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EVALUATION OF LABORATORY TEST RESULTS IN SUSPECTED PROSTATE CANCER.

PhD thesis

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Content

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List of abbreviations

ALT – alanine aminotransferase

CRP – C-reactive protein

eGFR – estimated glomerular filtration rate

IQR – interquartile range

NEAK - National Health Insurance Fund

NLR – neutrophil-to-lymphocyte ratio

OS – overall survival

PCA – prostate cancer

PLR – platelet to lymphocyte ratio

PSA – prostate specific antigen

QoL – quality of life

RBC – red blood cell

TRUS – transrectal ultrasound

TSH – thyroid stimulating hormone

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Foreword

Medicine is a very diverse discipline. From surgery to theoretical research, from radiology to ophthalmology, from pathology to general internal medicine, there are many areas in which young doctor with a degree in general medicine can start their careers. It was not an easy decision for me either: I was interested in physiology, surgery and internal medicine, but I also wanted to help the sick person. After a long struggle, I finally found the vocation that has defined my life to this day. Urology. It is a specialty that, although it focuses on a very small area of the human body, has had a profound impact on me since the beginning of my career, through its complexity, its challenges, its solutions and its impact on the quality of life of patients.

One of the main areas of my work is the diagnosis and therapeutic management of prostate malignancies. I have been interested in this disease since my university days and even wrote my thesis on this topic. Namely, the then new LHRH analogues used in androgen deprivation therapy. With great curiosity, I gathered expanding information about the hormone dependence of prostate cancer cells, the hormone treatments that exploited this, the "downregulation" phenomenon that is the basis of LHRH analogue therapy. Later, the challenge of castration resistance of hormone-treated prostate tumours became even more important. After university, I started working in the urology department of the Uzsoki Street Hospital (then Weil Emil Hospital) in Budapest. It was then that the first major paradigm shift in the therapy of PCA took place, with the introduction of the LHRH analogues mentioned above.

Evidence shows that early detection markedly improves the chances of treatment. Suspicion leading to early detection is raised by the physical examination and the patient's symptoms, as well as by prostate-specific antigen levels. At the beginning of the 1990s, Professor Imre Romics' pioneering work led to the introduction of PSA, a tumour marker that has revolutionised the diagnosis of PCA, into laboratory diagnostics in Hungary. [Stamey TA et al., 1987] After 35 years in urology, I am of the opinion that this has been one of the most significant changes in the care of PCA from an economic, social and therapeutic point of view. In just a few years, the number of male patients diagnosed with PCA has skyrocketed. The timing of diagnosis has changed: the disease is being diagnosed earlier, which has given us a much better chance of starting treatment. The number of patients detected at an operable stage has increased, so that more and more

radical prostatectomies have been performed in urological centres in developed countries with increasing success.

In a very short time, PSA measurement has become an everyday practice. As experience has grown, it has become increasingly clear that assessing results is not a simple task, and more and more factors have been identified that need to be taken into account when assessing PSA levels. I also became aware - in line with the conclusions of the major summary studies - that PSA levels can vary not only in cancer but also in other prostate diseases. [Merriel et al, 2022] If this is not taken into account, biopsy sampling is often unnecessary. Careful assessment of PSA levels is essential. This includes an analysis of whether PSA levels may be influenced by any internal disease other than the prostate; what factors may affect serum PSA levels.

The definitive diagnosis of PCA is based on the results of a histological examination. For this, a biopsy is taken. The tissue sections to be histologically processed are taken with a "TRU CUT" needle. The puncture itself can be made from the perineum and from the rectum. The introduction of transrectal ultrasound (TRUS) and TRUS-guided prostate biopsy, followed by increasingly specific MR scans and the combination of these procedures improved the effectiveness of prostate biopsy. However, the sampling remains invasive and involves considerable physical strain and stress for the patient. A serious complication is infection, which is even higher with TRUS-guided transrectal biopsy than with as perineal puncture. Therefore, perioperative antibiotic prophylaxis has been generally used in our practice to prevent this. This markedly reduces the risk of infection but may have side effects.

Micro-organisms in the body - a large proportion of which are bacteria - have a fundamental impact on health. The importance of this and its role in various pathological conditions is increasingly known. Therefore, I became concerned about the extent to which antibiotic treatment, so generously applied in the context of TRUS-guided transrectal prostate biopsy, modulates the microbiome composition of my patients.

In the care of the thousands of patients with PCA I have treated, I have often wondered whether it is possible to identify other biomarkers, beyond serum PSA and histological characteristics, that might predict the success or failure of therapy in the long term. I have therefore read with great interest the observations that some elements of routine blood counts may have predictive value in this respect. To answer this question, I analysed the

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long-term survival of patients I treated some 20 years ago and compared it with the blood count at the time of diagnosis.

