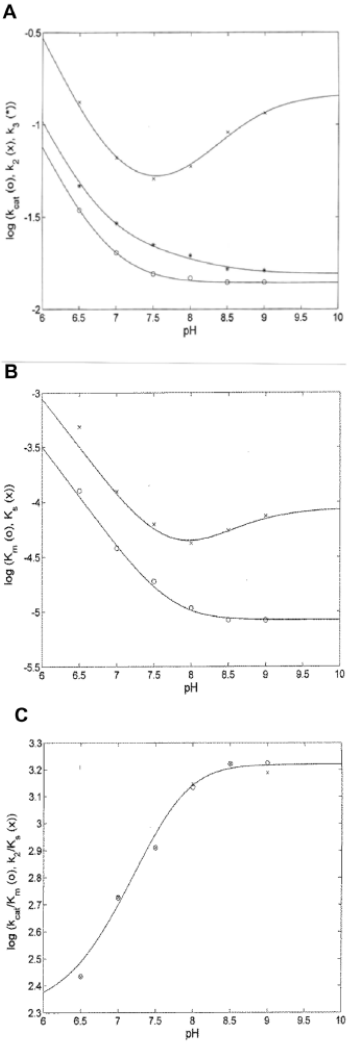

Figure 6. pH dependence of k_{cat} (o), k_2 (x), and k_3 (*) (panel A), of K_m (o) and K_s (x) (panel B), and of k_{cat}/K_m (o) and k_2/K_s (x) (panel C) for the PSA-catalyzed hydrolysis of Mu-HSSKLQ-AMC. The continuous lines have been obtained by non-linear least-squares fitting of data according to Eqs. 7–12 with parameters reported in Figure 6. The temperature was 37.0°C
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$$P_U = 1 + K_{U1}\cdot[H^+] + K_{U1}\cdot K_{U2}\cdot[H^+]^2 \quad (13)$$

$$P_{ES} = 1 + K_{ES1}\cdot[H^+] + K_{ES1}\cdot K_{ES2}\cdot[H^+]^2 \quad (14)$$

$$P_L = 1 + K_{L1}\cdot[H^+] + K_{L1}\cdot K_{L2}\cdot[H^+]^2 \quad (15)$$

^{obs}R refers to the observed parameter at a given pH value, ^{0}R refers to the parameter value of the unprotonated species, ^{1}R refers to the single-protonated species, and ^{2}R refers to the double-protonated species; K_{U1} and K_{U2} refer to the pK_a values (*i.e.*, $pK_{U1} = 10^{KU1}$ and $pK_{U2} = 10^{KU2}$) of protonating residues in the free enzyme, K_{ES1} and K_{ES2} refer to the pK_a values (*i.e.*, $pK_{ES1} = 10^{KES1}$ and $pK_{ES2} = 10^{KES2}$) of protonating residues in the *ES* complex and K_{L1} and K_{L2} refer to the pK_a values (*i.e.*, $pK_{L1} = 10^{KL1}$ and $pK_{L2} = 10^{KL2}$) of protonating residues in the *EP* form (see Figures 1 and 2).

Kinetics of the PSA-catalyzed hydrolysis of Mu-HSSKLQ-AMC were analyzed using the MatLab program (The Math Works Inc., Natick, MA, USA). The results are given as mean values of at least four experiments plus or minus the corresponding standard deviation.

Results and Discussion

Figure 4 shows a typical time course of the PSA-catalyzed hydrolysis of the fluorogenic substrate Mu-HSSKLQ-AMC (excitation wavelength = 380 nm; observation wavelength = 460 nm). This kinetic pattern, observed at all pH values, is characterized by the presence of the initial "burst" phase which precedes the insurgence of the steady-state phase. This feature, which can be described by Eqn 1, has been already observed for porcine pancreatic β-kallikrein [23] and it can be referred to a mechanism where the acylation and deacylation steps of the PSA-catalyzed hydrolysis of Mu-HSSKLQ-AMC (see Fig. 2) display different rate constants [19].

Figure 5 shows the substrate concentration dependence of k (according to Eqn, 3, see panel A) and v (according to Eqn. 4, see panel B), at different pH values. Of note, the two fitting procedures are interconnected and constrained according to the relationships depicted in Eqns. 3 and 4; therefore, they are mutually consistent, resulting in the parameters reported in Table 1.

The possibility of a quantitatively satisfactory description of the two processes by parameters which are mutually consistent indeed gives a great support to the fact that the mechanism described in Figure 2 is suitable to account for the observed behavior described in Figure 4. Furthermore, the difference between k_2 and k_3 at all investigated pH values (see Table 1) indicates that the rate-limiting step is not represented by the acylation reaction of the substrate (*i.e.*, the release of AMC, as observed in many proteolytic enzymes) [20], but it resides instead in the deacylation process (*i.e.*,

Table 2. pK_a values from the pH-dependence of various kinetic parameters.

pK_{U1}	8.02±0.16
pK_{U2}	7.61±0.18
pK_{ES1}	8.59±0.17
pK_{ES2}	5.11±0.16
pK_{L1}	8.01±0.17
pK_{L2}	5.11±0.18

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the release of Mu-HSSKLQ due to the low P_2 dissociation rate constant (*i.e.*, $k_2 \geq k_3 \approx k_{cat}$) (see Fig. 2).

Figure 6 shows the pH-dependence of the pre-steady-state and steady-state parameters for the PSA-catalyzed hydrolysis of Mu-HSSKLQ-AMC. The overall description of the proton linkage for the different parameters required the protonation/deprotonation of (at least) two groups with pK_a values reported in Table 2. In particular, the different pK_a values refer to either the protonation of the free enzyme (*i.e.*, E, characterized by pK_{U1} and pK_{U2}; see Fig. 3) or the protonation of the enzyme-substrate complex (*i.e.*, ES, characterized by pK_{ES1} and pK_{ES2}; see Fig. 3) or else the protonation of the acyl-enzyme intermediate (*i.e.*, EP, characterized by pK_{L1} and pK_{L2}; see Fig. 3). The global fitting of the pH-dependence of all parameters according to Eqns. 7–12 allows to define a set of six pK_a values (*i.e.*, pK_{U1}, pK_{U2}, pK_{ES1}, pK_{ES2}, pK_{L1}, and pK_{L2}; see Table 2) which satisfactorily describe all proton linkages modulating the enzymatic activity of PSA and reported in Figure 3. Of note, all these parameters and the relative pK_a values are interconnected, since the protonating groups appear to modulate different parameters, which then have to display similar pK_a values, as indicated by Eqns. 7–12 (*e.g.*, pK_U's regulate K_m, K_s and k_{cat}/K_m; pK_{ES}'s regulate both K_s and k_2, and pK_L's regulate both K_m, k_3 and k_{cat}); therefore, pK_a values reported in Table 2 reflect this global modulating role exerted by different protonating groups.

The inspection of parameters reported in Figure 7 envisages a complex network of interactions, such that protonation and/or deprotonation brings about modification of different catalytic parameters. In particular, the substrate affinity for the unprotonated enzyme (*i.e.*, E, expressed by $K_S = 8.8\times10^{-5}$ M; see Fig. 7) shows a four-fold increase upon protonation of a group (*i.e.*, EH, characterized by $K_{SH1} = 2.4\times10^{-5}$ M; see Fig. 7), displaying a $pK_a = 8.0$ in the free enzyme (*i.e.*, E, characterized by $K_{U1} = 1.1\times10^{8}$ M^{-1}; see Fig. 7), which shifts to $pK_a = 8.6$ after substrate binding (*i.e.*, ES, characterized by $K_{ES1} = 3.9\times10^{8}$ M^{-1}; see Fig. 7). On the other hand, this protonation process brings about a drastic five-fold reduction (from 0.15 s^{-1} to 0.036 s^{-1}; see Fig. 7) of the acylation rate constant k_2, which counterbalances the substrate affinity increase, ending up with a similar value of k_2/K_S (or k_{cat}/K_m) over the pH range between 8.0 and 9.0 (see Fig. 6, panel C). Because of this slowing down of the acylation rate constant (*i.e.*, k_2) in this single-protonated species, the difference with the deacylation rate is drastically reduced (thus $k_2 \approx k_3$; see Fig. 7). Further pH lowering brings about the protonation of a second functionally relevant residue, displaying a $pK_a = 7.6$ in the free enzyme (*i.e.*, E, characterized by $K_{U2} = 4.1\times10^{7}$ M^{-1}; see Fig. 7), which shifts to a $pK_a = 5.1$ upon substrate binding (*i.e.*,

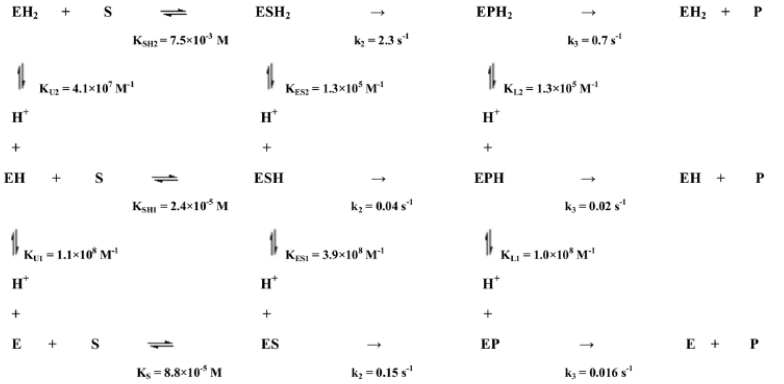

Figure 7. Proton-linked equilibria for the enzymatic activity of PSA at 37°C.
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$K_{ES2} = 1.3 \times 10^5$ M^{-1}; see Fig. 7). The protonation of this residue induces a drastic 250-fold decrease of the substrate affinity for the double-protonated enzyme (*i.e.*, EH_2, characterized by $K_{SH2} = 7.5 \times 10^{-3}$ M; see Fig. 7), even though it is accompanied by a 70-fold increase of the acylation rate constant k_2 ($= 2.3$ s^{-1}; see Fig. 7).

The identification of these two residues, characterized by substrate-linked pK_a shifts is not obvious, even though they are likely located in the kallikrein loop [24], which is known to restrict the access of the substrate to the active site and to undergo structural readjustment(s) upon substrate binding (see Fig. 1). In particular, a possible candidate for the first protonating residue ionizing at alkaline pH is the Lys95E of the kallikrein loop [24], which might be involved in the interaction with a carbonyl oxygen, orienting the substrate; this interaction could then distort the cleavage site, slowing down the acylation rate of the ESH (see Fig.7). On the other hand, the second protonating residue ionizing around neutrality may be a histidine (possibly even the catalytic His57), whose protonation dramatically lowers the substrate affinity, though facilitating the acylation step and the cleavage process. However, this identification cannot be considered unequivocal, since additional residues might be involved in the proton-linked modulation of substrate recognition and enzymatic catalysis, as envisaged in a structural modeling study [25], according to which, beside the His57 catalytic residue, a possible role might be played also by another histidyl group, possibly His172 (according to numbering in ref. [24]) (see Fig. 1).

Interestingly, after the acylation step and the cleavage of the substrate (with dissociation of the AMC substrate fragment), the pK_a value of the first protonating residue comes back to the value observed in the free enzyme, indeed suggesting that this ionizing group is interacting with the fluorogenic portion of the substrate which has dissociated after the acylation step (*i.e.*, P_1 in Figure 2), concomitantly to the formation of the EP complex; therefore this residue does not seem involved anymore in the interaction with the substrate, coming back to a situation similar to the free enzyme. On the other hand, the pK_a value of the second protonating residue ($= 5.1$) remains unchanged after the cleavage of the substrate observed in the EP complex, indicating that this group is instead involved in the interaction with the portion of the substrate which is transiently covalently-bound to the enzyme (possibly represented by the original *N*-terminus of the peptide), the dissociation (or deacylation) of the EP adduct representing the rate-limiting step in catalysis. Therefore, for this residue, ionizing around neutrality, the transformation of ES in EP does not bring about any modification of substrate interaction with the enzyme.

As a whole, from the mechanism depicted in Figure 7 it comes out that the enzymatic activity of PSA is mainly regulated by the proton-linked behavior of two residues, characterized in the free enzyme by $pK_{U1} = 8.0$ and $pK_{U2} = 7.6$, which change their protonation values upon interaction with the substrate. The evidence emerging is that these two residues interact with two different regions of the substrate, such that (*i*) the group characterized by pK_{U1}, which interacts with the portion released after the acylation process (probably corresponding to the original *C*-terminus of the substrate), displays a pK_a increase after substrate binding (likely reflecting the formation of an electrostatic favorable interaction in the ES complex), whereas (*ii*) the group characterized by pK_{U2}, which interacts with the portion released after the deacylation process, displays a pK_a decrease, clearly indicating that the corresponding residue tends to be deprotonated after substrate binding. The different modulatory role of the two residues, which sense in a distinct fashion the acylating and deacylating steps, is very interesting and may represent (i) an important mechanism to regulate in macromolecular substrates the release of different proteolytic products during the catalytic function of the enzyme and (ii) a relevant aspect to design enzyme inhibitors. In this respect, it is interesting to remark that the natural occurrence of a slow deacylating step in PSA might be exploited to design new potential inhibitors. Thus, appropriate modifications of the peptide sequence might be designed, so as to indefinitely slow down the deacylation step transforming he peptide in a "suicide" inhibitor, which completely abolishes the PSA activity.

Author Contributions

Conceived and designed the experiments: SM PA MC. Performed the experiments: LT DS MG ADM. Analyzed the data: LT DS MG ADM SM PA MC. Contributed reagents/materials/analysis tools: SM PA MC. Contributed to the writing of the manuscript: LT DS MG ADM SM PA MC.

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PCA3 in prostate cancer and tumor aggressiveness detection on 407 high-risk patients: a National Cancer Institute experience

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PCA3 in prostate cancer and tumor aggressiveness detection on 407 high-risk patients: a National Cancer Institute experience

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Abstract

Background: Prostate cancer (PCa) is the most common male cancer in Europe and the US. The early diagnosis relies on prostate specific antigen (PSA) serum test, even if it showed clear limits. Among the new tests currently under study, one of the most promising is the prostate cancer gene 3 (*PCA3*), a non-coding mRNA whose level increases up to 100 times in PCa tissues when compared to normal tissues. With the present study we contribute to the validation of the clinical utility of the PCA3 test and to the evaluation of its prognostic potential.

Methods: 407 Italian men, with two or more PCa risk factors and at least a previous negative biopsy, entering the Urology Unit of Regina Elena National Cancer Institute, were tested for PCA3, total PSA (tPSA) and free PSA (fPSA and f/tPSA) tests. Out of the 407 men enrolled, 195 were positive for PCa and 114 of them received an accurate staging with evaluation of the Gleason score (Gs). Then, the PCA3 score was correlated to biopsy outcome, and the diagnostic and prognostic utility were evaluated.

Results: Out of the 407 biopsies performed after the PCA3 test, 195 (48%) resulted positive for PCa; the PCA3 score was significantly higher in this population ($p < 0.0001$) differently to tPSA ($p = 0.87$). Moreover, the PCA3 test outperformed the f/tPSA ($p = 0.01$). The sensitivity (94.9) and specificity (60.1) of the PCA3 test showed a better balance for a threshold of 35 when compared to 20, even if the best result was achieved considering a cutoff of 51, with sensitivity and specificity of 82.1% and 79.3%, respectively. Finally, comparing values of the PCA3 test between two subgroups with increasing Gs ($Gs \leq 6$ *versus* $Gs \geq 7$) a significant association between PCA3 score and Gs was found ($p = 0.02$).

Conclusions: The PCA3 test showed the best diagnostic performance when compared to tPSA and f/tPSA, facilitating the selection of high-risk patients that may benefit from the execution of a saturation prostatic biopsy. Moreover, the PCA3 test showed a prognostic value, as higher PCA3 score values are associated to a greater tumor aggressiveness.

Keywords: Prostate cancer, Urine and blood biomarkers, Prostate Specific Antigen, Prostate Cancer gene 3, Tumor aggressiveness.

Background

Prostate cancer (PCa) is the most common malignancy in men of Western populations and one of the major burden in public health [1], despite numerous efforts were made attempting to clarify the various aspects of this disease [2-4]. During the last years an increasing PCa incidence has occurred, probably linked to the introduction of the prostate specific antigen (PSA) determination in terms of opportunistic screening [5]. The PSA test actually brought to the diagnosis of a high number of asymptomatic and preclinical forms of PCa, but it has not been associated with a decrease in mortality, opening a wide debate on the diagnostic utility of this test [6]. One of the main disadvantages of the PSA test is its low specificity, which causes the execution of a high percentage of negative biopsies (60-75%), especially in patients with total PSA (tPSA) levels between 4 and 10 ng/ml [7,8]. A great effort is therefore constantly turned to the research of new markers capable to improve the PCa diagnosis, to identify the asymptomatic and more aggressive forms and to reduce the number of biopsies, lowering the risk of pain, bleeding and infection to many patients [9]. Among the characterized biomarkers one of the most promising for its diagnostic potential, is the Prostate Cancer gene 3 (*PCA3*). *PCA3* (also known as *DD3* or *DD3PCA3*) is located on chromosome 9 and is transcribed into a non-coding prostate-specific mRNA which is overexpressed in tumor cells, from 60 to 100 times, when compared to the normal prostate tissue [10]. The PCA3 test is based on the quantification of the PCA3 mRNA on urine sample after digital-rectal examination (DRE), using the methodology of the transcription mediated amplification (TMA). The obtained result is then normalized to the amount of PSA mRNA, evaluated in the same urine sample, in order to calculate the PCA3 score (PCA3 mRNA / PSA mRNA ×1000). To date, many studies have been performed and most of them showed how the PCA3 test represents a useful tool to predict PCa, but questions about the optimal cutoff and the ability of PCA3 to predict tumor aggressiveness still remain highly controversial [11,12]. Here, we report the results of the PCA3 test among an Italian prospective cohort of high-risk PCa patients in

order to evaluate its actual clinical utility as a diagnostic test additional and/or alternative to the PSA test. Moreover, best PCA3 cutoff was assessed to better discriminate patients with and without PCa. Finally, the correlation between the results of the PCA3 test and the tumor aggressiveness has been evaluated.