In my PhD work, I sought to answer the above questions. I hope that my observations can contribute in some way to the development of urology, this beautiful profession, and ultimately to the effective treatment and recovery of patients.

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1. Introduction

1.1. The importance of prostate cancer

Prostate cancer (PCA) is the second most commonly diagnosed cancer in men, with an estimated 1.4 million cases diagnosed worldwide in 2020. [Culp et al, 2020] A systematic review of autopsy studies shows a steady increase in the prevalence of PCA with age. Under 30 years of age it is 5%, while over 79 years of age it is 48-71%, depending on the test. Of note, above age 80 years [Bell et al., 2015], the prevalence of autopsy-detected PCA varies between men of different ethnic backgrounds and geographic locations (e.g. 83% in white American men compared to 41% in Japan [Haas et al., 2008]). The prevalence of PCA diagnosis between different geographic areas is specifically dependent on the screening recommendations for prostate-specific antigen (PSA) testing in that area [Fleshner et al., 2017]. It is highest in Australia/New Zealand and North America (age-standardised rates [ASR] per 100 000 population 111.6 and 97.2, respectively), and in Western and Northern Europe (ASR 94.9 and 85, respectively). Incidence rates are low in East and South Central Asia (ASR 10.5 and 4.5), but are increasing [Kimura et al., 2021]. Rates in Eastern and Southern Europe were previously low, but have shown a steady increase here as well. In addition to PSA testing, the prevalence is also dependent on the age, geographic location and ethnicity of the population.

More accurate mortality data can be obtained from databases in Western developed countries. In the USA, about 190,000 new cases are registered each year. The number of deaths from prostate cancer is estimated at 28,000 per year. It is estimated that PCA is responsible for the deaths of about 220,000 people worldwide each year. [WHO data, 2020] No reliable data are available in Hungary. Between 4,500 and 4,800 new cases are detected each year, which is below the European average. However, prostate-cancer related mortality rate is above the average, which may be explained by the lack of early detection.

Early detection of PCA is curable, with a 5-year survival of almost 100%, compared to only 28% in the metastatic form. Family history and ethnic background are associated with an increased likelihood of PCA, suggesting a genetic predisposition [Hemminki, 2012]. Men of African descent living in the Western world are considered to be at increased risk due to a combination of biological, environmental, social and health factors

[Nyame et al., 2022]. They are more likely to be diagnosed with more advanced disease and are also more likely to experience "upstaging" after prostatectomy than Caucasian men (49% vs. 26%) [Sanchez-Ortiz et al., 2006]. A number of exogenous/environmental factors are known to be associated with the risk of developing PCA or to be etiologically important in the transition from latent to clinical PCA [Leitzmann et al., 2012]. Asians who immigrated to the US have approximately half the risk of PCA as their US-born Asian descendants, suggesting a role for environmental or nutritional factors. However, no effective preventive dietary or pharmacological interventions are currently known.

1.2. Screening for prostate cancer

Screening for PCA is still one of the most controversial topics in the urology literature [Etzioni et al., 2013]. Population or mass screening is defined as "the systematic screening of asymptomatic men to identify those at risk of a particular disease" and is usually initiated by health authorities.

The primary objectives are: reduction in mortality from PCA; maintained QoL, as expressed by QoL-adjusted life-years (QALY). Ilic et al. published a Cochrane review of randomised PCA screening trials in 2013 and updated it in 2018, with PCA mortality as the endpoint. The main findings of the publication, which assessed data from 5 randomized clinical trials involving 721718 men, are:

1. Screening carries a risk of overdiagnosis of PCA (incidence increases by 23%).
2. Screening is more useful for the diagnosis of more localised disease (relative risk: 1.39) than for advanced cancers (T3-4, N1, M1 stage).
3. No improvement in PCA-specific survival or change in overall survival was observed in the overall population screening.

With some disregard to the above data and findings, I consider screening for PCA important in my work, as do the vast majority of urological specialists in the country, and I follow the algorithm below.

It should be noted that before screening, subjects should be informed of the benefits and risks; the life expectancy of the screener should be at least 10 years. The target population is men over 50 years of age or those aged 45-50 years with a family history of PCA.)

A flowchart of the screening process is shown in Figure 1.

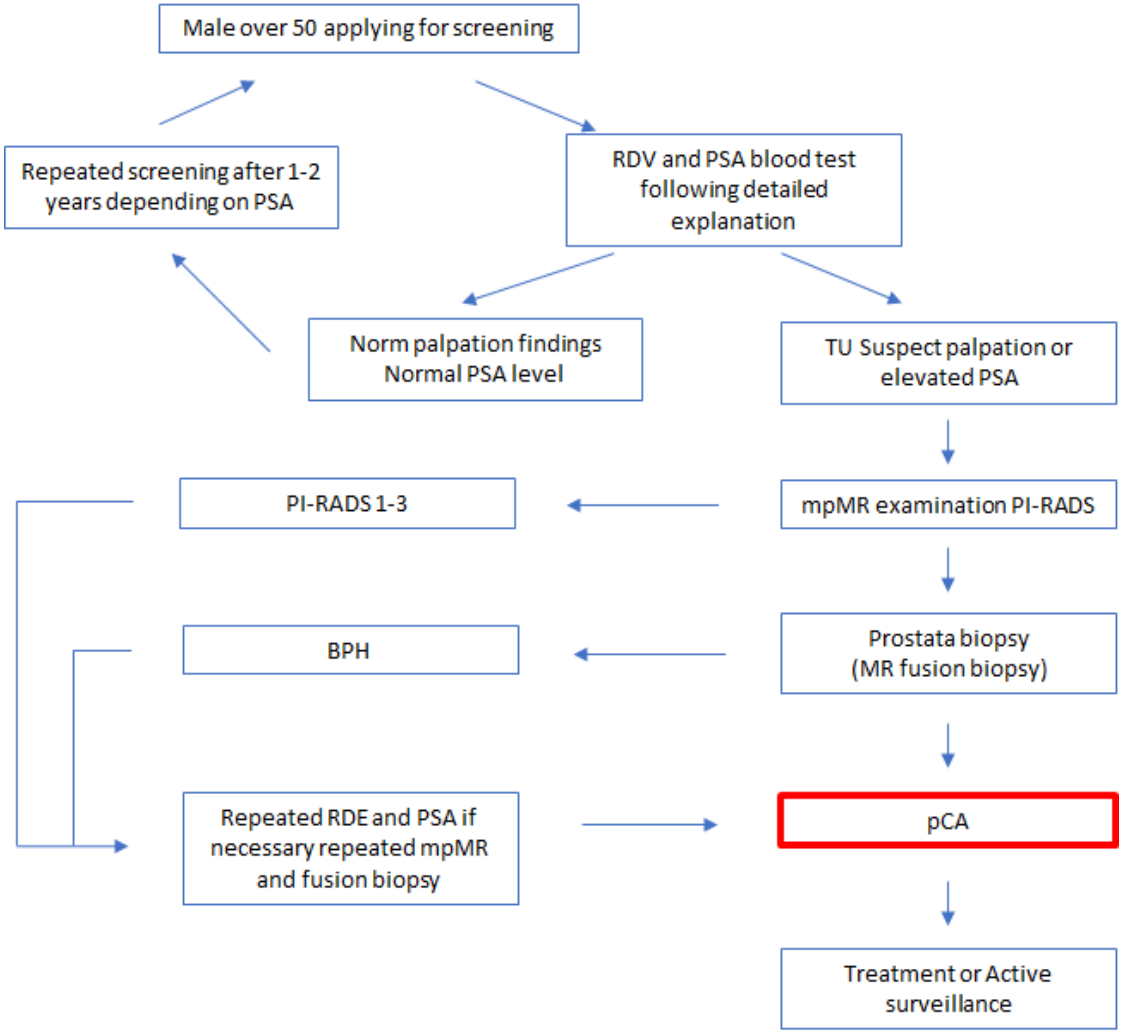

Figure 1. Flow diagram of PCA screening. Abbreviations: RDE: rectal digital examination; BPH: benign prostatic hypertrophy; MR: nuclear magnetic resonance; PCA: PCA; PI-RADS 1-3 Prostate Imaging-Reporting and Data System 1-3, mpMR: multiparametric nuclear magnetic resonance examination, The PI-RADS score of 3 for mpMR scan suggests uncertainty. In this case, indication or rejection of biopsy is difficult. In such cases, a risk estimate corrected for PSA density (i.e. the ratio of measured PSA value to prostate volume) is helpful [Randazzo et al., 2016][Stevens et al., 2020]

1.3. The prostate-specific antigen

Prostate-specific antigen (PSA) levels are commonly used as a laboratory marker for screening for PCA, but this is only recommended for a specific group of men [David et al., 2022]. In many countries, it is common practice to have a single PSA level

measurement between the ages of 40-45 years for screening purposes. For men at higher risk, annual testing from the age of 45 onwards is recommended. A rise of >0.75 μg/L per year or a change of >25% is considered suspicious for cancer. Routine screening is not recommended for men with a life expectancy (based on health status) of ten years or less (based on comorbidities). Patients who would not benefit from detection and treatment or would refuse the latter should not be screened either.