Methods

Patient selection

Between November 2009 and May 2011, 407 consecutive men with two or more risk factors for PCa and at least a previous negative biopsy entered the Urology Unit of Regina Elena National Cancer Institute. Risk factors for PCa could be: tPSA higher than 2,5 ng/ml, a family history of PCa, a borderline DRE and the presence of pre-neoplastic forms in a prior biopsy. None of the patients had a history for PCa and none was taking drugs able to lower PSA since at least one month. Biopsies evidencing pre-neoplastic forms , such as atypical acinar proliferation (ASAP), low-grade prostatic intraepithelial neoplasia (LGPIN) lesions or high grade PIN (HGPIN), were classified as negative. Once tests were carried out, patients were addressed more or less urgently towards a saturation prostatic biopsy. To date, all patients underwent a prostatic biopsy. This study was approved by the Ethics Committee of Regina Elena National Cancer Institute and a written informed consent was obtained from all participants.

Sample processing

Blood samples were collected in tubes containing gel and clot activator for serum separation (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ, USA). Samples were centrifuged within 1 h at 2500 g for 15 min and stored in aliquots at -80°C until processing. Serum tPSA and fPSA were

assessed with an electrochemiluminescence immunoassay (ECLIA) on fully-automated COBAS 6000 e601 module analyzer (Roche Diagnostics GmbH, Penzberg, Germany), according to the manufacturer's specifications and using proprietary reagents. After blood sampling, a prostatic massage was performed, always from the same urologist and consisting in three digital pressure per lobe, so 20-30 ml of urine were then collected in a sterile urine container (Nalgene, Rochester, NY, USA) and transferred into a specific transport tube (Progensa PCA3 Urine Specimen Transport Kit, San Diego, CA, USA) to be stored at -80°C until processing. The PROGENSA PCA3 assay (Gen-Probe Inc., San Diego, CA, USA) was used to evaluate the PCA3 and PSA mRNA expression levels in urine samples, in order to calculate the PCA3 score as the ratio of PCA3 to PSA mRNA ×1000. Both urine and serum samples were collected and processed at the Clinical Pathology Laboratories of the Regina Elena National Cancer Institute. After samples testing, all patients gradually performed a saturation prostatic biopsy. All tissue samples were collected and evaluated from the Pathological Anatomy Unit of the Regina Elena National Cancer Institute. If more than one neoplastic focus was detected in the same tumor, the highest Gs was reported.

Statistical analyses

The association between variables was tested by Pearson's Chi-square test or Fisher's Exact test, when appropriate. The continuous data as mean and standard deviation or median and range was reported. Binary data was reported as frequency and percentage values. Kruskal-Wallis or Mann-Whitney (adjusted for multiple comparison, when appropriate) were used for the comparisons. A p-value ≤ 0.05 was considered statistically significant.

The receiver operating characteristic (ROC) curve analysis was performed in order to find possible optimal cut-offs capable of splitting patients in two groups and for assess models predictive accuracy through the estimation of the area under the curve (AUC), providing specificity, sensitivity, negative and positive predictive value (NPV and PPV), and the 95% confidence interval

(CI) for all possible threshold values and differences between curves. The SPSS®(21.0) statistical program was used for all the analyses.

Results

Out of the 407 men enrolled, all were tested for tPSA, fPSA, and PCA3; moreover, all of them performed a subsequent biopsy that revealed 195 (48%) tumors. For both the PCa and non-PCa groups, data concerning the median age, tPSA, f/tPSA and PCA3 values were summarized in Table 1. Comparing PCa *versus* non-PCa men, no difference in tPSA values were found ($p = 0.87$), while men with PCa showed a lower median f/tPSA ($p = 0.01$) and a significantly higher median of the PCA3 score ($p < 0.0001$), compared to men without PCa (Figure 1). No association with age was found.

To further evaluate the clinical significance of the PCA3 test, six intervals of PCA3 score values *versus* biopsy outcomes were chosen (Figure 2). Specifically, PCA3 score values were parted in increasing ranges (0-20, 21-35, 36-50, 51-70, 71-100 and >100) so the number of PCa-positive biopsies for each interval was evaluated. The probability to find a positive biopsy strongly correlates with the PCA3 test, as the probability to find a PCa-positive biopsy is higher at increased PCA3 score values ($p < 0.0001$).

In order to characterize the best cutoff of the PCA3 test, the number of true negative (TN), true positive (TP), false negative (FN), and false positive (FP) at different PCA3 scores were evaluated. Consequently, sensitivity and specificity, for each considered threshold, as well as the PPV and NPV were calculated. Considering our cohort, 35 overcomes 20 as PCA3 score cutoff, because a better balance between sensitivity and specificity, as well as higher PPV and NPV, were observed. However, the best result was obtained from a PCA3 score threshold of 51, that showed the best sensitivity, specificity, PPV and NPV values (Table 2).

In addition, in order to compare the diagnostic performance of the PCA3 and PSA tests, a ROC analysis was performed (Figure 3). The area AUC was found to be higher for the PCA3 test (0.865) when compared to both tPSA (0.505) and f/tPSA (0.607).

Finally, the association between the PCA3 score and the tumor aggressiveness, expressed in terms of Gs score, was investigated (Table 3). The evaluation of the histologic grade was perfectly assessable on 114 PCa men. The tumor aggressiveness was split in two classes: Gs $\leq$ 6 (that includes the lower grades) and Gs $\geq$ 7 (representing the most clinically significant cases). The PCA3 score threshold of 51 (optimal for our cohort), was exceeded from the 69% of men with Gs $\leq$ 6, but this percentage was significantly higher (87.5%) for men with Gs $\geq$ 7 ($p = 0.02$).

Discussion

The PSA limitations in PCa detection and classification are well established [13,14]. Hereupon, the risk to underestimate patients with PCa because of normal PSA levels, and, more often, to guide patients toward specialized medical practices attempting to detect a small percentage of clinically significant cancers, is very high. Moreover, it has been shown how PSA fails to predict the lethal forms of PCa [15]. Therefore, many independent studies aimed to find and to validate new PCa biomarkers are being performed.

The present study is based on an Italian cohort of 407 men with one or more previous negative biopsies; all of them, belonging to a high risk population for PCa, were addressed to a saturation prostatic biopsy after the PCA3 test. This study succeeded in demonstrating that the PCA3 test is a more sensitive test than the tPSA and the f/tPSA tests in discriminating patients with and without PCa (Table 1 and Figure 1). In fact, for our cohort, the median tPSA value was similar between the two subgroups ($p = 0.87$), while a significant difference was found for the f/tPSA ($p = 0.01$);

however, the best result was obtained considering the different distribution of the PCA3 score ($p < 0.0001$) between PCa and non-PCa patients.

Although the PCA3 test seems to improve the probability to detect PCa, it is still unclear whether a not-optimal DRE can give false negative values of the PCA3 score, as well as if this test is able to detect a neoplasia at its very initial stage; on the other hand, some reports suggest that PCA3-mRNA can be also detected in HGPIN lesions [16-18]. Although in this study LGPIN and HGPIN reports were classified as negative, the present data support the hypothesis that the probability to find a PCa gets higher when the PCA3 score increases. At a low PCA3 score, in fact, the percentage of subjects with PCa was small (5.3% for PCA3 score between 0 and 20), while the percentage increased steadily to reach the maximum when the PCA3 score exceeded 100 ($p < 0.0001$); in this case, in fact, PCa was found in 79% of patients (Figure 2).

One of the major opened questions about the PCA3 test, on the other side, regards the optimal cutoff useful to discriminate patients with and without PCa. The optimal threshold proposed by Gen-Probe Inc., using the PROGENSA PCA3 assay, was 35, but several studies suggested that this value could be modified, getting lower or even higher, in a way that is probably dependent on the population features. In this respect, the cutoff value of 20 seems to increase the PCA3 test sensitivity without affecting the specificity [19-24]. Some studies demonstrated that PCA3 is effective only after the first negative biopsy, however, a recently published meta-analysis showed that PCA3 can be used for repeat biopsy to improve accuracy of PCa detection, since a large number of unnecessary biopsies can be avoided by using a PCA3 score cutoff of 20 [12,25]. To assess the best PCA3 score value, useful to discriminate those at a tumor stage, the most commonly used thresholds were examined. In our cohort, in which a division between men with one or more previous negative biopsies was not prevented, the lowest specificity was found for 20 (33.3%) when compared to 35 (60.1%), while the sensitivity resulted very similar (97.9% and 94.9%, respectively). Even if a threshold of 35 showed a better balance between sensitivity and specificity, the best performance was reached considering a threshold of 51, showing sensitivity and specificity

of 82.1% and 73.3%, respectively (Table 2). An optimal cutoff higher than 35 was found also in other independent prospective studies, where it showed the ability to prevent a larger number of unnecessary biopsies, highlighting more firmly on those patients who need a fast treatment [22,23,26]. These results were confirmed by the ROC analysis, as comparing the area under the curve for PCA3, tPSA, and f/tPSA tests we found values of 0.865, 0.505 and 0.607, respectively. These data indicate that the PCA3 test showed the best performance for the PCa diagnosis for our cohort of men (Figure 3).

Lastly, a possible correlation between the PCA3 score and the tumor aggressiveness, expressed in terms of Gs, was investigated. Subjects with organ-confined PCa and Gs ≥ 7 have a worst prognosis than those with Gs ≤ 6, even following radical prostatectomy or radiation therapy [27-29]. To recognize a low grade from a more aggressive PCa is therefore essential for therapeutic purposes, but currently the only way to discriminate patients with low or high grade PCa is to perform a biopsy. The possibility of using the PCA3 test as a prognostic marker is desirable, but the possibility to evaluate tumor aggressiveness by the PCA3 test is openly debated [17,21,23,26,30-34]. Indeed, the wide range of results obtained in previous studies may be due to different experimental conditions and may reflect the selected cohort features. In fact, the use of urine sediments or whole urine samples, collected before or without a previous DRE, can give rise to different results that are not often comparable in judging the prognostic value capabilities of the PCA3 test. On the other hand, the characteristics of the screened population could be important, indeed the choice to enroll only patients with a certain risk for PCa, or depending on the number of previous biopsies, can drive data towards an easier or less easy association between the result of the PCA3 test and the tumor aggressiveness.

The patients enrolled in this study were selected according to the presence of persistent risk factors for PCa with at least a previous negative biopsy. We evaluated, among patients with an assessable tumor grading ($n = 114$), those who exceeded the PCA3 score value of 51 (optimal for our cohort) showing, at the same time, a low grade PCa, *i.e.* Gs ≤ 6, or a higher grade PCa, represented by Gs $\geq$

7 (Figure 3). For our cohort of men, a correlation between the PCA3 level and the PCa grading was actually found; indeed, the percentage of patients with a PCA3 score higher than 51 and a Gs ≤ 6 was 69%, while the percentage of patients with a PCA3 score higher than 51 and a Gs ≥ 7 (87.5%) was significantly higher ($p = 0.02$). These data strengthen the hypothesis that the PCA3 test could recognize, among PCa subtypes, those more aggressive that may benefit from the resolutive radical prostatectomy surgery.

Conclusions

The present study was conducted on subjects with at least a previous negative prostatic biopsy and with two or more persistent risk factors for PCa, resulting therefore good candidates for a further biopsy. Here, we report that the PCA3 score shows a great diagnostic accuracy compared to both tPSA and f/tPSA tests; moreover, a high PCA3 score corresponds to an increased probability to find a positive biopsy. Our data suggest that the PCA3 test could predict a PCa and allow urologists to more easily select, among high-risk patients, those who may benefit from a saturation prostatic biopsy. Even more interesting is the finding of a correlation between PCA3 score and tumor aggressiveness, expressed in terms of Gleason score, that strengthened the hypothesis of PCA3 as an effective prognostic marker, able to discriminate, among cancers, those less significant that may directly enter the active surveillance protocols, lowering the economic effort for PCa diagnosis supported from public health.

Abbreviations

ASAP: atypical acinar proliferation; AUC: area under the curve; BPH: benign prostatic hyperplasia; DRE: digital-rectal examination; ECLIA: electrochemiluminescence immunoassay; f/tPSA:

fPSA/tPSA ratio; FN: false negative; FP: false positive; fPSA: free PSA; Gs: Gleason score; HGPIN: high grade prostatic intraepithelial neoplasia; LGPIN: low-grade prostatic intraepithelial neoplasia; NPV: negative predictive value; PCa: prostate cancer; PCA3: prostate cancer gene 3; PPV: positive predictive value; PSA: prostate specific antigen; ROC: receiver operating characteristics; TMA: transcription mediated amplification; TN: true negative; TP: true positive; tPSA: total PSA.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the study: GM, CL

Performed the laboratory analysis: MR, TL, AA, MC, MS, OG

Performed the urological analysis: PR, GS, CM, CG

Performed the histopathological analysis: SS

Analyzed the data: SI

Contributed reagents/materials/analysis tools: SS, CG, AP, GM, CL

Wrote the manuscript: MR, TL, SI, AP, GM, LC

Acknowledgments

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Figure legends

Figure 1. tPSA (A), f/tPSA (B), and PCA3 score (C) values for patients negative and positive for PCa.

Figure 2. Relationship between PCA3 score and the percentage of positive biopsies.

Figure 3. ROC analysis with evaluation of the corresponding AUC for tPSA (0.505), f/tPSA (0.607) and PCA3 score (0.865).

Tables and captions

Table 1. Number of PCa-positive and PCa-negative patients and evaluation of the related distribution in terms of median age, tPSA, f/tPSA and PCA3 score values.

	PCa	non-PCa	*p* value
Number (%)	195 (48)	212 (52)	/
Age (median±SD)	71±27	69±31	0.33
tPSA (ng/ml) (median±SD)	7.53±4.88	7.34±5.87	0.87
f/tPSA (median±SD)	0.15±0.07	0.18±0.07	0.01
PCA3 score (median±SD)	82±45	33±26	<0.0001

Table 2. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of different PCA3 score cutoff.

	PCA3 score cutoff		
	20	**35**	**51**
Sensitivity	97.9	94.9	82.1
Specificity	33.3	60.1	79.3
PPV	47.8	68.5	78.4
NPV	57.4	92.8	82.8

Table 3. Correlation between tumor aggressiveness, expressed in terms of Gleason score (Gs), and the PCA3 score (p=0.02) in a subgroup of patients with PCa assessable histological characterization (n = 114).

	PCA3 score	
	≤ 51	> 51
Gs ≤ 6 (%)	13 (31)	29 (69)
Gs ≥ 7 (%)	9 (12.5)	63 (87.5)

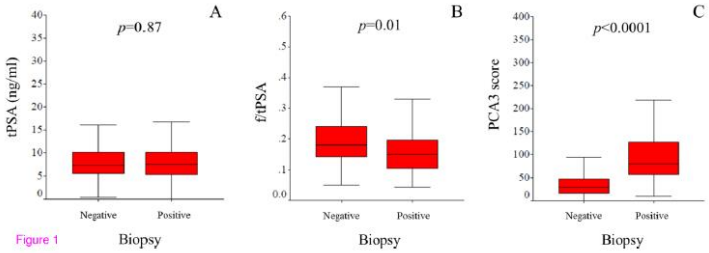

Figure 1

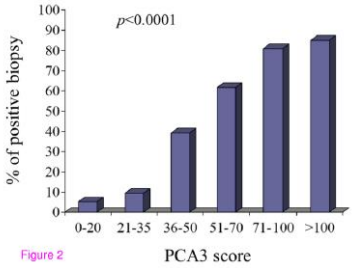

Figure 2

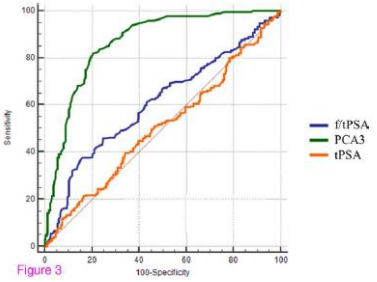

Figure 3

Diagnostic and prognostic value of serum [-2]proPSA and galectin-3 related indices in prostate cancer: a retrospective study

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Abstract

Background: Prostate cancer (PCa) early diagnosis relies on prostate specific antigen (PSA) serum test, even if it showed clear limits. Among the new tests currently under study, one of the most promising is the [-2]proPSA (p2PSA) serum test and the related prostate health index (PHI); moreover, in the last period, Galectin-3 (Gal3) is emerging as a possible complementary marker for PCa The present study aims to validate the clinical utility of the p2PSA and Gal3 tests along with their related indices.

Methods: Total PSA (tPSA), free PSA (fPSA and %fPSA), p2PSA (%p2PSA and PHI) and Gal3 (Galphi = (tPSA × Gal3) / fPSA) were evaluated on serum samples of 47 men with PCa, 25 men with benign prostatic hyperplasia (BPH) and 15 healthy patients. All PCa and BPH patients had tPSA between 2 and 10 ng/ml. Values of tPSA, %fPSA, %p2PSA, PHI, Gal3, and Galphi were compared between groups and their relative diagnostic accuracy was evaluated. Finally, tumor grading (T) and tumor aggressiveness, expressed as Gleason score (Gs), were matched with all the analyzed parameters, so their prognostic value was assessed.

Results: Comparing PCa versus BPH men, there was no statistically significant difference in tPSA ($p = 0.93$) and Gal3 ($p = 0.09$) levels; however, PCa men had a significantly lower %fPSA median ($p = 0.001$) and a significantly higher %p2PSA ($p = 0.001$), PHI ($p \leq 0.0001$), and Galphi ($p = 0.001$) median values. The AUC values for tPSA, %fPSA, %p2PSA, PHI, Gal3, and Galphi were 0.509, 0.729, 0.734, 0.760, 0.626, and 0.740, respectively, highlighting no significant difference between PHI and Galphi ($p = 0.81$). Finally, matching all the analyzed variables with tumor pathological characteristics in patients affected by PCa, no significant differences were found between patients with high or low tumor extent (pT2 *vs* pT3) and aggressiveness ($Gs \leq 6$ *vs* $Gs \geq 7$).

Conclusions: The same cohort of patients, here used to validate the clinical usefulness of p2PSA related indices (%p2PSA and PHI), have been used to test the diagnostic performances of a novel PCa biomarkers of our creation calculated by serum Gal3, tPSA, and fPSA (the Galphi). This index

showed light and dark sides similar to those of markers currently used in clinical practice and demonstrated to deserve further investigations to reveal definitively the utility of its introduction in the management of patients with prostate pathologies.

Keywords: Prostate cancer, Serum diagnostic and prognostic biomarkers, Prostate Specific Antigen and related index, [-2]proPSA and related indices, Galectin-3 and related index.

Background

Prostate cancer (PCa) is the most common male malignancy in western populations and it is the second leading cause of cancer related death in men [1]. During the last decades an increasing PCa incidence has been observed, probably due to the introduction of prostate specific antigen (PSA) determination in the form of opportunistic screening. PSA serum test actually brought to the diagnosis of a higher number of asymptomatic and preclinical forms of PCa, but this has not been associated with a decrease in mortality levels, suggesting that most of the PCa cases identified by the PSA test are not intended to be clinically manifest [2]. One of the main disadvantages of the PSA test is its low specificity, which involves the execution of a high percentage of negative biopsies (60-75%), especially in patients with total PSA (tPSA) levels between 2 and 10 ng/ml [3,4]. This is because the increase of PSA serum level is not an event that closely reflects the presence of a PCa, but it is also linked to benign prostatic hyperplasia (BPH) and prostatitis events. Therefore, the strategy to perform a biopsy whenever tPSA levels get higher exposes male population to undergo an unnecessary procedure that often does not exclude medical complications [5].

A great effort is therefore constantly turned to the research of new biomarkers in order to improve the PCa diagnosis and to identify the most aggressive forms. Therefore, several biomarkers for PCa have been intensely examined, over the past ten years. However, despite the new findings and the prospects of obtaining good performance from them, the clinical usefulness of biomarkers is still widely debated [6]. Most studies have been focused on the evaluation of the clinical usefulness of tPSA derivatives, such as the free PSA (fPSA) serum test and its percentage (%fPSA), that is generally lower in patients with PCa compared to subjects with BPH [7]. Further attention has been devoted to fPSA, which includes the inactive pro-enzyme, the proPSA, and the PSA isoform containing the inhibitory sequence formed by two amino acids, *i.e.* [-2]proPSA (p2PSA) [8].

Immunohistochemical studies showed that p2PSA is the most abundant form of proPSA present in tumor tissues [9], while prospective studies have shown that the percentage of p2PSA (%p2PSA) exceeds the %fPSA diagnostic accuracy [10-14].

Recently, p2PSA has been included in the "Beckman Coulter Prostate Health Index" (PHI), that corresponds to %p2PSA × √tPSA. This index seems to show the best sensitivity and specificity values compared to any single test with which is calculated [15-17].

Over the last two decades, the role of galectin-3 (Gal3), an extra- and intra-cellular β-galactoside-binding protein located in several tissues and organs, has been deeply investigated in inflammation as well as in cell proliferation, motility, and apoptosis [18-20]. Gal3 expression levels (both in terms of up and down-regulation) have been associated to a variety of pathological conditions such as heart failure, infection by microorganisms, diabetes, and cancer progression [21-24]. However, only few and conflicting data concerning the relationship between Gal3 levels and PCa are available [25-28].

The present study aims to determine the actual clinical utility of p2PSA, Gal3 and their related indices as additional and/or alternative assessments to tPSA and fPSA tests. Therefore, the results obtained in a population of PCa men, attending radical prostatectomy (RP) surgery, have been compared to a group of BPH men, suitable for a TransUrethral Resection of the Prostate (TURP) surgery. In parallel, the prognostic value for p2PSA and Gal3 have be evaluated by correlating the results obtained from PCa patients and the tumor extent and aggressiveness, as determined histopathologically.

Methods

Patients selection

Between October 2012 and May 2014, 85 patients were enrolled: 47 with a PCa diagnosis, 25 with a BPH diagnosis, and 15 healthy donors. The inclusion criteria for the study consisted in having a tPSA between 2 and 10 ng/ml (for PCa and BPH groups) and no prostate manipulation since two months or previous chemo/hormone/radio therapy before serum samples collection (for all groups). After PSA and Gal3 testing, patients with PCa underwent RP, whereas, on BPH patients a TURP was performed. The initial diagnosis was confirmed by the post-surgery histopathological analysis. For the PCa group, tumor staging was evaluated by TNM classification and its grading was expressed in terms of Gs. This study was approved by the Ethics Committee of the "Regina Elena" National Cancer Institute and a written informed patient consent was obtained from all participants.

Sample processing and indices calculation

Blood samples were collected in tubes containing gel and clot activator for serum separation (Vacutainer, Becton- Dickinson, Franklin Lakes, NJ, USA) during the pre-hospital period for all patients (maximum one month before surgery). Samples were centrifuged within 1h at 2500g for 15 min and stored in aliquots at -80°C until processing. Serum tPSA, fPSA, and p2PSA levels were assessed with a chemiluminescence immunoassay (CLIA) on fully-automated Access 2® analyzer (Beckman Coulter Inc., Brea, CA, USA), according to the manufacturer's specifications. The obtained values were used for the calculation of the %p2PSA (= p2PSA / fPSA) and PHI (= %p2PSA × √tPSA) indices. Serum Gal3 levels were determined by CLIA on fully-automated Architect 1000i analyzer (Abbott Laboratories, Abbott Park, IL, USA), according to the manufacturer's specifications. Gal3 level was accounted to combine this ssessment with tPSA and fPSA tests and try to obtain a more powerful marker, the Galphi, calculated as (tPSA × Gal3) / fPSA. In this case, serum tPSA and fPSA levels were assessed with an electrochemiluminescence immunoassay (ECLIA) on fully-automated COBAS 6000 e601 module analyzer (Roche Diagnostics GmbH, Penzberg, Germany), according to the manufacturer's specifications. Serum tPSA and fPSA levels values were used also for the determination of %fPSA (= fPSA / tPSA).

Statistical analysis

The association between variables was tested by Pearson's Chi-square test or Fisher's Exact test, when appropriate. The continuous data as mean and standard deviation or median and range was reported. Binary data were reported as frequency and percentage values. The Mann-Whitney U text was used for comparisons between subgroups. A p-value ≤ 0.05 was considered statistically significant.

The Odds Ratio (OR) and the 95% confidence intervals (95% CI) were estimated for each variable. A multivariate logistic regression model was also developed using stepwise regression (forward selection) to compare the diagnostic accuracy of different factors. Enter limit and remove limit were $p = 0.10$ and 0.15, respectively.

The receiver operating characteristic (ROC) curve analysis was performed in order to find possible optimal cut-offs capable of splitting patients in two groups and for assess models predictive accuracy through the estimation of the area under the curve (AUC), providing sensitivity, specificity for all possible threshold values and differences between curves. The SPSS® (21.0) statistical program (IBM, Armonk, New York, US) was used for all the analyses.

Results

Out of the 47 PCa men enrolled, all were tested for tPSA, fPSA, and pPSA, while 45 were tested for Gal3; the median age was 62 years old with a median tPSA, %fPSA, %p2PSA, PHI, Gal3, and Galphi values of 5.61 ng/ml, 13, 1.94, 41.5, 13.3 ng/ml, and 107.2, respectively. Out of the 25 BPH men enrolled, all were tested for tPSA, fPSA, and pPSA, while 24 were tested for Gal3. The median age was 60 years old with a median tPSA, %fPSA, %p2PSA, PHI, Gal3, and Galphi values of 6.11 ng/ml, 19, 1.38, 32.8, 12.4 ng/ml, and 69.1, respectively. Out of the 15 healthy men enrolled, all

were tested for tPSA and Gal3. The median age was 61 with a median tPSA and Gal3 concentration of 0.7 ng/ml and 12.6 ng/ml respectively (Table 1). Comparing PCa *versus* BPH men, there was no statistically significant difference in tPSA ($p = 0.93$) and Gal3 ($p = 0.09$) levels; however, PCa men had a significantly lower %fPSA median ($p = 0.001$) and a significantly higher %p2PSA ($p = 0.001$), PHI ($p \leq 0.0001$) and Galphi ($p = 0.001$) median compared to men with BPH (Figure 1). Considering the control population, a significant difference in tPSA was found *versus* both PCa ($p < 0.0001$) and BPH ($p<0.0001$) groups, whereas, as regarding Gal3, the difference was significant only *versus* the PCa group ($p = 0.002$). No association with age was found.

In order to clarify the diagnostic accuracy of the selected tests, a ROC analysis was performed (Figure 2). Of note, tPSA has low sensitivity and specificity in the 2–10 ng/ml range (AUC = 0,509), while %fPSA demonstrates a greater performance (AUC = 0.729). In contrast, the p2PSA related indices (*i.e.*, %p2PSA (AUC = 0.734) and PHI (AUC = 0.760)) are the most accurate predictors of PCa. Finally, the Gal3 test (AUC = 0.626) failed in outperforming tPSA as the PCa marker ($p = 0.25$), however the Galphi index (AUC = 0.74) showed favorable performance characteristics similar to those obtained from PHI ($p = 0.81$). The sensitivity and specificity of all considered parameters are reported in Table 2. Setting an optimal cutoff, useful to better discriminate PCa and BPH patients, for all the analyzed variables, it was evident how both tPSA and Gal3 alone showed the worst balance in terms of sensitivity and specificity. A better result was obtained considering all the other markers, even if whenever the sensitivity is high (as for %fPSA and PHI), the specificity turned out to be lower. At the same time %p2PSA and Galphi, that showed the best specificity, showed low sensitivity values.

In order to deepen the diagnostic potential of the parameters considered in the present study, a multivariable analysis was performed. Taking all those markers that showed a significantly different distribution between PCa and BPH patients (*i.e.*, %fPSA, %p2PSA, PHI and Galphi) the variable that showed the best accuracy was PHI, that revealed the 52.2% of BPH and the 85.4% of PCa, with a global precision of 73.4%. The only other variable that resulted significative after its inclusion in

the multivariable model was Galphi, arising as the only variable PHI-indipendent. Consequently, considering PHI and Galphi together the percentage of BPH and PCa revealed was 56.4 and 87.8 respectively, with a global precision of 76.6% (data not shown).

In parallel, the marker *versus* tumor histopathological study was conducted accounting for only the 47 PCa men. All tumors, after histological analysis, were reported as without regional lymph node metastasis (N0) and without distant metastasis (M0), while the extent of the tumor (T) was pT2 for 42 men and pT3 for 5 men. Comparing the distribution of all parameters between men with a low (pT2) and a high (pT3) tumor staging, no significant difference was found (Table 3). In parallel, the association between the analyzed variables and the tumor aggressiveness, expressed in terms of Gs, was investigated. The evaluation of the histologic grade was assessable for all specimens and the tumor aggressiveness was split in two classes: Gs ≤ 6 (that includes the lower grades, $n = 13$) and Gs ≥ 7 (representing the most clinically significant cases, $n = 34$). Also in this case none of the examined variables showed a different distribution between the two groups (Table 4).

Discussion

The PSA limitations in PCa detection and classification are well established [29,30]. Hereupon, the risk to underestimate patients with PCa because of normal PSA levels, and, more often, to guide patients toward specialized medical practices attempting to detect a small percentage of clinically significant cancers is very high This problem is faced especially from those patients who have serum tPSA values between 2 and 10 ng/ml, representing the so called gray zone, where the challenge to discriminate between benign and malignant prostate pathologies reaches its maximum.

The present retrospective study is based on two groups of men, one with a diagnosis of PCa and another with BPH, all of which had a serum tPSA level between 2 and 10 ng/ml. Present data confirm the %fPSA, %p2PSA, and PHI diagnostic performances, incrementing the ability to

discriminate PCa from BPH patients [7,10,13-17]. In fact, for our cohort, the median tPSA value was similar between the two subgroups ($p = 0.93$), while a significant difference was found considering the %fPSA ($p = 0.001$) and %p2PSA ($p = 0.001$) values. The best result was obtained considering the different distribution of the PHI values ($p < 0.0001$) between PCa and BPH patients (Table 1 and Figure 1).

In parallel, we tried to assess whether Gal3, which emerged as a possible complementary marker to the serum PSA test [26], was able to discriminate patients with and without PCa. In fact, Gal3 expression has been reported to vary between healthy and tumor conditions,[22-27]. The present study represents the first case in which serum Gal3 was compared between PCa patients, none of which at a metastatic stage, and patients with a benign prostatic condition. In this view, also a group of healthy patients was screened in order to reveal the basal Gal3 distribution. Present data indicate that the serum Gal3 value is significantly higher in localized-PCa patients than in healthy patients ($p = 0.02$), while no difference occours between BPH and healthy patients ($p = 0.1$. However, the difference in the Gal3 distribution between the PCa and the BPH groups is not statistically significant ($p = 0.09$); this suggests that this test could be useful to assess a prostatic pathology, but not a PCa. Findings deeply change when Gal3 values are considered in the determination of the Galphi index (= (tPSA × Gal3) / fPSA) of our conception. This equation has been thought postulating that men with high Gal3 and tPSA and low fPSA serum levels are more likely to have PCa. In fact, evaluating the present results, we found a significantly different distribution between the two subgroups ($p = 0.001$), indicating that high values of this marker could strongly predict the presence of a PCa (Table 1 and Figure 1). Accordingly, it could be worth outlining that the contemporary validation of the PSA and p2PSA related indices is fundamental to understand the robustness of our data as regards the new biomarkers. In fact, since data obtained from tPSA, %fPSA, %p2PSA, and PHI resulted coherent with the literature, they represent a good quality control of how much the results obtained with the Gal3 and its related index could be solid and consistent. However, one of the major opened questions about PHI as a marker of PCa regards the

optimal cutoff useful to discriminate patients with benign or malignant prostate pathologies. The indication proposed by Beckman Coulter suggested that for values between 21 and 40 the risk of PCa is medium. In this regards, basically all the performed studies demonstrated that the PHI value, reporting the best balance in terms of sensitivity and specificity, fails in this range [14,15,17,31,32].

In order to depict the diagnostic accuracy of the screened predictors of PCa, a ROC analysis was performed (Figure 2). As regards PHI, it indicated that 35 resulted the best cutoff useful to discriminate PCa from BPH patients, showing sensitivity and specificity values of 80.9 and 62.0, respectively. Sensitivity and specificity values were 89.4 and 60.0 for %fPSA, respectively, and 57.4 and 92.0 for %p2PSA, respectively. This indicates that when sensitivity is high the specificity is low and *vice versa*. As regards tPSA, instead, specificity and sensitivity turned out to be both low, being 68.1 and 44.0, respectively. At the same time, AUC for tPSA, %fPSA, %p2PSA, and PHI was 0.509, 0.729, 0.734 and 0.760, respectively. Even in this case, PHI overcomes all the other markers, indicating that this test reveals the best diagnostic performance for PCa diagnosis (Figure 2 and Table 2).

As regards Gal3 and Galphi, Gal3 failed in showing good diagnostic performance, with sensitivity, specificity, and AUC of 57.8, 66.7, and 0.626, respectively. Conversely, Galphi shows characteristics similar to those expressed by %fPSA and %p2PSA, with sensitivity and specificity values of 51.1 and 87.5, respectively; the AUC value (= 0.740) is very close closest to that of PHI ($p = 0.81$; see Figure 2 and Table 2).

Taken together, present results indicate that the Gal3 related index, Galphi, could be taken seriously in exam as a PCa biomarker as it shows good diagnostic performances and accuracy, also in comparison with the most quoted serum tests used today in clinical practice.

Lastly, a possible correlation between all the screened biomarkers and tumor pathological characteristics was investigated. Subjects with organ-confined PCa and Gs $\geq$ 7 have a worst prognosis than those with Gs $\leq$ 6, even following radical prostatectomy or radiation therapy [33-35]. To recognize a low grade from a more aggressive PCa is therefore essential for therapeutic

purposes, but currently the only way to discriminate patients with low or high grade PCa is to perform a biopsy. The possibility of using p2PSA and its related indices as prognostic markers is desirable, but the possibility to evaluate tumor staging and aggressiveness by these tests is openly debated [15,16,31,32,36-38]. In our case, we considered all the 47 men with PCa, all with an organ-confined cancer, and split this groups in two classes of increasing tumor extent: pT2 (n = 42) and pT3 (n = 5). Comparing the distribution of all parameters between the two subgroups, no significant difference was found (Table 3); however, looking at the distribution of PHI and Galphi, it could be noted that both markers tend to rise moving from the pT2 towards the pT3 group, resulting Galphi the more sensitive (p = 0.44 *versus* 0.36).

The same 47 PCa patients were then split in two classes of increasing tumor aggressiveness: Gs $\leq$ 6 (n = 13) and Gs $\geq$ 7 (n = 34). Also in this case none of the examined variables showed a different distribution between the two groups (Table 4). However, focusing on the PHI and Galphi distribution, only the Galphi parameter increases from $\leq$ 6 to $\geq$ 7 subgroups, even if the difference was not statistically significant (p = 0.17). All these data suggest that none of the selected markers could recognize, among PCa subtypes, the more extended and aggressive forms; however it could be a false result, driven from the low number of patients, principally belonging to the pT3 and Gs $\geq$ 7 groups. Ultimately, the relationship between tumor extent and aggressiveness versus p2PSA, Gal3 and their related indices should be reviewed in the light of larger studies, aimed at the research of a hypothetical prognostic value of these markers in the PCa pathology.

Conclusions

Early diagnosis for PCa is currently based, along with other diagnostic tools, on the PSA serum test. This marker, however, showed clear limits because it is not cancer specific and it is abundantly released in the bloodstream even in case of BPH and prostatitis. Several studies revealed that the

p2PSA derivatives %p2PSA and PHI are able to improve the PSA performance, demostrating a better discrimination between PCa and BPH. In parallel, only few studies have been conducted on Gal3 as a possible PCa-related marker and no evidence of its use as a diagnostic tool in clinical practice was firmly highlighted. The present retrospective study was conducted on subjects with a diagnosis of PCa or BPH that was confirmed by the histopathological analysis conducted after RP or TURP surgery. Our data show that, for men with tPSA between 2 and 10 ng/ml, the p2PSA derivatives have a diagnostic accuracy greater than tPSA and f/tPSA tests, with PHI resulting the most accurate. Moreover, the Gal3 serum test fails to predict PCa, but the index of our conception, the Galphi, is able to reach good diagnostic performances, in some respects comparable to that obtained from p2PSA related indices. Our data suggest that the p2PSA derivatives show good performances in descriminating patients with malignant or benign prostatic pathologies and, for the first time, that a Gal3 related index may be used for the same purposes.