PSA is a serine protease enzyme produced in the cylindrical epithelial cells of prostate tissue. The PSA precursor secreted from here, pro-PSA, is converted to active PSA at the basal and endothelial cell layers and then into the prostate's ejection tubes. Its function in the semen is to degrade semenogelin proteins in the semen and thus promote semen fluidity [Balk et al., 2003]. However, a small fraction of it is released into the capillaries and then into the circulation. [Schedlich et al., 1987].

In PCA, PSA production does not increase, but rather decreases. However, the structure of the prostate is altered and the basal layer is damaged, which makes it easier for the PSA produced to reach the blood vessels. In addition, the increased vascularisation and the greater permeability of the blood vessels also leads to a higher proportion of PSA entering the bloodstream with PCA. (It should be noted that there are three isoforms of PSA in serum: intact PSA (some of which is present in free form, the majority of which is bound to protein), pro-PSA and BPH-associated PSA (BPSA). The use of all three isoforms as biomarkers has been suggested to distinguish between the causes of PSA elevation. Currently, I have more than two decades of experience with the PSA level, which is the only one I have dealt with in my clinical and PhD work.)

It follows from the above that the increase in PSA levels is not specific for PCA. An increase can also occur acutely after ejaculation in healthy, asymptomatic men, as well as in the presence of infection, trauma (even marked constipation), inflammation and benign prostatic hyperplasia. Studies suggest that in the latter case, PSA levels are elevated in the vast majority of patients [Stamey et al., 1987].

PSA test results may show a high intra-individual variability due to the above pre-analytical factors. Therefore, in the case of abnormal PSA levels, it is worth repeating the sampling and measurement. Repeated blood sampling after 1 month for an initial PSA result of 3-10 μg/L will significantly influence the next step in the diagnostic process. It has been observed that repeat testing alone significantly reduces the number of

unnecessary prostate biopsies. [Norström et al. 2016] As PSA levels in prostatitis can also increase, sometimes completely asymptomatically, there are also recommendations that patients with higher PSA levels should routinely receive several weeks of antibiotic treatment to clear the infection [Toktas et al. 2013]. (The results of my PhD work, which indicate the long-term effects of antibiotic administration on gut flora, call into question the appropriateness of this practice.) The enormous burden of PSA assessment on the urologist is alleviated, without the need for completeness or detailed description of methods, by PSA density, PSA change velocity and PSA/fPSA ratio [Omri, N., et al. 2020].

However, despite its limitations, there is a general consensus that PSA is a more effective means of detecting PCA than digital rectal examination and/or TRUS alone. Using a cut-off value of 4 μg/L, the specificity of serum PSA for PCA is 91% [Catalona et al., 1991] The sensitivity of PSA is much lower at this cut-off value, varying between 9% and 33% depending on age. In other words, the positive predictive value of the test in the general population is low, sometimes less than 10% [Leal et al., 2018] In the clinical patient population (i.e. the group of patients presenting to a urologist with a urological complaint), the probability of PCA in men with elevated PSA is about 30%. It should be noted that an increase in PSA levels is statistically associated with the severity of PCA and the occurrence of metastases. For metastatic disease, the positive predictive value for a PSA cut-off >20 μg/L is 65% and for a PSA cut-off >100 μg/L is 86% [Lojanapiwat et al., 2014].

The majority of pCAs detected in the context of elevated PSA levels are low risk, often requiring only active surveillance. In such cases, only a quarter of patients progress to a condition requiring definitive therapy. Therefore, above a certain age, when the expected survival is less than 10 years due to the patient's age or comorbidities, PSA measurement is not recommended to avoid overdiagnosis and the associated unnecessary therapeutic efforts. (Another way of avoiding and reducing overdiagnosis is to educate patients: PSA testing is not an urgent test, no immediate clinical decision depends on the result, and patients should be given time to consider their options [Moynihan et al., 2012].)

In clinical practice, the general consensus is that the upper limit of the reference range for PSA is, in a very simplified approach, 4 μg/L. However, the assessment of the first PSA laboratory result during a medical consultation is a very complex task, because it requires

the simultaneous evaluation of several circumstances. An elevated PSA level raises the suspicion of PCA and thus indicated biopsy sampling. As this intervention is not without risk, there is a fundamental need to reduce the number of unnecessary biopsies. The introduction of personalised PSA reference ranges could help in this respect: it would be worthwhile to identify and take into account in the assessment those conditions that may affect PSA levels independently of prostate pathology.

The most important of these is age. There is a direct correlation between age and serum PSA concentration, with PSA levels in healthy men expected to increase by 3.2% per year after the age of 60 [Oesterling et al., 1993], and therefore it is recommended that age-adjusted ranges are used when analysing serum PSA levels [Partin et al., 1996]. PSA levels have been shown to be affected by a number of drugs, including statins, NSAIDs and thiazides [Chang et al., 2010; 29]. High body mass index also leads to lower PSA levels [Banez et al., 2007].

It is worth noting that PSA values depend – to a lesser extent – on the method used and the platform used for measurement. [Loeb et al., 2008] It is therefore advisable to use the same manufacturer's PSA measurement system when following up patients.

1.4. Prostate biopsy and preventive antibiotic treatment

If the clinical picture, laboratory tests or imaging studies raise the suspicion of PCA, histological examination of the lesion is necessary to establish the diagnosis. This involves biopsy sampling.

I have been performing transrectal prostate biopsies under TRUS guidance since 2003. Sampling has remained unchanged since the introduction of this method: 13 samples are taken in 8 positions. Besides its higher accuracy, the known risk of the procedure is acute prostatitis with severe symptoms. To avoid this, sampling should only be performed in truly justified cases and it is of paramount importance to reduce the possibility of asepsis, bacterial exposure.

The process of intervention:

Previously, for about 15 years, the procedure was performed under anaesthesia. Since 2018, following international recommendations, we have abandoned routine intravenous anaesthesia and only use it at the patient's explicit request, especially for repeat

procedures if the first procedure proved to be very painful. In my experience, patients' fear of the procedure is much greater than the actual stress of the procedure. The procedure is performed in the standard incisional position (Figure 2)

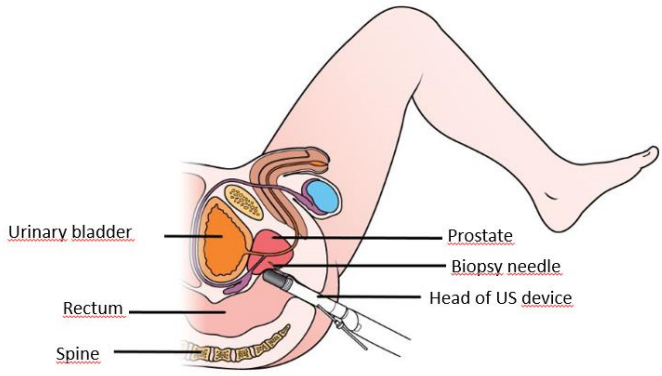

Figure 2 Schematic diagram of the ultrasound-guided transrectal prostate biopsy [https://www.uzsoki.hu/sites/default/files/Prosztata%20biopszia_TRUS.pdf]

To avoid complications (prostatitis), I have used several preventive measures. For many years, all my patients were prepared with antibiotic prophylaxis and enemas. Antibiotic treatment around biopsy is part of the efforts to reduce bacterial exposure. In our practice, all cases were given 2*500 mg of ciprofloxacin daily and 2*150 mg of clindamycin daily for 5 days starting from the day of biopsy.

This combination of antibiotics was shown to be effective in reducing the incidence of prostatitis, with a clear short-term benefit in terms of hospitalisation. Over the past decade, more and more data have accumulated on the importance of the microbiome in predisposing to disease, including prostate disease. More and more data have emerged on the marked effects of antibiotics on the microbiome.

The human microbiome is a complex community of bacteria, viruses and fungi, made up of thousands of different microorganisms. They modulate the immune system, in particular through bacterial lipopolysaccharides; they produce metabolites, including short-chain fatty acids, vitamins and bile acids, which influence homeostasis and are essential for various cellular functions. Therefore, the dynamic interaction between the microbiome and the host is increasingly recognised as a key player in health and disease [Hou et al., 2022].

The role of the colonic microbiome in prostate disease has been extensively studied; specific patterns of the microbiome have been identified in PCA and the microbiome has been shown to influence the success of anticancer treatment [Banerjee et al., 2019; Wheeler and Liss, 2019]. The composition of the urogenital microbiome may also indirectly reflect and be influenced by the gut microbiome. This is of particular relevance in urology. A recent review presents the relevant data collected so far in this field [Miyake et al., 2022]. Therefore, changes in the microbiome in an unfavourable way, even as a result of antibiotic administration for preventive purposes, both before and after biopsy for a short period, may pose a serious health risk. However, there were no data on this until our study.

1.5 Blood count and prostate cancer outcome prediction

Observations suggest a link between prostate cancer and inflammation. Both local and systemic inflammation may influence tumour development, promotion and metastatic progression [Grivennikov et al., 2010]. It is known that systemic inflammation is also indicated by blood count abnormalities: changes in white blood cell count, lymphocyte-to-monocyte ratio (LMR), neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) within white blood cells. Several studies have indicated that these parameters may also have predictive value in a number of cancers (e.g. gastrointestinal malignancies [Zhang et al., 2023][Misiewicz et al., 2023], breast cancer [Savioli et al., 2022]). Based on these findings, it is hypothesized that blood counts may help to determine the progression of PCA and to predict outcome.