For the future, an increase in the number of PCa and BPH screened patients will be needed, as well as the organization of several independent studies, in order to confirm the diagnostic usefulness of Galphi, to upgrade its prognostic role and to understand if its application could be extended to patients who show tPSA values out of the gray zone.

Abbreviations

95% CI: 95% confidence intervals; %fPSA: (fPSA / tPSA) × 100; %p2PSA: (p2PSA / fPSA) × 100; AUC: area under the curve; BPH: benign prostatic hyperplasia; CLIA: chemiluminescence immunoassay; ECLIA: electrochemiluminescence immunoassay; fPSA: free PSA; Gal3: Galectin-3; Galphi: Gal3 related prostate health index, (tPSA × Gal3) / fPSA; Gs: Gleason score; M0: tumors without distant metastasis; N0: tumors without regional lymph node metastasis; OR: Odds Ratio; p2PSA: [-2]proPSA; PCa: prostate cancer; PHI: prostate health index; PSA: prostate specific

antigen; ROC: receiver operating characteristics; RP: radical prostatectomy; SE: standard error; T: tumor grading; tPSA: total PSA; TURP: TransUrethral Resection of the Prostate.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the study: LT, PA, MG, GD, LC

Performed the laboratory analysis: LT, AA, FDB

Performed the urological analysis: MG

Performed the histopathological analysis: SS

Analyzed the data: IS

Contributed reagents/materials/analysis tools: SS, PA, MG, GD, LC

Wrote the manuscript: LT

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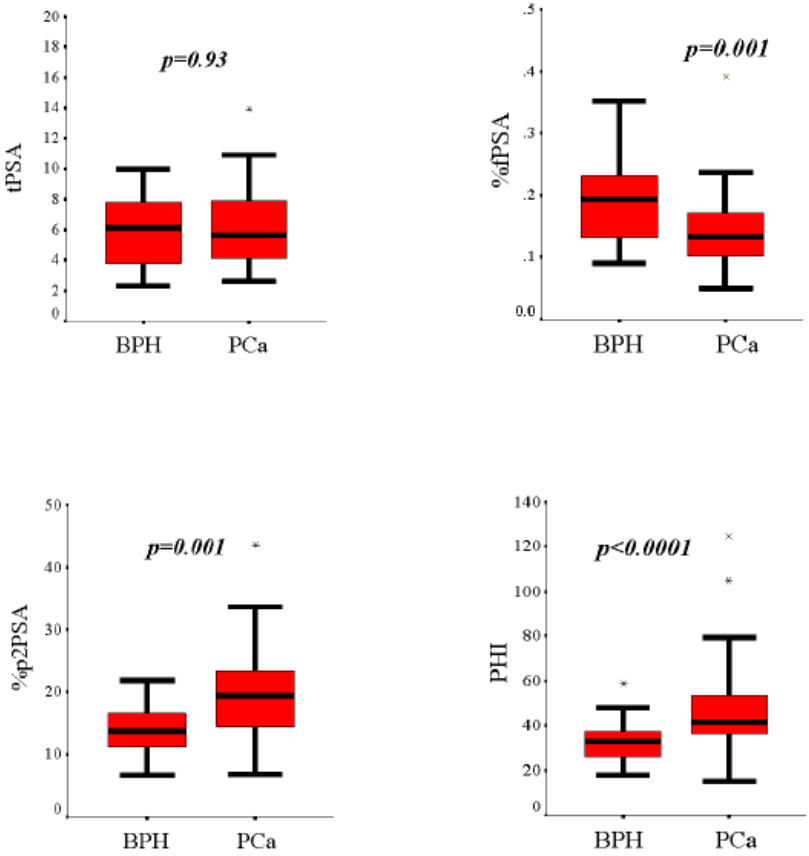
Torre P, Fowler L, Roux J, Guazzoni G. Clinical performance of serum prostate-specific antigen isoform [-2]proPSA (p2PSA) and its derivatives, %p2PSA and the prostate health index (PHI), in men with a family history of prostate cancer: results from a multicentre European study, the PROMEtheuS project. BJU Int. 2013 Aug;112(3):313-21.

Figures

Figure 1. tPSA (A), %fPSA (B), %p2PSA (C), PHI (D), Gal3 (E), and Galphi (F) values for patients with PCa and BPH.



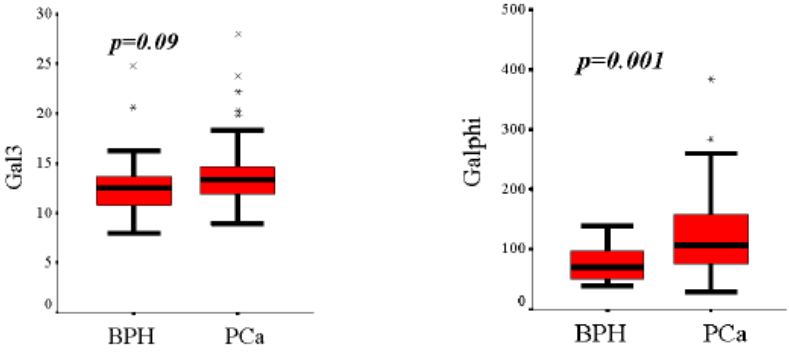
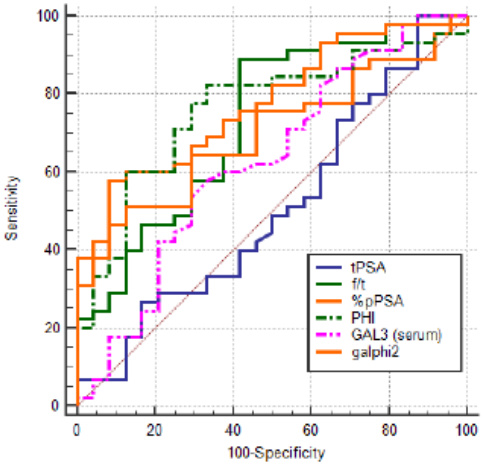


Figure 2. AUC values of tPSA (0.509), %fPSA (0.729), %p2PSA (0.734), PHI (0.760), Gal3 (0.626), and Galphi (0.740) by ROC analysis.



Tables

Table 1. Age, tPSA, %fPSA, %p2PSA, PHI, Gal3, and Galphi median values of PCa, BPH, and healthy patients.

	PCa	BPH	healthy	*p* value		
				PCa vs BPH	PCa vs healthy	BPH vs healthy
Number	47	25	15	/	/	/
Age (median±SD)	62±12	60±13	61±9	0.31	0.39	0.37
tPSA (ng/ml) (median±SD)	5.61±2.49	6.11±2.38	0.7±0.3	0.93	<0.0001	<0.0001
%fPSA (median±SD)	13±6	19±7	/	0.001	/	/
%p2PSA (median±SD)	1.94±0.73	1.38±0.36	/	0.001	/	/
PHI (median±SD)	41.5±20.4	32.8±9.2	/	<0.0001	/	/
Gal3 (ng/ml) (median±SD)	13.3±3.8	12.4±3.7	12.6±3.5	0.09	0.002	0.1
Galphi (median±SD)	107.2±72.3	69.1±28.2	/	0.001	/	/

Table 2. Area under the curve (AUC), with standard error (SE), sensitivity, and specificity of tPSA, %fPSA, %p2PSA, PHI, Gal3, and Galphi.

Variable	AUC	SE	Criterion	Sensitivity	Specificity
tPSA (ng/ml)	0.509	0.0757	≤7.09	68.1	44.0
%fPSA	0.729	0.0655	≤18.4	89.4	60.0
%pPSA	0.734	0.0593	>1.77	57.4	92.0
PHI	0.760	0.0600	>34.8	80.9	62.0
Gal3 (ng/ml)	0.626	0.0738	>13	57.8	66.7
Galphi	0.740	0.0605	>104.2	51.1	87.5

Table 3. Correlation between tumor staging (T) and all parameters in the subgroup of patients with PCa (n = 47).

	T2	T3	p value
Number	42	5	/
tPSA (ng/ml) median±SD	5.7±2.4	4.53±2.9	0.44
fPSA (ng/ml) median±SD	0.76±0.4	0.75±0.3	0.18
p2PSA (pg/ml) median±SD	14.5±5.7	12.0±5.3	0.27
%fPSA median±SD	14±6	10±4	0.16
%p2PSA median±SD	1.84±0.76	2.20±0.46	0.34
PHI median±SD	41.1±21.4	42.4±11.5	0.44
Gal3 (ng/ml) median±SD	13.3±3.9	12.0±1.9	0.1
Galphi median±SD	94.6±75.7	109.2±38.0	0.36

Table 4. Correlation between tumor aggressiveness, expressed in terms of Gleason score (Gs), and the analyzed parameters in the subgroup of patients with PCa (n = 47).

	Gs ≤ 6	Gs ≥ 7	*p* value
Number	13	34	/
tPSA (ng/ml) median±SD	4.65±2.1	5.79±2.6	0.10
fPSA (ng/ml) median±SD	0.74±0.3	0.76 (0.4)	0.30
p2PSA (pg/ml) median±SD	14.1±5.7	14.4±5.7	0.34
%fPSA median±SD	14±4	12±6	0.32
%p2PSA median±SD	1.83±0.58	1.98±0.79	0.40
PHI median±SD	43.9±13	41.0±22.6	0.19
Gal3 (ng/ml) median±SD	13.3±5.4	13.2±3.1	0.13
Galphi median±SD	78.2±63.8	109.9±65.7	0.17

Perioperative changes in pro and anticoagulant factors in prostate cancer patients undergoing laparoscopic and robotic radical prostatectomy with different anaesthetic techniques

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Perioperative changes in pro and anticoagulant factors in prostate cancer patients undergoing laparoscopic and robotic radical prostatectomy with different anaesthetic techniques

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Abstract

Background: Laparoscopic prostatectomy (LRP) may activate clotting system influencing the risk of perioperative thrombosis in patients with prostate cancer. Moreover, different anaesthetic techniques can also modify coagulant factors. Thus, the aim of this study was to investigate the effects on pro- and anti-coagulant and fibrinolytic factors of two established types of anaesthesia in patients with prostate cancer undergoing elective LRP.

Methods: 102 patients with primary prostate cancer, who underwent conventional LRP or robot-assisted laparoscopic prostatectomy (RALP), were studied and divided into 2 groups to receive total intravenous anesthesia with target-controlled infusion (TIVA-TCI) or balanced inhalation anaesthesia (BAL) prior to surgery. Before the induction of anaesthesia (T0), 1 hr (T1) and 24 hrs post-surgery (T2), some pro-coagulant factors, fibronolysis markers, p-selectin and haemostatic system inhibitors were evaluated.

Results: Both TIVA-TCI and BAL patients showed a marked and significant increase in pro-coagulant factors and consequent reduction in haemostatic system inhibitors in the early post operative period ($p \leq 0.004$ for each markers). Use of RALP showed a significant increase in prothrombotic markers as compared to LRP. In TIVA patients undergoing LRP, a significant reduction of p-selectin levels between T0 and T2 ($p = 0.001$) was observed as compared to BAL, suggesting a better protective effect on platelet activation of anaesthetic agents used for TIVA.

Conclusions: Both anaesthetic techniques significantly seem to increase the risk of thrombosis in prostate cancer patients undergoing LRP, mainly when the robotic device was utilized, encouraging the use of a peri-operative thromboembolic prophylaxis in these patients.

Keywords: TIVA-TCI anaesthesia, BAL anaesthesia, Thrombotic factors, Prostate cancer, Prostatectomy

Background

Several epidemiological studies have shown that a strong correlation exists between cancer and haemostatic system [1-4]. The interaction between cancer and the coagulation system perturbs and stimulates pro-coagulant activity, consequently inducing a pro-thrombotic state [5] and increasing the risk of thromboembolic disease (TED) [6]. Interestingly in cancer patients a systemic activation of blood coagulation has frequently been observed even in the absence of TED [2,7].

Cancer cells can activate the clotting system directly, thereby generating thrombin, or indirectly by stimulating mononuclear cells, platelets and endothelial cells to synthesize and express a variety of procoagulants [8]. The consequent formation of a fibrin matrix appears to promote tumor growth by favoring neoangiogenesis and shielding tumor cells against attack from immuno-competent cells [5]. Thrombin also works as a potent promoter of cancer growth and spread via an increase in tumor cell adhesion [9]. Some biomarkers have been

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specifically investigated for their capacity to predict TED during the course of cancer disease. Associations between elevated levels and future TED have been found for D-Dimer, prothrombin fragment 1 + 2 (F1 + 2), thrombin-antithrombin complexes (TAT), plasminogen activator inhibitor type 1 (PAI-1), clotting factor VIII (FVIII) and soluble P-selectin [10]. These markers, not sufficiently validated in patients undergoing different intraoperative anaesthetic regimens, reflect different steps of the coagulation cascade (Figure 1). In particular, F1 + 2 is released when activated factor X cleaves prothrombin into active thrombin and the fragment formation is a key event in the coagulation cascade. The formation of TAT complexes represents an indirect measure for the activation of the coagulatory system, because is the first amount of thrombin that binds to antithrombin (AT). Elevated FVIII levels are a well-established risk factor for first manifestation and for recurrence of TED. PAI-1 is a potent inhibitor of the fibrinolytic system while d-dimer is a stable end product of fibrin degradation and is elevated by enhanced fibrin formation and fibrinolysis [10-12]. P-selectin, a member of cell adhesion molecules, is released from the α-granules of activated platelets and from Weibel-Palade bodies of endothelial cells. P-selectin plays a crucial role in thrombogenesis and induces a prothrombotic state by the adhesion of platelets and leukocytes to cancer cells. Levels of soluble P-selectin are elevated in patients with acute TED [13].

Surgical tissue trauma also leads to an increased risk of TED [14] even though the incidence of TED is closely related to the organ involved. The tumor sites most at risk of developing TED seem to be the pancreas, brain, and stomach [14]. In patients with advanced prostate cancers, the incidence of TED is controversial, ranging from 0.5% to 40% in the first month after surgery [3,15-17]. The increased risk of TED in prostate cancer patients undergoing radical prostatectomy recommends administering a pharmacologic anti-thrombotic prophylaxis [18-22], though the latter may cause an increase in intra-operative bleeding [23,24] .

To date, factors influencing the risk of perioperative thrombosis in patients undergoing prostate cancer surgery have not been identified yet. At present, we do not know whether, in addition to the risk factors already known, the use of different techniques of anesthesia may increase the risk of thrombosis in cancer patients undergoing surgery. Therefore, the main aim of this prospective study was to investigate changes in the markers most sensitive to detecting activation of the haemostatic system in patients with prostate cancer undergoing elective laparoscopic prostatectomy with two different intra-operative anaesthetic regimens, target-controlled infusion (TIVA-TCI) and balanced inhalation anaesthesia (BAL). A secondary aim was to evaluate whether using a robot device in the laparoscopic prostatectomy influences the effect of different anesthetic techniques applied.

Methods

Patient population

Between October 2009 and June 2012, 400 consecutive patients with primary prostate cancer, undergoing general anaesthesia and conventional laparoscopic radical

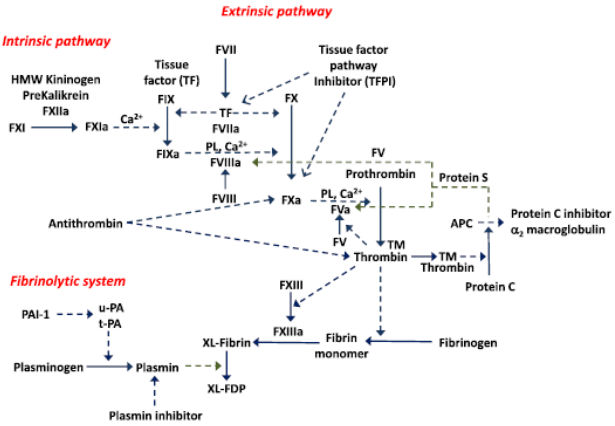

Figure 1 Coagulation cascade. The solid lines indicate a activating function, while the dashed lines a inhibitory action.

prostatectomy (LRP) or robot-assisted laparoscopic prostatectomy (RALP), were considered eligible for the study (Figure 2). This study was approved by the Ethics Committee of the Regina Elena National Cancer Institute, Rome (Prot.CE/550), and a written informed patient consent was obtained from all participants. Protocol was registered in *Clinical trials.gov* (NCT01998685). The inclusion criteria for the study were a newly diagnosed cancer of the prostate with histological Gleason score evaluation. Exclusion criteria included: (a) ASA >2, (b) metabolic equivalent task < 4, (c) BMI > 30, (d) no pre-operative pharmacological thromboprophylaxis and/or anti-coagulant therapy, (e) history of abnormal bleeding, or abnormal coagulant factors, (f) sepsis within the last 2 weeks, (g) previous new adjuvant treatments (chemo, hormone, and radiotherapy), (h) non-steroid, anti-inflammatory and statin drugs for at least 2 wks before surgery, (i) venous or arterial thromboembolism within the last 3 months, peripheral venous disease, (l) neurological disease with extremity paresis, (m) chronic liver disease, (n) pre-operative haemoglobin concentration < 9 mg dl^{-1}, (o) prolonged duration of surgery (>3 hrs); (p) peri-operative blood transfusion, (q) inadequate material for laboratory testing. One exclusion criterion sufficed exclusion.

Out of the 400 patients with primary prostate cancer who underwent laparoscopic prostatectomy, 244 were excluded from the study for the following reasons: 218 for ASA ≥ 3, 4 for previous new adjuvant treatments, 22 for anti-inflammatory and statin therapy before surgery.

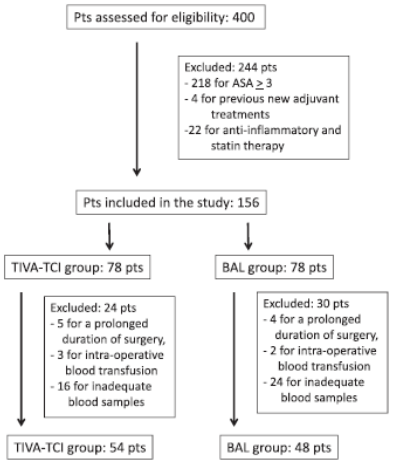

Figure 2 Design of the study: patient selection.

Thus, 156 patients with primary prostate cancer constituted the patient population of this randomized study and were alternatively divided into 2 groups to receive TIVA-TCI or BAL anaesthesia prior to surgery. Then, a further 54 patients were excluded: 9 for a prolonged duration of surgery, 5 for intra-operative blood transfusion and 40 for inadequate blood samples. Finally, 102 patients with primary prostate cancer comprised the patient population of the study: 54 received TIVA-TCI and 48 BAL anesthesia prior to surgery.

All patients with high-risk prostate cancer (according to Guidelines on Prostate Cancer of European Association of Urology, 2012) underwent LRP with extended pelvic lymph node dissection. Patients with intermediate risk underwent LRP or RALP .

Anesthetic protocol

The patients did not receive premedication. In the TIVA-TCI group, anaesthesia was induced with propofol (Diprivan™, ASTRA-Zeneca, Milano, Italy) 6 μg ml^{-1} and remifentanyl (Ultiva™, GlaxoSmith-Kline AB, Verona, Italy) 0.4-1 μg kg^{-1} min, simultaneously administered using two separate modules of a continuous computer-assisted TCI system. Anaesthesia was maintained with propofol 4 μg ml^{-1} and remifentanil 0.25 μg Kg^{-1} min. This infusion was modified by 0.05 μg kg^{-1} min steps according to analgesic needs. In the BAL group, anaesthesia was induced with midazolam (Hameln pharmaceuticals Gmbh, Hameln, Germany) 0.1 mg kg^{-1} and fentanyl (Fentanest™, Pfizer, Latina, Italy) 1.5 μg kg^{-1} Anaesthesia was maintained with sevoflurane (Sevorane™, Abbott, Latina, Italy) 2.0% , oxygen 40% and air 70% with positive pressure ventilation in a circle system, in order to achieve normocapnia.

In both groups, cisatracurium besylate (Nimbex™, Glaxo Smith Kline) 0.1-0.5 mg kg^{-1} was given to facilitate orotracheal intubation with a cuffed tube, followed by the continuous application of 0.06-0.12 mg kg^{-1} h^{-1} via infusion pumps. Pneumoperitoneum was created by intraperitoneal insufflation of CO_2 with an insufflation pressure of 13–15 mmHg and patient in the supine position. Patients were then placed in the steep Trendelenburg position (30° from horizontal). Intraperitoneal pressure was maintained at 15 mmHg during the induced pneumoperitoneum. A routine anaesthesia monitoring was performed on all patients (Table 1).

During anaesthesia all patients received warm venous infusion of saline solution (0.9% NaCl) 3 ml Kg^{-1} h^{-1} and thermal mattresses. Systolic arterial pressure was maintained at 100 mm Hg or 70% of the preoperative value. Hypotension was treated with crystalloid fluid infusion or intravenous boluses of ephedrine.

After surgery the residual neuromuscular blockade was reversed with a mixture of atropine (Galenica Senese, Siena, Italy) 1.5 mg and neostigmine (Intrastigmina™,

Table 1 Clinical characteristics and peri-operative data of patients with prostate cancer who underwent surgery with TIVA-TCI or BAL anaesthesia

	TIVA-TCI (n. 54)	BAL (n. 48)	P
Clinical data			
Age (yrs)	60.66 (5.91)	62.16 (6.23)	0.31
Venous thromboembolism risk			
Highest risk	54 (100%)	48 (100%)	1
Prostate cancer risk*			
Intermediate-risk	26 (48.1%)	30 (62.5%)	
High-risk	28 (51.8%)	18 (37.5%)	0.17
ASA, n (%):			
I	4 (7.4%)	6 (12.5%)	
II	50 (92.6%)	42 (87.5%)	0.39
Histological grade of cancer			
G2 (Gleason 5–6)	15 (27.8%)	14 (29.2)	
G3 (Gleason 7–10)	39 (72.2%)	34 (70.8%)	0.88
pT, n (%)			
2	30 (55.6%)	32 (66.7%)	0.25
3	24 (44.4%)	16 (33.3%)	
pN, n (%) #			
0	17 (85.0%)	24 (96.0%)	0.20
1	3 (15.0%)	1 (4.0%)	
Peri-operative data			
Type of surgery			
LRP	36 (66.7%)	34 (70.8%)	0.65
RALP	18 (33.3%)	14 (29.2%)	
Time of anaesthesia (min)	107.5 (16.8)	101.4 (26.2)	0.26
Blood loss (ml)	123.3 (131.1)	121.4 (110.6)	0.81
Total amount of crystalloid received (ml)	468.5 (110.21)	496.8 (198.5)	0.27
Intra-operative body temperature	36.2 (0.3)	36.1 (0.2)	0.83
Intra-operative MAP (mmHg)	104.6 (10.5)	106.2 (10.2)	0.61
Intra-operative SpO2 (%)	96.7 (0.9)	97.8 (1.8)	0.75
Arterial lactate level (mmol/l)			
1 h post-surgery	0.7 (0.2)	0.6 (0.4)	0.32
24 h post-sugery	1.7 (0.2)	1.8 (0.2)	0.82
Intra-operative BE (mmol/l)	0.3 (0.4)	0.4 (0.4)	0.62
Intra-operative PaO2 (mmHg)	219.4 (11.2)	216.5 (16.8)	0.72

Values are expressed in absolute values or mean (SD).
Abbreviations: *TIVA-TCI* total intravenous anaesthesia with target-controlled infusion, *BAL* balanced inhalation anaesthesia, *LRP* conventional laparoscopic radical prostatectomy, *RALP* robot-assisted laparoscopic prostatectomy.
*According to Guidelines on Prostate Cancer, European Association of Urology, 2012.
#Lymph node dissection was made in 45 out of 102 pts.

Lusofarmaco, Milano, Italy) 2.5 mg. Anaesthetic agents were switched off, and 100% O_2 was given with 8 l min fresh gas flow for 1 min. In addition, a forced-air warming blanket was used post-surgery (Equator Covective Warming ^{TM}, Smith Medical Italia, Milano, Italy).

After tracheal extubation all patients received ketoralac trometamina (Toradol, Recordati, Milano, Italy) 30 mg, ranitidine (Ranidil^{TM}, Menarini, Firenze, Italy) 50 mg and morphine (Recordati) 2 mg in bolus and then by a controlled analgesia device (Deltec^{TM}, Smiths Medical ASD, St Paul, MN).

Clinical parameters

The risk of venous thromboembolism was evaluated according to the model proposed by Caprini et al. [25] and Bergqvist et al. [26]. Patients were divided into 4 different levels of risk: low (score 0–1), moderate (score 2), high (score 3–4), highest (score >4). The following clinical parameters were also evaluated: (a) global assessment of anesthetic risk (ASA), (b) grading of prostate cancer (Gleason score), (c) pathological tumor-node-metastasis stage, (d) time of surgery, (e) quantity and type of liquids administered, (f) blood loss, (g) peri-operative complications such as hypertension, hyperglycemia, hypothermia, infections and pain (evaluated by a 6-point verbal rating scale: 0: no pain to 5: most severe pain imaginable).

In all patients, the presence of venous thrombosis by clinical observation, venous and pelvic ultrasound were evaluated in the peri-operative period and on days 8 and 21 after surgery.

Prophylaxis anti-thrombosis

Since in most of our patients changes in pro- and anti-coagulant and fibrinolytic markers were observed in the peri-operative period, an anti-thrombotic prophylaxis was made 24 hrs post surgery, for 4 weeks, by using Enoxaparina (Clexane^{TM}, Sanofi-Aventis, Milano) 4000 UI/die .

Prothrombotic markers

Before the induction of anaesthesia (T0), 1 hr post-surgery (T1) and 24 hrs post-surgery (T2), the following factors were evaluated: (a) procoagulant markers: fibrinogen, TAT, F1 + 2 and FVIII; (b) fibrinolysis markers: PAI-1, D-dimer; (c) platelet-aggregating properties: p-selectin; (d) hemostatic system inhibitors: AT, protein C (PC) and protein S (PS) activity.

Blood samples were collected in tubes without additives containing 3.2% sodium citrate (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ USA). Samples were centrifuged within 1 h at 2500 g for 20 min, to obtain platelet-poor plasma. The plasmas were immediately tested. Moreover, plasma and serum samples were separated and stored in multiple aliquots at −80°C for subsequent testing. All coagulation parameters (PT,

aPTT, fibrinogen, AT, D-dimer, PC, PS, FVIII) were assayed by clotting, chromogenic and immunological methods on fully-automated ACL TOP analyzer using HemosIL® commercial kits (Instrumentation Laboratory Company, Bedford, MA USA). Abnormal values were defined by the clinical laboratory or manufacturer's assay. Plasma levels of TAT and F1 + 2 were measured by enzyme-linked immunosorbent assay Enzygnost® TAT micro and Enzygnost® F1 + 2 mono kits, respectively (Siemens Healthcare Diagnostics Inc, NY USA), according to the manufacturer's instructions. Both assays employ the quantitative sandwich enzyme immunoassay technique. All samples showing values above the standard curve were re-tested with appropriate dilutions. Plasma levels of PAI-1 were measured with the enzyme-linked immunosorbent assay Asserachrom® kit (Diagnostica Stago, Asnieres, France), according to the manufacturer's instructions. Plasma p-selectina levels were determined by Human sP-Selectin enzyme immunoassay (R&D Systems, Inc Minneapolis, MN USA), according to the manufacturer's instructions, employing the quantitative sandwich enzyme immunoassay technique.

Statistical analysis

Data were analyzed with Statistical Package for the Social Sciences (SPSS) 14.0 software. Continuous and categorical variables were expressed as the mean ± standard deviation or standard error and as frequency values and proportions, respectively. Pearson's chi-square test was used to assess possible differences in dichotomous variables between the various groups examined. The means of normally distributed data were compared with the Student's *t*-test. In other cases, the groups were compared with the Mann-Whitney's *U* test. P values of the tests were adjusted using the Bonferroni method. Paired samples were analyzed by *t*-test and Wilcoxon Signed Ranks Test. Multiple linear regression was used in order to test the effect of anaesthesia, surgery and clinical characteristics of patients on changes of prothrombotic markers 24 h post-surgery (T2 time). A p-value of <0.05 was considered statistically significant.

Results

Clinical characteristics of the patients

The clinical characteristics of the patients enrolled in the study are reported in Tables 1 and 2. No significant differences were observed regarding age between TIVA-TCI and BAL patients.

Thirty-two out of 102 patients (31.4%) underwent RALP and were equally distributed between the TIVA-TCI and BAL. The lymph node dissection was made in 45 out of 102 pts (44.1%).

All patients were at highest risk of venous thromboembolism, according to the model proposed by Caprini et al. [25] and Bergqvist et al. [26] (being all neoplastic and undergoing surgery); 10 of these (9.8%) had an ASA I whereas 92 (90.2%) an ASA II.

Thirty-nine patients of TIVA-TCI group (72.2%) and 34 of BAL group (70.8%) showed a high grade prostatic carcinoma (G3) with Gleason score ≥7.

Patients undergoing LRP showed a locally more advanced tumor (pT3) as compared to those treated with RALP (Table 2). No significant differences were observed regarding lymph node involvement (pN). The mean duration of anesthesia was 103.8 ± 26.1 min, with no differences between the TIVA-TCI and BAL groups ($p = 0.26$).

During surgery a light decrease in hematocrit and hemoglobin concentration was observed in both groups, but intra-operative blood loss was similar. Also, the volume of crystalloid administered during anaesthesia was similar in both groups. Similarly, no statistical differences were observed regarding hemodynamic and respiratory parameters. None of the patients experienced adverse clinical events during their postoperative course.

In all patients no TED was observed in the postoperative period and in a 2-yr follow-up. This is probably due to the anti-thrombotic prophylaxis which was carried out for ethical reasons in all patients 24 hrs post surgery because intra-operative changes of some pro-coagulant markers were observed. Lymph node metastases were detected in only 4 out of 45 patients with lymph node dissection (8.9%): one in the TIVA-TCI group and 3 in the BAL group ($p = 0.32$).

Types of anaesthesia and prothrombotic markers

Changes of prothrombotic markers associated with the use of different techniques of anesthesia are reported in Tables 3 and 4. No statistically significant differences were observed in the baseline values of biomarkers (at T0) between TIVA-TCI and BAL groups, even when we considered the type of surgery. In both TIVA-TCI and BAL patients a significant and continuous reduction in screen clotting time PT (given as percentage) was observed during post-surgery period (T2) as compared to T0 ($p = 0.001$), while aPTT was shortened at T1 and then normalised on the first postoperative day (T2).

At the end of surgery (T1), both TIVA-TCI and BAL patients showed a marked and significant increase in pro-coagulant factors (TAT, F1 + 2 and FVIII) and consequent reduction in haemostatic system inhibitors (AT, PC and PS) compared to the values measured prior to surgery ($p \leq 0.004$ for each markers). The greatest increase was observed in the values of TAT and F1 + 2 (about 3 times compared to T0), while the values of FVIII increased approximately 30%. F1 + 2 and FVIII slightly reduced at T2 but remained significantly higher than basal levels ($p \leq 0.04$ for each markers). Only TAT values returned to pre-anaesthesia values. We observed

Table 2 Clinical characteristics and peri-operative data of patients with prostate cancer, divided in 4 subgroups according type of anesthesia and surgery

	TIVA-TCI LRP (n. 36 pts)	TIVA-TCI RALP (n. 18 pts)	BAL LRP (n. 34 pts)	BAL RALP (n. 14 pts)	P
Clinical data					
Age (yrs)	61.4 (5.7)	59.5 (6.7)	63.2 (5.8)	60.1 (7.7)	0.25
ASA, n (%):					
I	3 (8.3%)	1 (5.6%)	5 (14.7%)	1 (7.1%)	0.68
II	33 (91.7%)	17 (94.4%)	29 (85.3%)	13 (92.9%)	
Histological grade of cancer					
G2 (Gleason 5–6)	9 (25.0%)	6 (33.3%)	10 (29.4)	4 (28.6)	0.93
G3 (Gleason 7–10)	27 (75.0%)	12 (66.7%)	24 (70.6%)	10 (71.4%)	
pT, n (%)					
2	12 (33.3%)	18 (100%)	18 (52.9%)	14 (100%)	0.001
3	24 (66.7%)	0	16 (47.1%)	0	
pN, n (%)*					
0	11 (84.6%)	6 (85.7%)	14 (93.3%)	10 (100%)	0.57
1	2 (15.4%)	1 (14.3%)	1 (6.7%)	0	
Perioperative data					
Time of anaesthesia (min)	104.0 (21.3)	109.7 (24.4)	98.8 (30.2)	105.2 (24.8)	0.32
Blood loss (ml)	119.2 (140.3)	128.3 (150.1)	118.2 (121.4)	125.2 (131.5)	0.30
Total amount of crystalloid received (ml)	475.4 (100.4)	460.8 (118.4)	486.1 (166.4)	499.8 (200.2)	0.21
Intra-operative body temperature	36.2 (0.3)	36.1 (0.4)	36.1 (0.2)	36.1 (0.3)	0.87
Intra-operative MAP (mmHg)	103.8 (11.8)	105.3 (12.5)	105.4 (12.4)	106.8 (12.2)	0.54
Intra-operative SpO2 (%)	96.7 (0.9)	96.7 (0.9)	97.8 (1.8)	97.8 (1.8)	0.75
Arterial lactate level (mmol/l)					
1 h post-surgery	0.7 (0.2)	0.7 (0.3)	0.6 (0.3)	0.6 (0.4)	0.81
24 h post-sugery	1.8 (0.3)	1.7 (0.2)	1.7 (0.3)	1.8 (0.3)	0.77
Intra-operative BE (mmol/l)	0.3 (0.4)	0.4 (0.3)	0.3 (0.4)	0.4 (0.3)	0.78
Intra-operative PaO2 (mmHg)	220.6 (13.2)	218.8 (13.4)	214.6 (18.6)	219.5 (19.0)	0.22

Values are expressed in absolute values or mean (SD).
Abbreviations: *TIVA-TCI* total intravenous anaesthesia with target-controlled infusion, *BAL* balanced inhalation anaesthesia, *LRP* laparoscopic radical prostatectomy, *RALP* robot-assisted laparoscopic prostatectomy.
*Lymph node dissection was made in 45 out of 102 pts.

a corresponding increase in anti-coagulant factors that remains significantly lower than prior to surgery ($p = 0.001$).

Fibrinogen levels significantly decreased at T1 in comparison to the initial values, but rose significantly 24 hours post-surgery in both groups, showing an increase of about 20-30% as compared to T0 values ($p = 0.001$).

Changes in pro-coagulant factors and haemostatic system inhibitors were similar in both TIVA-TCI and BAL patients with no significant differences between the two groups of patients. In regards to the fibrinolysis system, D-dimer concentration in TIVA-TCI group, levels increased about 6-fold at T1 compared to baseline level ($p = 0.001$, Table 3), while in BAL patients it showed an increase of about 4-fold ($p = 0.001$, Table 4). Both groups showed a decrease of D-dimer at T2 even if the concentration remained higher than baseline levels ($p = 0.001$), with no significant differences between TIVA-TCI and BAL patients.

Levels of the PAI-1, the principal inhibitor of the fibrinolysis system, and D-dimer remained constant between T0 and T1 but significantly increased at T2 in both groups.

Grading of prostate cancer evaluated by Gleason score and pathological tumor stages showed no significant effects on changes in prothrombotic markers observed both in the TIVA and BAL groups. Similarly, it was observed for all other clinical parameters analyzed.