The prognostic value of NLR (worse overall survival with high NLR) was confirmed in metastatic castration-resistant PCA. In contrast, NLR was not associated with the prognosis of patients with localised PCA [Yin et al., 2016]. In PCA, high PLR was significantly associated with shorter OS and increased cancer-specific mortality [Wang et al., 2017]. in PCA treated with irradiation, the risk of mortality was also increased when PLR, NLR, and white blood cell count increased. However, the growing number of publications have predominantly used data from well-defined homogeneous clinical patient groups (e.g. data from medical trials at admission), without taking the heterogeneity typical of general hospital practice into account, and it is questionable to what extent these observations can be used in Hungarian patient care.

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2. Objectives

1. Changing the PSA cut-off values has far-reaching effects on the sensitivity and specificity of laboratory-based PCA screening [Hoffman, 2011] The reference range of PSA varies depending on certain biological factors (e.g. age), while other factors, such as diurnal or seasonal fluctuations, have only recently been identified [Connolly et al., 2011]. Liver and kidney disease, low levels of systemic inflammation, thyroid abnormalities or even vitamin D deficiency affect a significant proportion of patients.

Our work used a large laboratory database to evaluate whether the distribution of PSA levels changes in the presence of laboratory abnormalities suggestive of these conditions; whether we should expect the reference value for PSA levels to differ.

2. The body regions and organ systems (predominantly the gut and skin, but also the prostate) have a local microbiome, the presence of which is an essential element for maintaining local homeostasis [Hou et al., 2022]. Changes in the microbiome are called dysbiosis, which may be related to the dysfunction of the organism. Transrectal ultrasound-guided biopsy sampling is a commonly used procedure to collect tissue samples to investigate suspected PCA. To prevent infectious complications, patients receive a short course of antibiotics before the procedure.

Our work investigated whether the combination of ciprofloxacin and clindamycin given for this purpose affects the colonic microbiome.

3. Early detection and treatment are essential for the progression of PCA. The latter is determined by the characteristics of the tumour. The development of therapeutic options has led to a marked increase in survival and improved quality of life, even at an advanced stage. However, in routine clinical work, there is still a need for simple biomarkers that are easily accessible for daily practice and that can provide information on the prognosis of the disease. In recent years, several observations have reported that certain blood parameters or their ratios may be associated with survival in certain cancers.

Our work has sought to answer the question of whether blood counts taken at the time of PCA diagnosis can help predict patients' long-term overall survival.

3. Methods

3.1 Distribution of PSA levels as a function of other laboratory parameters

3.1.1. Database selection

The Institute of Laboratory Medicine at Semmelweis University provides laboratory services, including PSA level measurements, to a number of healthcare institutions at Semmelweis University and in the region. The Laboratory Information Management System (GLIMS) archives the results; between 2011 and 2019, about 200 million entries were generated. [Tóth Z, et al, 2020(a)] [Tóth Z, et al, 2020(b)] [Tóth Z, Gyarmati B et al, 2019]

In collaboration with the Institute, the database was analysed (Ethics approval: 52331/2019 TUKEB). The database contains records, each of which consists of the following elements: anonymised patient identifier; gender; age; name of the measured parameter; date of the test, result of the measurement. From the database, we selected by patient identifier the laboratory results of men who had a PSA level measurement and had any of the following parameters available within ±1 month: estimated glomerular filtration rate (eGFR), alanine transaminase (ALT), C-reactive protein (CRP) and vitamin D, respectively, or available within ±2 weeks: thyroid-stimulating hormone (TSH). All laboratory parameters were determined using CE IVD-rated commercially available tests on clinical chemistry and immunochemistry automats.

As results from patients with severe prostate disease (PCA) may have skewed the distributions analysed, we arbitrarily excluded individuals with PSA levels above 20 μg/L.

In order to avoid repeated measurements of a patient distorting the results, only records for the first time point PSA measurement were used.

3.1.2 Statistical analysis

We created subgroups and cohorts according to ALT, eGFR and CRP and calculated PSA values of 5, 25, 50 (median), 75 and 95 percent. In addition, logistic regression analysis of logarithmic data was used to determine the independent effects of eGFR, ALT and CRP, and age on PSA levels. The direct correlation between age and each analyte was also tested. For the analyses, we performed logarithmic transformations of PSA and

laboratory parameters to obtain a near-normal distribution in each dimension. Because of the characteristics of the PSA test, values <0.1 μg/L were treated uniformly as 0.1 μg/L. We also created subgroups according to vitamin D levels and age, with the 95th percentile PSA values as the upper end of the reference range. We examined the proportion of subjects with elevated PSA (>4 μg/L) in each vitamin D subgroup. Logistic regression analysis of the logarithmic data was used to determine the independent effect of vitamin D level and age on PSA levels. Pearson's correlation coefficient (R) was used to characterize the direct correlation between log vitamin D and PSA levels. The two-dimensional distribution and correlation were calculated for the whole patient population and for patients with PSA levels above 4 μg/L only. Due to the characteristics of the PSA test, values <0.1 μg/L were treated uniformly as 0.1 μg/L. However, this bias was corrected by kernel density estimation.