Surgery and prothrombotic markers

Multivariate analysis demonstrated that only p-selectin was significantly correlated to the type of anesthesia and

Table 3 Changes of prothrombotic markers in patients with prostate cancer who underwent surgery with total intravenous anesthesia with target-controlled infusion (TIVA-TCI) before the induction of anaesthesia (T0), 1 hr post-surgery (T1) and 24 hrs post-surgery (T2)

	T0	T1	T2	P		
				T0 vs T1	T1 vs T2	T0 vs T2
Screen clotting time						
- PT (%)	93.1 (1.3)	85.6 (1.2)	82.5 (1.2)	0.001	0.21	0.001
- PTT (sec)	29.6 (0.6)	26.8 (0.7)	27.6 (0.8)	0.003	0.07	0.18
Procoagulant markers						
- Fibrinogen (mg/dL)	285.5 (7.1)	262.3 (6.6)	353.3 (8.8)	0.004	0.001	0.001
- TAT (ng/L)	9.1 (1.9)	22.8 (3.2)	9.7 (2.4)	0.002	0.004	0.79
- F1 + 2 (pmol/L)	210.8 (27.3)	622.1 (64.2)	364.4 (45.6)	0.001	0.001	0.007
- FVIII (%)	142.9 (8.1)	194.2 (9.3)	162.3 (5.6)	0.001	0.004	0.04
Fibrinolysis markers						
- PAI-1 (ng/ml)	15.2 (1.4)	21.9 (5.8)	36.1 (9.8)	0.41	0.20	0.04
- D-dimer (μg/L)	127.1 (12.8)	721.4 (170.4)	364.2 (28.3)	0.001	0.02	0.001
Haemostatic system inhibitors						
- AT (%)	102.1 (1.8)	90.6 (1.9)	87.4 (2.4)	0.001	0.38	0.001
- protein C (%)	109.6 (2.8)	95.4 (2.8)	87.8 (2.8)	0.004	0.03	0.001
- protein S (%)	93.8 (3.1)	84.2 (2.8)	82.4 (2.4)	0.01	0.56	0.001
Platelet-aggregating properties						
- p-selectin (ng/ml)	37.9 (2.0)	36.8 (2.4)	33.5 (2.6)	0.78	0.37	0.28

Values are mean (SD).

surgery ($p = 0.01$). It is very important to note that the TIVA-TCI patients undergoing LRP showed a significant reduction in p-selectin levels between T0 and T2 ($p = 0.001$) while no changes were observed in the BAL group that did not use the robotic device (Figure 3). In contrast, a significant increase of p-selectin value was observed in patients undergoing RALP, regardless of the type of anesthesia, both 1 and 24 hours after surgery.

Patients undergoing RALP showed also 24 hrs after surgery (T2), at univariate analysis, a greater reduction of PS, an inhibitor of haemostatic system, as compared to patients undergoing LRP ($p = 0.02$) independent of the type of anaesthesia applied.

Discussion

Results of our study have demonstrated that both anaesthetic techniques seem to increase the risk of TED in prostate cancer patients undergoing LRP, mainly when the robot device was utilized, suggesting, therefore, the utility of a peri-operative thromboembolic prophylaxis. In fact, both TIVA-TCI and BAL patients showed a marked and significant increase in pro-coagulant factors and consequent reduction in haemostatic system inhibitors in the early post operative period ($p \leq 0.004$ for each markers). However, this effect could be linked also to surgical stress, although the latter seems to have an independent effect only for p-selectin, as demonstrated by multivariate analysis. Moreover, the significant reduction of p-selectin levels between T0 and T2 ($p = 0.001$) observed in TIVA patients undergoing LRP, although this group of patients was composed mainly of patients at high-risk prostate cancer (as reported in Table 1), demonstrated that general anaesthetic agents used for TIVA have a better protective effect on the platelet activation in this subgroup of patients.

The evaluation of markers detecting activation of the hemostatic system represents a more sensitive way to assess the risk of thromboembolism as compared to the clinical assessment of TED. In our study, the activation of haemostatic system associated with thromboembolic risk was estimated by measuring levels of thrombin activation markers. TAT, PF1 + 2 and FVIII increased in the immediate post operative period and gradually returned to near baseline levels. The peri-operative activation of coagulation also caused an increased of peri-operative PAI-1 levels, a potent inhibitor of fibrinolysis. The activation state persists during surgery and is independent of the anaesthetic agents used. These results confirm previous studies performed on patients undergoing major abdominal surgery for colon-rectal cancer [27], hepatic cancer resection [28], pneumonectomy for lung cancer [29].

No studies had previously examined whether different intra-operative anaesthetic regimens (TIVA-TCI *vs.* BAL) could cause different intra-operative profiles of highly

Table 4 Changes of prothrombotic markers in patients with prostate cancer who underwent surgery with balanced inhalation anaesthesia (BAL) before the induction of anaesthesia (T0), 1 hr post-surgery (T1) and 24 hrs post-surgery (T2)

	T0	T1	T2	P		
				T0 vs T1	T1 vs T2	T0 vs T2
Screening clotting time						
- PT (%)	91.4 (1.4)	86.8 (1.6)	81.8 (1.4)	0.007	0.02	0.001
- PTT (sec)	30.1 (0.4)	26.2 (0.7)	28.3 (0.6)	0.001	0.02	0.01
Procoagulant markers						
- Fibrinogen (mg/dL)	318.5 (8.6)	301.3 (10.9)	372.4 (11.2)	0.21	0.001	0.001
- TAT (ng/L)	6.2 (0.8)	19.2 (3.1)	6.7 (0.8)	0.002	0.002	0.42
- F1 + 2 (pmol/L)	182.4 (11.8)	558.1 (65.6)	266.8 (19.2)	0.001	0.001	0.001
- FVIII (%)	123.4 (4.8)	228.2 (15.8)	169.2 (6.2)	0.001	0.001	0.001
Fibrinolysis markers						
- PAI-1 (ng/ml)	14.1 (1.4)	21.7 (15.8)	22.6 (2.4)	0.16	0.86	0.002
- D-dimer (μg/L)	175.5 (22.6)	622.1 (175.4)	421.3 (30.6)	0.003	0.07	0.001
Haemostatic system inhibitors						
- AT (%)	97.8 (1.7)	92.0 (1.7)	89.1 (1.8)	0.04	0.25	0.001
- protein C (%)	105.2 (3.8)	99.3 (2.7)	88.5 (2.7)	0.18	0.03	0.001
- protein S (%)	95.6 (2.4)	91.2 (2.4)	81.8 (2.6)	0.08	0.01	0.001
Platelet-aggregating properties						
- p-selectin (ng/ml)	41.5 (2.7)	40.7 (2.9)	40.2 (2.8)	0.65	0.88	0.18

Values are mean (SD).

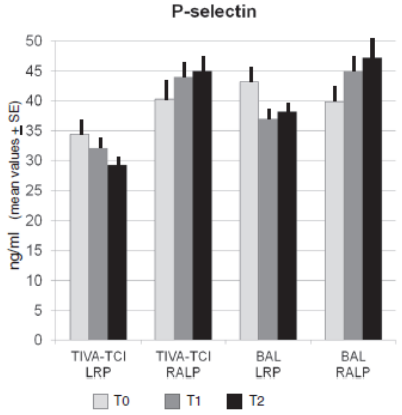

Figure 3 Changes of p-selectin levels between T0 (before the induction of anaesthesia) and T2 (24 hrs post-surgery) in patients undergoing conventional laparoscopic radical prostatectomy (LRP) or robot-assisted laparoscopic prostatectomy (RALP). TIVA-TCI patients undergoing LRP showed a significant reduction in p-selectin levels between T0 and T2 ($p = 0.001$) while no changes were observed in the BAL group. In contrast, a significant increase of p-selectin value was observed 24 hours after surgery (T2) in patients undergoing RALP, regardless of the type of anaesthesia.

sensitive and specific coagulation and fibrinolysis markers in prostate cancer patients undergoing a highly standardized type of surgery (LRP or RALP). In this context, the results of our study seem to provide useful information in reducing the peri-operative trombo-embolic risk and improving the prognosis of cancer patients undergoing LRP and RALP.

Even though cancer patients who undergo surgery are targeted for thromboprophylaxis, widespread use of prophylaxis could determine the risk of intra-operative bleeding [23,24] and a detrimental effect rather than a benefit. This problem is evident in prostate cancer patients undergoing surgery, especially in view of the increasingly frequent use of the robotic technique that has resulted in a significant reduction of surgical complications [30,31]. Although the American and European guidelines recommend prophylaxis in patients with prostate cancer [18-22], its use is currently widely debated given the different incidence of TED observed by several authors. A multicentric analysis of a number of institutions from both Europe and the United States showed a very low incidence of TED (about 0.5%) [32]. A similar incidence (0.9%) was reported from the California Cancer Registry [4]. Conversely, Osborne et al. [14] consider patients with prostate cancer at intermediate risk of TED similar to patients with uterine, rectal, colon and liver cancer.

Prostatectomy significantly increases the incidence of TED up to 2.9% and 3.9%, as reported by Hu JC et al.

[17], irrespective of the surgical approach. Tewari et al. [33] in a recent meta-analysis on 400 original research articles on surgical treatment for prostate cancer and its complications reported that the rate of deep vein thrombosis was significantly lowest for RALP (0.3%), intermediate for LRP (0.5%) and highest for open surgery (1.0%). More recently, Van Hemelrijck et al. [16] analysed thromboembolic events following prostatectomy in about 45.000 men collected in the Prostate Cancer Database Sweden. Risk of venous thromboembolism and pulmonary embolism occurred especially in the first 2 months after surgery with the highest risk in patients undergoing open or laparoscopic surgery with pelvic lymph node dissection while laparoscopic procedures without lymph node dissection were at lowest risk. Unfortunately, in this study authors did not created separate categories for LRP and RALP as the majority of laparoscopic surgery was performed with robotic assistance. In our case series, dissection of pelvic lymph node was not an independent risk factor for TED because no significant differences were demonstrated in the values of the markers analyzed among the various subgroups of patients studied. Moreover, it should be noted that in previous studies only the clinical incidence of venous thromboembolism was measured, but not the changes of coagulation factors. In other studies many biomarkers were specifically checked for their capacity to predict venous thromboembolism during the course of cancer disease [10], but changes in these markers due to different types of surgery, such as LRP or RALP, were not evaluated. Our results are even more surprising when we consider that the anesthetic drugs used both in TIVA-TCI and BAL, in particular propofol [34] and sevoflurane [35], act by inhibiting the platelet aggregation, although with different mechanisms.

Patients underwent RALP, compared to LRP group, showed a greater reduction of inhibitors of haemostatic system, such as protein S, and the increase of p-selectin, a cell adhesion molecule on the surface of activated endothelial cells and activated platelets [13]. Data present in the literature regarding the different risk of thrombosis in patients submitted to LRP or RALP are very few. In a recent study Saily et al. [36] observed that RALP activates coagulation, and thromboprophylaxis for high-risk patients even after minimally invasive surgery may be beneficial. In particular, patients undergoing RALP showed postoperatively increased levels of fibrinogen, factor VIII, d-dimer associated to a thrombocytosis, reflecting a coagulation activity. The greater risk of thrombosis with the RALP could be also related to the surgical stress that leads RALP to a major release of inflammatory mediators [37] or a greater oxidative stress induced by ischemia–reperfusion [38], determining the endothelial dysfunction and hypercoagulability [27]. This hypothesis is outlined by the fact that no differences were observed in other factors that may cause an activation of the haemostatic system in the peri-operative period such as anemia, hypoxia, hypothermia, hemodilution, hypotension, peritoneal insufflation, and Trendelenburg position [39,40]. We do not know whether changes in pro-coagulant factors may determine the occurrence of thrombotic complications since an anti-thrombotic prophylaxis was administered for ethical reasons 24 hrs after surgery.

Our results suggest the use of a prophylaxis in all patients undergoing laparoscopic prostatectomy, in particular RALP, regardless of the type of anesthesia. Prophylaxis could not be required only in patients undergoing LRP with TIVA-TCI anaesthesia since a significant reduction in p-selectin levels between T0 and T2 ($p = 0.001$) was observed in this subgroup of patients. On the contrary, p-selectin did not change in patients undergoing LRP with BAL. Thus, the results we obtained suggest a greater inhibition effect of propofol, as compared to sevofluorane, on platelet aggregation p-selectin mediated. The different effect of propofol and sevofluorane on p-selectin levels observed in our study is in agreement with previous observations reporting that sevofluorane inhibits human platelet aggregation induced by weak antagonists such as adenosine diphosphate, but not by strong agonists like thrombin [41,42]. Propofol, on the contrary, inhibits platelet aggregation mediated by thrombin [43] that regulates also the expression of p-selectin on platelets.

Conclusions

The marked and significant increase in pro-coagulant factors and consequent reduction in haemostatic system inhibitors we observed in the early post operative period suggests that a peri-operative thromboprophylaxis may be beneficial in cancer patients undergoing laparoscopic radical prostatectomy especially when a robot-assistance is used.

Abbreviations

LRP: Laparoscopic prostatectomy; RALP: Robot-assisted laparoscopic prostatectomy; TIVA-TCI: Total intravenous anesthesia with target-controlled infusion; BAL: Balanced inhalation anaesthesia; TED: Thromboembolic disease; F1 + 2: Prothrombin fragment 1 + 2; TAT: Thrombin-antithrombin complexes; PAI-1: Plasminogen activator inhibitor type 1; FVIII: Factor VII; AT: Antithrombin; PC: Protein C; PS: Protein S.

Competing interests

Sofra M, Antenucci A, Gallucci M, Mandoj C, Papalia R, Claroni C, Monteferrante I, Torregiani G, Gianaroli V, Sperduti I and Forastiere E: No interest declared.

Authors' contributions

MS and EF contributed to conception and design of the study, acquisition, analysis and interpretation of data. AA, MG, CM and IS worked on the acquisition, analysis and interpretation of data. RP, CC, IM, GT and VG contributed to acquisition of data. All Authors were involved in drafting the manuscript or revising it critically for important intellectual content and gave final approval of the version to be published.

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The impact of different anaesthetic and surgery protocols in peri-operative variations of VEGF and vWf in patients with prostate cancer

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Abstract

Background: Angiogenesis is a highly complex and dynamic event regulated by a number of pro- and anti-angiogenic molecules. Surely one of the major pathways involved in this process is represented by the vascular endothelial growth factor (VEGF) family and its receptors. In the last years a negative regulation of VEGF pathway from von Willebrand factor (vWf) was revealed, as well as the evidence that peri-operative patients manipulation protocols are able to trigger the activation of vascularization molecules, opening a debate on whether some anaesthetic drugs or surgery approaches could increase the risk of tumor spreading and metastasization.

Methods: Peri-operative variations in serum VEGF (sVEGF), plasma VEGF (pVEGF) and plasma vWf antigen (vWf:Ag) were evaluated in 87, 73 and 45, respectively, patients with prostate cancer (PCa) who underwent laparoscopic radical prostatectomy (LRP) or robot-assisted laparoscopic prostatectomy (RALP) after total intravenous anaesthesia with target-controlled infusion (TIVA-TCI) or balanced inhalation anaesthesia (BAL). Plasma and serum sample were collected immediately before anaesthesia induction (T0) and 24 hours after radical prostatectomy (T2).

Results: Considering the variations of sVEGF, pVEGF and vWf:Ag between T0 and T2 in patients divided only for the anaesthetic regimen, no differences were found (p=0.892, 0.233 and 0.342, respectively). Dividing patients only for the surgery approach, an higher variations of pVEGF in the LRP group was found (p=0.862, 0.005 and 0.560, respectively). This difference was confirmed for the LRP/TIVA-TCI group after the division of patients for both anaesthesia and surgery (p=0.995, 0.008 and 0.086, respectively). Moreover considering the variation of pVEGF and vWf:Ag levels in all the considered group conditions, an opposite trend of the two markers was always observed.

Conclusions: This is the first study that focused on the contemporary impact of anaesthesia and surgery on *in vivo* peri-operative variations of vascularization and endothelial activation markers in PCa patients. Our data showed that the surgery approach, but not the anaesthetic regimen, could generate a significant variation of pVEGF levels during radical prostatectomy; moreover they

supported the evidence of a relationship between VEGF and vWf molecules in endothelial activation, that could be important during the cancer-related angiogenic process.

Keywords: Prostate cancer, laparoscopic radical prostatectomy, anaesthesia, vascular endothelial growth factor, von Willebrand factor.

Background

The dependence of tumor growth on the neovascularization process is a well-established aspect of cancer biology (Folkman, 1971). Angiogenesis is important for oxygen, nutrients, growth factors, hormones and proteolytic enzymes supply; moreover it influences the haemostatic factors that control the coagulation and fibrinolytic system, as well as dissemination of tumor cells to distal sites (Berger *et al.*, 2003; Mahabeleshwar *et al.*, 2007; Cairns *et al.* 2011; Hanahan *et al.,* 2011). The angiogenic process is a highly complex, dynamic event regulated by a number of pro- and anti-angiogenic molecules. Surely one of the major pathways involved in this process is represented by the vascular endothelial growth factor (VEGF) family and its receptors (Terman *et al.,* 2001; Takahashi *et al.,* 2005; Roberts *et al.,* 2013). At the same time, a second pathway that seems to be involved in cancer progression is that enhanced by the von Willebrand factor (vWf), a pro-coagulant multimeric plasma protein synthesized by endothelial cells and megakaryocytes (Franchini *et al.,* 2008). Because of its ability in promoting platelet adhesion to the subendothelium and platelet aggregation (Ruggeri, 2001), vWf has been proposed as a pro-metastasization molecule, considering that tumor cells actively interact with coagulation cascade factors and platelets to pass through the vascular endothelium and reach tissues (Nierodzik *et al.,* 1995). Results obtained more recently, however, are basically concordant in emphasizing an antitumor function of vWf, exerted by its demonstrated anti-angiogenic and pro-apoptotic effects (Shavit *et al.,* 2006) (Starke *et al.,* 2011) (Franchini *et al.,* 2013). In this light, tumor vascularization processes and tumor expression of pro- and anti-angiogenic factors are very important events, as they are strongly associated with staging and prognosis in a variety of human cancers. The vascularization process, in fact, is deeply related to the switch towards a metastatic tumor phenotype (Berger *et al.*, 2003;. De *et al.*, 2003).

It's well known, as well, that the surgical resection of a tumor in absence of distal lymph node metastases is generally resolutive. However some neoplasia, including prostate cancer (PCa), are

able to generate metastases after a few years, even if they are not detected at the moment of the surgery (Roato *et al*., 2008; Gupta *et al*., 2010; Pietras *et al.,* 2010, Meyer *et al*., 2010; Roberts *et al*., 2013). In this light, some investigations have suggested that a number of factors in the perioperative period could promote metastasization. These include surgery and its associated stress response, anaesthesia, acute pain and opioid analgesics, all of which could induce the liberation of angiogenic factors (Condon *et al*., 2004; Lee *et al.,* 2009; Gottschalk *et al*., 2010; Mao *et al*., 2013). From this point of view, one of the most affective factor seems to be the anaesthetic procedure and several works have opened a wide debate as regards the use of a regional anaesthesia (RA) in place of the classic general anaesthesia (GA). In particular, RA, aside from reducing the amount of intra-operatively required GA and postoperative opioid consumption, has been consistently shown to attenuate, respect to systemic opioid administration (*i.e.* GA), the neuroendocrine response to surgery and, therefore, peri-operative immunosuppression (Melamed *et al.,* 2003; Snyder *et al.,* 2010; Mao *et al.,* 2013). Recent retrospective analyses indicate that RA for breast and prostate cancer surgery is associated with a markedly reduced risk of tumor recurrence and metastasization compared to systemic opioid administration (Exadaktylos *et al*., 2006; Biki *et al*., 2008). This finding strengthen the hypothesis according to which an anaesthetic technique, consisting of paravertebral RA, might reduce the incidence of metastases and recurrences compared with standard volatile agent-opioid anaesthesia and analgesia (*i.e.* GA). Deepening these issues could be very important to understand if some anaesthetic drugs or technique could promote angiogenesis *in vivo* and if the administration of anti-angiogenic agents could be helpful in preventing the pro-metastasization events derived from the peri-operative manipulations. In view of these opened questions, the main aim of this study was to investigate whether two group of PCa patients, undergoing conventional laparoscopic radical prostatectomy (LRP) or robot assisted laparoscopic prostatectomy (RALP), with two different intra-operative anaesthetic regimens, total intravenous anesthesia with target-controlled infusion (TIVA-TCI) and balanced inhalation anaesthesia (BAL), showed changes in serum and plasma VEGF (sVEGF and pVEGF, respectively) and plasma vWf

antigen (vWf:Ag) amount in the peri-operative period. At the same time, a secondary aim was to evaluate an eventual prognostic value for VEGF and vWF by correlating the obtained results and the tumor aggressiveness, determined histopathologically after prostate resection.

Methods

Patients selection

Between October 2009 and June 2012, 400 consecutive patients with primary prostate cancer, undergoing BAL (*i.e.* GA) or TIVA-TCI (*i.e.* RA) anaesthesia and conventional laparoscopic radical prostatectomy (LRP) or robot-assisted laparoscopic prostatectomy (RALP), were enrolled. This study was approved by the Ethics Committee of the Regina Elena National Cancer Institute, Rome (Prot.CE/550), and a written informed patient consent was obtained from all participants. Protocol was registered in *Clinical trials.gov* (NCT01998685). The inclusion and exclusion criteria for this study are listed in the previous published work from Sofra *et al*., 2014. Out of the 400 patients enrolled 102 fitted all criteria and 87 of them were selected for the present study.

Anaesthetic and surgery protocols

All patients with high-risk prostate cancer (according to Guidelines on Prostate Cancer of European Association of Urology, 2012) underwent LRP with extended pelvic lymph node dissection, while patients with intermediate risk underwent LRP or RALP. All patients did not receive premedication. In the TIVA-TCI group, anaesthesia was induced with propofol (Diprivan™, ASTRA-Zeneca, Milano, Italy) 6 $\mu g\ ml^{-1}$ and remifentanyl (Ultiva™, GlaxoSmith-Kline AB, Verona, Italy) 0.4-1 $\mu g\ kg^{-1}\ min$, simultaneously administered using two separate modules of a continuous computer-assisted TCI system. Anaesthesia was maintained with propofol 4 $\mu g\ ml^{-1}$ and remifentanil 0.25 $\mu g\ Kg^{-1}\ min$. This infusion was modified by 0.05 $\mu g\ kg^{-1}\ min$ steps according to analgesic needs. In the

BAL group, anaesthesia was induced with midazolam (Hameln pharmaceuticals Gmbh, Hameln, Germany) 0.1mg kg^{-1} and fentanyl (Fentanest™, Pftzer, Latina, Italy) 1.5 μg kg^{-1}. Anaesthesia was maintained with sevoflurane (Sevorane™, Abbott, Latina, Italy) 2.0% , oxygen 40% and air 70% with positive pressure ventilation in a circle system, in order to achieve normocapnia.

Sample collection and processing

Before the induction of anaesthesia (T0) and 24 hours post-surgery (T2), sVEGF, pVEGF and plasma vWf:Ag levels were evaluated. Blood samples in T0 and T2 were collected in tubes without additives and in tubes containing 3.2% sodium citrate (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ USA). Samples were centrifuged within 1h at 2500g for 20min and then, plasma and serum aliquots were separated and stored at -80°C for subsequent testing. For the quantitative determination of serum and plasma VEGF levels the Human VEGF Quantikine Kit (R&D Systems, Inc Minneapolis, MN USA) was used, according to the manufacturer's instructions, employing a quantitative enzyme-linked immunosorbent assay (ELISA) technique. The vWf:Ag was tested by a latex enhanced immunoassay method on fully-automated ACL TOP analyzer using HemosIL® commercial kits (Instrumentation Laboratory Company, Bedford, MA USA) according to the manufacturer's specifications and using proprietary reagents.

Data analysis

Data were analyzed with Statistical Package for the Social Sciences (SPSS) 21.0 software (IBM, Armonk, New York, US). Continuous and categorical variables were expressed as the mean ± standard deviation (SD) or standard error (SE) and as frequency values and proportions, respectively. Pearson's chi-square test was used to assess possible differences in dichotomous variables between the various groups examined. The means of normally distributed data were compared with the Student's t-test. In other cases, the groups were compared with the Mann-Whitney's U test. P values of the tests were adjusted using Bonferroni method. Paired samples were

analyzed by t-test and Wilcoxon Signed Ranks Test. Multiple linear regression was used in order to test the effect of anaesthesia and surgery on changes of sVEGF, pVEGF and vWf:Ag levels 24h after radical prostatectomy (T2). A p-value of <0.05 was considered statistically significant.

Results

Baseline patients characteristics are shown in Table 1. Out of the 87 screened patients, all were tested for sVEGF, 73 for pVEGF and 45 for vWf:Ag, before the induction of anaesthesia (T0) and 24 hrs post-surgery (T2). No significant differences in terms of age, tumor extent, tumor grading, type of surgery and anaesthesia duration were found in patients treated with TIVA-TCI or BAL protocol. Considering only the baseline mean levels (T0), apart from the anaesthetic and surgery approach, no significant difference between patients with different tumor extent (pT2 vs pT3) or tumor aggressiveness ($Gs\leq6$ vs $Gs\geq7$) was found for all the tested biomarkers (data not shown).

The impact of the anaesthetic regimen

Dividing the screened patients only for the anaesthetic regimen, in the BAL group 36 patients were tested for pVEGF, 42 for sVEGF and 25 for vWf:Ag, while in the TIVA-TCI group 37 patients were tested for pVEGF, 45 for sVEGF and 20 for vWf:Ag. In this regards, we hypothesized that changes in pVEGF, sVEGF and vWf:Ag could reflect biologic response to anaesthesia, so we examined whether the two groups of patients with a different anaesthetic treatment showed differences between T0 and T2 marker levels. Our data showed that an increase in sVEGF, pVEGF and vWf:Ag levels at T2 in both TIVA-TCI and BAL-treated patients actually occurred, but the difference between the two groups failed to be statistically significant ($p=0.89$, $p=0.233$ and $p=0.342$, respectively) (Table 2 and Figure 1).

The impact of the surgery approach

On the other side, dividing the screened patients only for the type of surgery they underwent, in the LRP group 49 patients were analysed for pVEGF, 62 for sVEGF and 24 for vWf:Ag, while in the RALP group 24 patients were analysed for pVEGF, 25 for sVEGF and 20 for vWf:Ag. In this regards, we hypothesized that changes in pVEGF, sVEGF and vWf:Ag could reflect biologic response to different surgery protocols, so we examined whether the two groups of patients, treated with a more or less invasive manipulation, showed differences between T0 and T2 marker levels. In our study, both LRP and RALP-treated patients showed an increase in sVEGF, pVEGF and vWf:Ag levels at T2, but only in pVEGF levels a significant difference between the two groups was founf, as it resulted much higher after the LRP surgery (p=0.862, p=0.005 and p=0.560, respectively) (Table 3 and Figure 2).

The impact of anaesthesia associated to surgery

Finally, dividing the screened patients considering both anaesthesia and surgery, in the LRP/BAL group 25 patients were analysed for pVEGF, 30 for sVEGF and 16 for vWf:Ag, while in the LRP/TIVA-TCI group 24 patients were analysed for pVEGF, 32 for sVEGF and 8 for vWf:Ag. Parallely in the RALP/BAL group 11 patients were analysed for pVEGF, 12 for sVEGF and 9 for vWf:Ag, while in the RALP/TIVA-TCI group 13 patients were analysed for pVEGF, 13 for sVEGF and 11 for vWf:Ag. In this regards, we hypothesized that changes in pVEGF, sVEGF and vWf:Ag could be enhanced combining a particular surgery technique (*i.e.* LRP or RALP) with a certain anaesthetic regimen (*i.e.* BAL or TIVA-TCI), so we examined whether the four groups of patients, representative of all the possible combinations, showed differences between T0 and T2 marker levels. In this case, an increase in sVEGF and pVEGF levels in T2 was found for all groups and the same seemed to occur for the vWf:Ag, even if, for the LRP/TIVA-TCI group, this marker resulted to remain particularly stable between T0 and T2. For the same group a significant difference was found comparing the distribution of pVEGF levels, as the increase of this marker in T2 resulted

much higher respect to all the other screened groups (p=0.955, p=0.008 and p=0.086, respectively) (Table 4 and Figure 3).

Discussion

Since different peri-operative protocols in PCa treatment have been approved, researcher started to question if a RP surgery performed with RA was able to generate a lower risk of future cancer recurrence. A particular work evaluated PCa recurrence in patients who underwent RP with epidural anaesthesia/analgesia (*i.e.* RA) or GA and opioid analgesia, coming to the conclusion that risk of cancer recurrence is 57% lower in patients treated with RA (Biki *et al.*,2008). On the other side, in another study, a group of patients was followed for five years after RP and no difference between the RA and GA group in terms of disease-free survival was found (Tsui *et al.*, 2010). In this scenario, the main goal of our study was to evaluate the peri-operative changes in serum and plasma markers that, according to literature, could contribute to facilitate cancer dissemination in patients with PC treated with RP surgery. For this purpose, pVEGF, sVEGF and plasma vWf:Ag levels were taken in exam as risk factors for cancer progression and metastasization. A recent study demonstrated a significant decrease in sVEGF levels in women undergoing surgery for primary breast cancer using propofol-paravertebral anaesthesia (*i.e.* RA) in place of GA (Looney *at al.*, 2010). This drug attenuated post-operative changes in the angiogenic factors to a greater extent than GA. Our data showed as sVEGF and pVEGF levels increased in both TIVA-TCI (*i.e.* RA) and BAL (*i.e.* GA) anaesthetic protocols, but their variation between the two groups did not differ significantly (Table 2 and Figure 1). So, on the basis of the observed trends, it wasn't possible to affirm which anaesthetic drug could be better in lowering future cancer-related complications in patients treated with RP surgery.

Another open question in today's clinical practice is linked to the optimal specimen to be used for measuring VEGF in human blood. Some studies showed that an amount of sVEGF derives from platelets activation and that this part could hide the little variations of this marker that could result as the most significant. For this reason pVEGF is considered, from many authors, a more sensitive markers than sVEGF and so the one to be preferred (Lee *et al.*, 2000). In our study pVEGF seemed to show with more sensitivity the angiogenic changes when we considered two different anaesthetic protocols, even if the difference between BAL and TIVA-TCI groups wasn't significant (Table 2).

On the other side, high plasma levels of vWf:Ag have been observed in patients with several types of malignant diseases, such as head and neck, laryngeal and prostatic cancer, and this event has been associated with tumor-induced endothelial growth during the angiogenic process (Schellierer *et al.*, 2011, Wang *et al.*, 2005, Zietek *et al.*, 1996, Gadducci *et al.*, 1993, Kim *et al.*, 2009). This increase may reflect endothelial proliferation and/or may be part of the acute phase reaction in response to vascular abnormalities (Lenting *et al.*, 2013). The mechanisms involved in this process are not completely understood, but there is evidence that the activation of this pathway may be related to accelerated endothelial synthesis associated with tumor-dependent angiogenesis (Franchini *et al.*, 2013). It was recently hypothesized, in fact, that vWf could have a regulatory role in angiogenesis. For instance, a recent work demonstrated an increased vascularization process in vWf-deficient mice, an effect that seemed to involve the angiopoietin 1 and 2 pathway (Starke *et al.*, 2011). Findings of another study, moreover, are basically in accordance with the experimental data that emphasized the anti-angiogenic function of vWf (Gritti *et al.*, 2011). Our data showed as vWf:Ag levels increased in both TIVA-TCI and BAL anaesthetic protocols, but the variation between the two groups didn't result statistically significant (Table 2). However, our data are in accordance, in some way, with the previous mentioned studies that tried to clarify the relationship between the vWf and the angiogenic process. Actually, looking at the variation of pVEGF and vWf:Ag levels between patients belonging to BAL and TIVA-TCI, it could be observed that a greater variation in one parameter corresponded to a lower variation of the other, for the same

group, and *vice versa*. This trend was evident also when we split the screened patients in two groups exclusively on the basis of the surgery protocol they underwent (Table 3). Considering the increase in sVEGF between T0 and T2 for LRP and RALP groups, in fact, we failed to find some difference, but, on the other side, taking in exam the pVEGF levels, a significant increase in T2 for the LRP group was highlighted when compared to the RALP group (p=0.005) (Figure 2). On the other side, vWf:Ag levels increased in both groups and we failed to find a significant difference. Interestingly, however, we observed that also in this case the group in which the higher vWf:Ag variation was observed (*i.e.* the RALP group) is the same in which the lowest variation in pVEGF values was found (Table 3). Our data strongly confirm that pVEGF is able to show with more sensitivity the angiogenic changes in blood specimens and, even more interestingly, that RALP approach probably stimulates the angiogenic process in a less relevant manner than the conventional LRP surgery, so, from this point of view, it could be considered the method of election for RP protocols. Moreover another indirect association of the relationship between vWf and the angiogenic process *in vivo* was observed.

To conclude our evaluation of the impact of anaesthesia and surgery on angiogenesis and endothelial activation in PCa patients treated with RP, we considered the association of both variables creating, therefore, four groups of patients representing all the possible combination between BAL/TIVA-TCI and LRP/RALP protocols (Table 4). Considering sVEGF levels, an increase in all groups was found but the difference between the four conditions failed to be significant. Things deeply changed when we evaluated the pVEGF levels. In this case, in fact, besides the detection of an increase in its amount for all groups, we observed, for the group of patients who underwent LRP surgery under TIVA-TCI anaesthetic protocols, a significantly greater variation compared to all the other conditions (p=0.008) (Figure 3). On the other side, vWf:Ag levels increased for all groups too, however also in this case a correlation with pVEGF variations was found. In fact, for the same group in which the pVEGF increase was higher (*i.e.* the LRP-TIVA-TCI), vWf:Ag levels remained almost stable between T0 and T2, while for the other groups,

in which the pVEGF variations were modest, the vWf:Ag increase resulted more intense, even if not significant (p=0.086) (Figure 3). Ultimately, the explanation of the observed trend could be related to that pathway in which in the absence of vWF an enhanced VEGF signaling occurs, so, as a consequence, proliferation, migration and angiogenesis events are triggered. Our results, however, should be confirmed increasing the number of screened patients in order to reveal the close correlation between vWf and VEGF in controlling the angiogenic process *in vivo*.In conclusion, this randomized controlled study, conducted on PCa patients candidate to RP, demonstrated that a robotic laparoscopic approach (*i.e.* RALP) was able to induce a significant lower variation of angiogenic molecules (*i.e.* pVEGF) giving the idea that this type of surgery could be preferred to avoid eventual dissemination of cancer cells. Moreover, our results are consistent with the hypothesis of a close correlation between vWf and VEGF. In fact, the theory of a negative regulation of VEGF by vWf during angiogenic process could explain the particular trend observed in our study, in which a greater variation in one of these two parameters corresponded to a lower variation of the other, for the same screened group, and *vice versa* Additional large-scale prospective trials, involving a great number of patients in each groups, are required to determine the significance of our observations.

Our study, however, has some limitations. First of all, numerous peri-operative factors may influence angiogenesis, including the intensity of the surgical stress and other post-operative therapy, which we did not control. Secondly, our study was limited to data collected 1 day after surgery, but peri-operative phenomena may be observed up to 30 days, or more, after surgery. Third, our patients follow-up was not long enough to determine the presence of PCa recurrence. Lastly, it could be interesting to evaluate not only plasma concentrations of VEGF and vWf:Ag levels but also plasma levels of angiopoietin 1 and 2 that are associated with the vWf/VEGF-dependent angiogenic pathway. On the other hand, our work represents the first study focusing on the contemporary impact of anaesthesia and surgery on *in vivo* peri-operative variations of vascularization and endothelial activation markers in PCa patients.

Abbreviations: BAL, balanced inhalation anaesthesia; ELISA, enzyme-linked immunosorbent assay; GA, general anaesthesia; LRP, laparoscopic radical prostatectomy; PCa, prostate cancer; pVEGF, plasma VEGF; RA, regional anaesthesia; RALP, robot-assisted laparoscopic prostatectomy; SD, standard deviation; SE, standard error; sVEGF, serum VEGF; TIVA-TCI ,total intravenous anaesthesia with target-controlled infusion; VEGF, vascular endothelial growth factor; vWf, von Willebrand factor; vWf:Ag vWf antigen.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the study: MS, PA, EF, LC

Performed the laboratory analysis: AA, LT

Performed the anesthetic and pre-surgery treatments analysis: MS, CC,IM, GT, VG, EF

Performed the urological analysis: MG, RP

Analyzed the data: IS

Contributed reagents/materials/analysis tools: MS, MG, PA, EF, LC

Wrote the manuscript: AA, LT, MS

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Tables and captions

Table 1. Clinical characteristics and peri-operative data of patients with prostate cancer who underwent LRP or RALP surgery after TIVA-TCI or BAL anaesthesia.