To analyse the relationship between TSH and PSA levels, we used the natural logarithm (ln) of TSH and PSA levels to normalise them (for PSA levels below the measurement limit, we used a uniform value of 0.001 μg/L). The relationship between lnPSA and lnTSH levels was assessed by multiple regression. In addition, patients were divided into hyperthyroid (TSH <0.35 mU/L) and euthyroid (TSH 0.35-4.95 mU/L) groups based on TSH levels. PSA levels were compared by Wilcoxon test.

Statistical analyses were commonly performed using the R software package.

3.2. Impact of antibiotic prophylaxis in prostate biopsy on the colonic microbiome

3.2.1. Patients

The study included 9 patients (age (median [range]: 67 [57-75 years]) who underwent TRUS-guided transrectal prostatic needle biopsy for suspected PCA (mainly indicated by elevated PSA levels) between August and November 2018. The patients had not received antibiotics in the six months prior to the study and reported not to have taken probiotics. [Tóth Z et al, 2022]

Patients were in good general health; they were seen by a urologist for complaints of moderate dysuria or for screening and had elevated PSA levels (lowest PSA was 5.65 μg/L, highest 14 μg/L, median 9 μg/L). Four subjects were also hypertensive. Five patients were receiving alpha-blockers for urological complaints and 1 patient was also taking alpha-reductase blockers.

To prevent infectious complications of the procedure, as part of the practice in 2018, patients received 2*500 mg of ciprofloxacin and 2*150 mg of clindamycin from the day before the procedure. The combination therapy lasted until day 5 after the biopsy; thereafter, patients received only 2*150 mg clindamycin daily for three more days. No TRUS-associated complications were reported in any of the patients. It should be noted that pre-treatment included cleansing of the rectal region with povidone-iodine according to European guidelines [Mottet et al., 2021]. The study was approved by the institutional ethics committee (Ethics Committee of Uzsoki Hospital, Budapest, Hungary) according to the principles of the Declaration of Helsinki; all patients gave verbal and written informed consent to participate in the study.

3.2.2. Sampling to determine the gut microbiome

All participants took stool samples themselves at home 1 day before the antibiotics were given and 14 days after the biopsy. Sampling was performed using standard sterile uBiome RNA/DNA-free swabs (uBiome, Ca, USA) according to the standardised sampling procedure recommended by the laboratory [McInnes and Cutting, 2010]. This involved transferring a small amount of stool material into a sample collection vial containing lysis and stabilisation buffer using the sterile swab provided with the kit. The samples were sent via a mail order service to the uBiome processing laboratory (uBiome, San Francisco, Ca, USA).

3.2.3. Laboratory analysis of the gut microbiome

For DNA extraction in the laboratory, samples were first lysed with beads and then DNA was extracted using a purification method based on a guanidine-thiocyanate silica column [Hummel and Kula, 1989] [Cady et al., 2003].

The variant region V4 of the 16S rRNA gene was amplified by PCR using universal primers (515F: GTGCCCCCCAGCMGCCGCCGCCGCGGTAA and 806R: GGACTACHVGGGTWTWTCTAAT) [Caporaso et al., 2011]. The candidate PCR products were column purified after pooling and size-selected by microfluidic DNA fractionation [Minalla et al., 2001]. Libraries were quantified by quantitative PCR using a Kapa Bi6o-Rad iCycler qPCR kit on a Bio- Rad MyiQ instrument; sequencing was performed on an Illumina NextSeq 500 platform, which measured 2150 bp paired-end sequences. Sequencing of the 16S rRNA gene and taxonomic annotation were

performed according to the standardized fecal microbiome identification procedure routinely used in the laboratory, based on information collected by the Human Microbiome Project (HMP) [2012].

After sequencing, BCL2FASTQ software was used to demultiplex the data and generate individual fastq files per sample. Reads with a Q-score <30 were excluded from the analysis. Primers were removed, paired forward and reverse reads were matched. The resulting amplicons were clustered using the Swarm V.2.1.5 algorithm, using a nucleotide distance and the 'fastidious' and 'u-search-abundance' flags. The sequence with the highest frequency per cluster was considered the true biological sequence and the total number of reads in the cluster was added. Chimera sequences were removed using the VSEARCH algorithm. All reads passing through the above filter were aligned to a manually compiled database of 16S rRNA gene target sequences and taxonomic annotations from the SILVA V.132 database using 99% identity at 100% of length. Only taxa for which V4 amplicons allowed unambiguous taxonomic classification were included in the analysis. For analysis, raw counts were transformed using a centred log ratio (CLR).