	TIVA-TCI (n=45)	**BAL (n=42)**	***p*-value**
Age (yrs)	60.66 (5.91)	62.16 (6.23)	0.31
Histological Tumor grade			
G2 (Gleason score 5-6)	10	12	0.39
G3 (Gleason score 7-10)	35	30	0.88
Histological Tumor extent			
pT2	32	33	0.25
pT3	13	9	0.20
Type of surgery			
LRP	32	30	0.65
RALP	13	12	0.37
Time of anaesthesia (min)	107.5 (16.8)	101.4 (26.2)	0.26

Values are expressed in absolute values or mean (SD)

Table 2. Variation of sVEGF, pVEGF and vWf:Ag levels in patients sorted for the anaesthetic regimen (TIVA-TCI and BAL)

	Baseline (T0)	24- hours (T2)	*p*-value
sVEGF (pg/mL)	Mean±SD	Mean±SD	
BAL	347±198	370±172	
TIVA-TCI	339±216	365±207	0.892
pVEGF (pg/mL)			
BAL	57±46	64±49	
TIVA-TCI	62±44	81±48	0.233
vWf:Ag (%)			
BAL	142±42	207±58	
TIVA-TCI	146±49	180±38	0.342

Table 3. Variation of sVEGF, pVEGF and vWf:Ag levels in patients sorted for the type of radical prostatectomy approach (LRP and RALP)

	Baseline (T0)	24- hours (T2)	*p*-value
sVEGF (pg/mL)	Mean±SD	Mean±SD	
LRP	349±223	372±197	
RALP	327±160	355±174	0.862
pVEGF (pg/mL)			
LRP	67±50	82±54	
RALP	43±26	52±26	0.005
vWf:Ag (%)			
LRP	151±49	195±38	
RALP	135±39	195±67	0.56

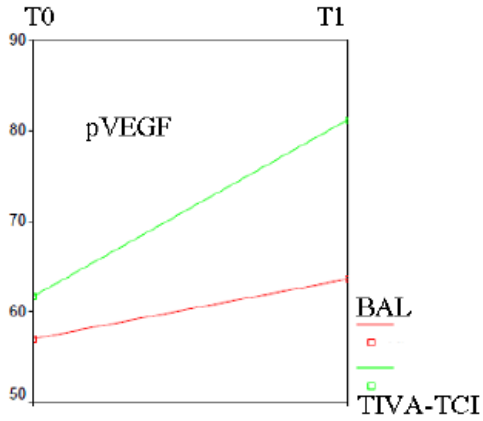
Table 4. Variation of sVEGF, pVEGF and vWf:Ag levels in patients sorted for the anaesthetic protocol together with the radical prostatectomy approach (LRP/BAL, LRP/TIVA-TCI, RALP/BAL and RALP/TIVA-TCI)

	Baseline (T0)	24- hours (T2)	*p*-value
sVEGF (pg/mL)	Mean±SD	Mean±SD	
LRP/BAL	348±220	369±183	
LRP/TIVA-TCI	350±229	375±213	
RALP/BAL	342±137	370±149	
RALP/TIVA-TCI	313±184	340±199	0.995
pVEGF (pg/mL)			
LRP/BAL	61±52	68±55	
LRP/TIVA-TCI	67±50	98±50	
RALP/BAL	48±28	54±31	
RALP/TIVA-TCI	39±23	51±22	0.008
vWf:Ag (%)			
LRP/BAL	135±45	199±51	
LRP/TIVA-TCI	183±43	189±51	
RALP/BAL	156±34	223±89	
RALP/TIVA-TCI	118±37	173±29	0.086

Figures

Figure 1. Peri-operative variation of (a) pVEGF (p=0.233) and (b) vWf:Ag (p=0.342) levels in patients sorted for the anaesthetic regimen (TIVA-TCI and BAL)

a)



b)

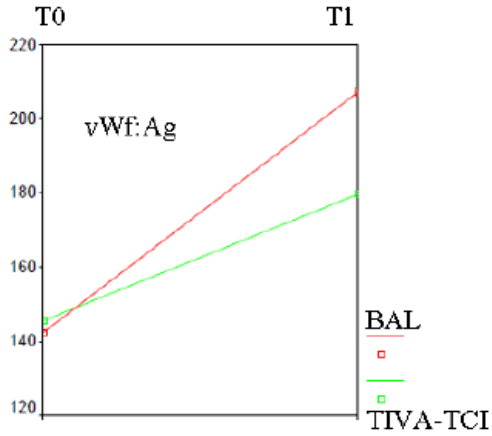
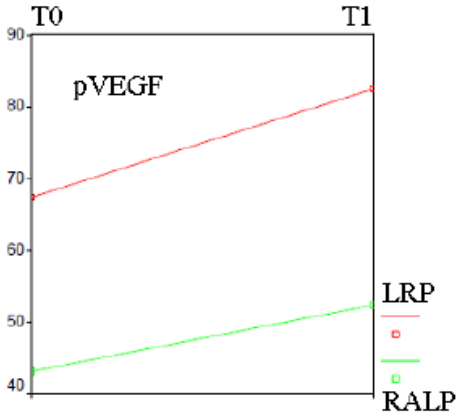


Figure 2. Peri-operative variation of (a) pVEGF (p=0.005) and (b) vWf:Ag (p=0.560) levels in patients sorted for the type of radical prostatectomy approach (LRP and RALP)

a)



b)

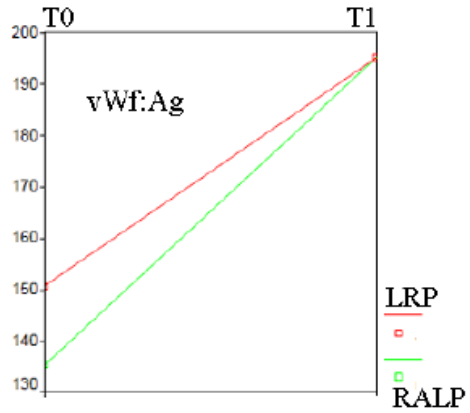
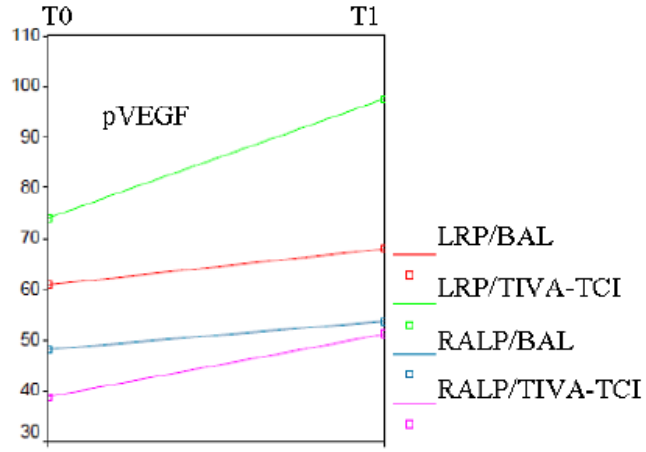
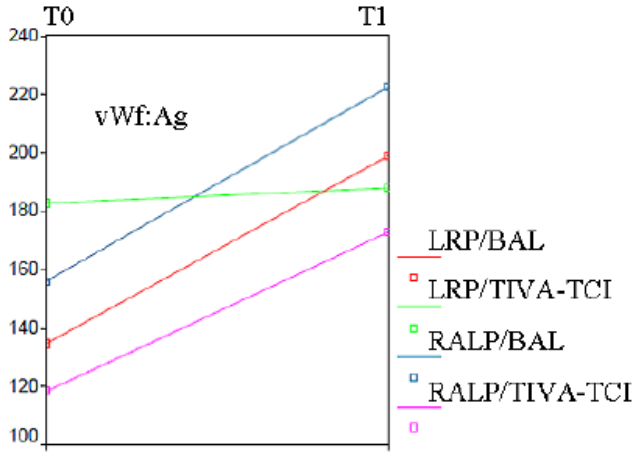


Figure 3. Peri-operative variation of (a) pVEGF (p=0.008) and (b) vWf:Ag (p=0.086) levels in patients sorted for the anaesthetic protocol together with the radical prostatectomy approach (LRP/BAL, LRP/TIVA-TCI, RALP/BAL and RALP/TIVA-TCI)

a)



b)



Concluding remarks

Characteristic of prostate cancer (PCa) is the selective production of unique prostate tissue differentiation markers. In particular, prostate cancer cells, like normal prostate epithelial cells, produce high levels of prostate-specific antigen (PSA), an enzyme belonging to kallikrein family [Williams et al., 2007]. All of the kallikreins are serine proteases that are produced as pre-pro enzymes and all have conserved position of the aspartate /histidine/ serine catalytic triad. Of the kallikreins that have been characterized, most have trypsin-like proteolytic activity. PSA, however, has a chymotrypsin-like substrate specificity. In addition, the ability to cleave after the amino acid glutamine appears to be unique to PSA [Goettig et al., 2010]. This protein is synthesized as a zymogen (or pre-pro-peptide) consisting of a 17 amino acid pre-sequence, that is removed intracellularly by signal peptidases, and a 7 amino acid pro-sequence that is totally or partially removed extracellularly, generating different form of proPSA [Mikolajczyk et al., 2001]. In proPSA, the smaller the part bound to the peptide in the leader region, the more difficult it is to activate. This makes the isoform of proPSA containing 2 residues in the leader region (the [-2]proPSA) the most stable component of proPSA in the serum [Mikolajczyk et al., 2003]. PSA is present in the serum in a number of different forms, all of which are enzymatically inactive. These forms can be classified into two general categories: complexed PSA (i.e., initially enzymatically active but now inactive due to binding to serum protease inhibitors) and free PSA (i.e., unbound, never activated PSA) [Kouriefs et al., 2009]. On the other hand, several studies have documented that PSA in the extracellular fluid surrounding prostate cells is enzymatically active and that this enzyme is able to process fibronectin and laminin as well as other molecules involved in growth stimulation and inflammation [Webber et al., 1995]. These results suggest that PSA, besides being useful to identify men at risk for the development of prostate cancer, itself may be causally involved in the development of localized prostate cancer and its progression to metastatic disease. In this light, part of my PhD thesis has been focused on deepening the catalytic activity of PSA in order to clarify the hypothetical role of this enzyme in tumor progression and metastasization. Therefore, the steady state and pre-steady state kinetics of the PSA-catalyzed hydrolysis of a fluorogenic substrate (with amino-acid glutamine in P1 to give more specificity to the reaction) has been determined between pH 6.5 and 9.0 at 37°C temperature [Tomao et al., 2014]. The obtained kinetic pattern was characterized by the presence of an initial burst phase which precedes the insurgence of the steady-state phase. This feature could be referred to a mechanism where the acylation and deacylation steps of the PSA-catalyzed

cleavage of the fluorescent substrate displayed different rate constants (k_2 and k_3, respectively). The possibility of a quantitatively satisfactory description of the two processes by parameters which are mutually consistent (i.e., k_{cat}, k_2, k_3, K_m and K_s) gave a great support to the fact that the mechanism described was suitable to account for the observed behavior. Furthermore, the difference between k_2 and k_3 at all investigated pH values strengthened the idea that the feature described for the enzymatic mechanism of PSA was actually referable to the fact that the rate-limiting step was not represented by the acylation reaction of the substrate but it resides instead in the deacylation. Fitting the obtained data the pH-dependence of the pre-steady-state and steady-state parameters for the PSA-catalyzed hydrolysis of the fluorescent substrate was demonstrated. The overall description of the proton linkage for the different parameters required the protonation / deprotonation of (at least) two groups. The global fitting of the pH-dependence of all parameters has allowed to define a set of six pKa values (i.e., pK_{U1}, pK_{U2}, pKE_{S1}, pKE_{S2}, pK_{L1}, and pK_{L2}) which satisfactorily describe all proton linkages modulating the enzymatic activity of PSA. Another goal was to identify two hypothetical residues involved in enzyme-substrate interaction. A possible candidate for the first protonating residue ionizing at alkaline pH is the Lys95E of the kallikrein loop [Menez et al., 2008], which might be involved in the interaction with a carbonyl oxygen, orienting the substrate; this interaction could then distort the cleavage site, slowing down the acylation rate (i.e., k_2). On the other hand, the second protonating residue ionizing around neutrality may be a histidine (possibly even the catalytic His57), whose protonation dramatically lowers the substrate affinity, though facilitating the acylation step and the cleavage process.

As a PCa-related diagnostic marker, the PSA serum test causes every year a large number of unnecessary biopsies [Hessels et al., 2009]. The PSA test low specificity is linked to the fact that its increase in serum is not an event that closely reflects the presence of a PCa, but it can also be found in patients with benign prostatic hyperplasia (BPH) and prostatitis [Nogueira et al., 2010]. In this scenario, part of my PhD thesis aimed to evaluate the performance characteristics of three of the most promising new PCa-related biomarkers: the prostate cancer gene 3(PCA3) urine test, the [-2]proPSA (p2PSA) and Galectin-3 (Gal3) serum tests. In particular the main purpose was to determine the characteristics of sensitivity, specificity and the diagnostic accuracy of the PSA, PCA3, proPSA and Gal3 tests, as well as their prognostic value, based on the results of two studies that separately compared the new vs the old markers. Through a prospective study, first of all, the different distribution in PSA, expressed as total PSA (tPSA) and free PSA (fPSA) to tPSA ratio (f/tPSA), and PCA3 score in two groups of subjects with negative (n=212) and positive biopsy (n=195) was evaluated [Merola and Tomao et al., 2014]. tPSA values overlapped between the two

groups indicating that this parameter cannot be used to discriminate PCa from non-PCa patients. Considering instead the f/tPSA ratio, a most powerful marker was found, as it resulted significantly lower in subjects with PCa. However, the best result was obtained from the PCA3 urine test, that shows the highest difference between the two groups, demonstrating to be a more sensitive test, resulting strongly associated to the prostate oncologic pathology. Conversely to the PSA, the PCA3 test showed also a good prognostic performance, since the percentage of patients with more aggressive PCa (Gleason score ≥ 7) had significantly higher PCA3 score values respect to patients with lower grade PCa (Gleason score ≤ 6).

With a second retrospective study, the clinical utility of PSA, p2PSA and Gal3 serum test was investigated [Tomao et al., 2015], comparing the different distribution of these markers along two populations, one with PCa and a second with BPH. The obtained results showed that none of these markers was able to discriminate the malignant from the benign prostatic pathology. However, things deeply changed when these parameters were combined together to calculate different indices, such as the PSA ratio (fPSA/tPSA), the percentage of p2PSA (p2PSA/fPSA), the prostate health index ($p2PSA/fPSA*tPSA^{1/2}$) and the Galphi (Gal3*tPSA/fPSA), a novel index of our creation here investigated for the first time. In this case, interestingly, all the considered indices were able to predict PCa in a good manner. In particular, the p2PSA derivatives (i.e. the percentage of p2PSA and the prostate health index) showed a diagnostic accuracy greater than tPSA and f/tPSA tests, with prostate health index resulting the most accurate. At the same time, the Galphi resulted able to reach good diagnostic performances, in some respects comparable to that obtained from p2PSA related indices, showing, for the first time, that a Gal3 related index may be used for the same purpose of other quoted PCa biomarkers. Also in this case a possible association between the screened biomarkers and tumor aggressiveness was investigated; however, no association with the Gleason score was found for all the analyzed markers and their related indexes.

The early diagnosis of a PCa is often linked to a subsequent surgical resection of the tumor, a procedure that, in the absence of distal lymph node metastases, is generally resolutive. However, in some cases, PCa tends to generate metastases after a few years, even if they are not detected during the surgery [Roberts et al., 2013]. Accordingly, some investigations have suggested that a number of factors in the perioperative period could promote metastasization. These include the surgery approach and its associated stress response, the anaesthetic regimen, the acute pain, and the administration of opioid analgesics [Mao et al., 2013]. The hypothesis is that different anesthetic protocols and surgery techniques can differently activate the clotting system, thereby generating thrombin, or stimulate mononuclear cells, platelets and endothelial cells. The consequent formation of a fibrin matrix, together with cell activation, appear to promote tumor

growth and neo-angiogenic processes [Falanga et al., 2013; Franchini et al., 2013]. In this view part of this PhD thesis was dedicated to investigate whether two group of PCa patients, undergoing conventional laparoscopic radical prostatectomy (LRP) or robot assisted laparoscopic prostatectomy (RALP), with two different intra-operative anaesthetic regimens, total intravenous anesthesia with target-controlled infusion (TIVA-TCI) and balanced inhalation anaesthesia (BAL), showed different changes in coagulation, cell activation and angiogenesis activation markers during the perioperative period [Sofra et al., 2014; Antenucci and Tomao et al., 2015]. Both TIVA-TCI and BAL patients showed a marked and significant increase in pro-coagulant factors, with consequent reduction in haemostatic system inhibitors, in the early post-operative period, while the RALP approach showed a significant increase in pro-thrombotic markers as compared to LRP. In TIVA patients undergoing LRP, instead, a lower variation of p-selectin levels, compared to BAL, was observed. Considering the variations of pro-angiogenesis and endothelial activation markers a significant increment in vascular endothelial growth factor (VEGF) and von Willebrand factor (vWf), in the perioperative period, was found for all conditions. At the same time, patients undergoing LRP showed a significantly higher variation in VEGF levels. Interestingly, in all the analyzed groups, an opposite trend in VEGF and vWf variations was always observed, manifesting, for the first time *in vivo*, the possible negative regulation of angiogenic processes by vWf.

The obtained results fundamentally support the hypothesis that the administration of anti- thrombotic and/or anti-angiogenic agents could be helpful in preventing the pro-metastasization events derived from some of the perioperative manipulations received from PCa patients during the radical prostatectomy.

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