3.2.4. Statistical analysis

The results for the samples were collected from the results files generated by the uBiome laboratory. Sequencing results were used to compare microbiome components before and after biopsy at strain, order and genus level. Only those strains, orders and genera that had a median frequency of at least 1.0% of the total microbiome before and/or after biopsy were considered for analysis. The detection level was considered as an abundance ratio of 0.1%; abundance values below this level were used as '0' in the statistical analysis. Differences in abundance ratios of the microbiome components before and after biopsy were analysed using the Wilcoxon test.

3.3. Predictive value of general blood count parameters for overall survival in PCA

Data from patients diagnosed with PCA at the Urology Department of the Uzsoki Street Hospital in Budapest between 2000 and 2005 were included in the analysis. The blood count parameters (RBC, WBC, PLT, NLR, PLR), PSA level, TNM score, histological result (Gleason score) and co-morbidities determined at the time of diagnosis between 2000 and 2005 (±1 week) were extracted and recorded from the patients' medical records.

Co-morbidities were diagnosed hypertension, diabetes mellitus, history of cardiovascular event, heart failure, neurological disease. Depending on their condition, patients may have undergone surgery and/or chemotherapy and/or irradiation and/or hormone therapy. A significant proportion received palliative treatment only.

A 3 -part-differential haematology machine (type not identifiable) was used for blood counting to isolate the major blood cell types at the time of diagnosis. PSA levels were measured by Abbott immunoassay. I made the diagnosis, performed any surgery and managed the treatment for all patients.

We retrieved the date of death from the NEAK database based on the patients' social security number. If this was not available in the NEAK database, the patients were classified as living. Statistical analysis was performed using descriptive statistics to characterize the clinical parameters of living and non-living patients. The effect of blood count parameters, Gleason score, PSA level, comorbidities and age on overall survival in the group of patients who died was evaluated by Cox regression analysis.

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4. Results

4.1 Distribution of PSA levels as a function of other laboratory parameters

Mean PSA levels were nominally lower in patients with high ALT and low eGFR. PSA levels were higher with high CRP levels. [Tóth Z et al, 2020 (b)]

Table 1 Prostate-specific antigen (PSA) per centile values among patients grouped by laboratory abnormalities. PSA values below the limit of quantification were treated as 0.1 µg/L.

Parameter	Case number	Average age of patients (years)	Percentile PSA value (µg/L)				
			5	25	50 (median)	75	95
ALT. IU/L							
8	19887	61.5	0.1	0.54	1	2.16	10.94
40-<80	3675	55.3	0.1	0.47	0.83	1.6	8.71
80-120	653	55.5	0.1	0.43	0.77	1.4	7.79
>120	552	57.2	0.1	0.4	0.73	1.61	9.92
eGFR ml/min/1.73m2							
<15	296	62.3	0.1	0.67	1.12	2.77	13.43
15-<30	596	68.8	0.1	0.58	1.3	3.14	12.57
30-<60	4412	68.5	0.1	0.61	1.26	2.94	12.85
60-90	14146	60.5	0.1	0.52	0.96	2.05	10.37
>90	4743	52.4	0.1	0.48	0.80	1.43	6.97
CRP. mg/L							
<5	11487	58	0.1	0.53	0.92	1.8	9.04
5-<10	2401	61.8	0.1	0.50	0.97	2.1	10.12
10-<20	1786	64.5	0.1	0.52	1.03	2.39	12.17
20-50	1740	66	0.1	0.49	1.08	2.73	11.99
>50	2238	66.9	0.1	0.48	1.16	2.9	14.25

A significant correlation was found between PSA and ALT, eGFR and CRP levels. When the association was adjusted for age, the association between ALT or eGFR and PSA disappeared, but high CRP levels remained significantly associated. The estimate, standard error and probability values are shown in Table 2. [Tóth Z et al., 2020]

Table 2. Assessment of factors affecting PSA: results of our logistic regression analysis

Coefficients:	Estimate	Standard error	P value
when age was NOT included in the analysis			
log ALT	-0.125	0.013	< 2e-16 *
log eGFR	-0.147	0.024	1.12e-09*
log CRP	0.024	0.005	9.70e-06 *
when the age is REQUIRED in the analysis			
age	0.0088	0.0003	<2e-16*
log ALT	-0.0068	0.014	0.642
log eGFR	-0.0459	0.025	0.076
log CRP	0.002*	-0.0335	0.011
relationship between age AND			
log ALT	-0.025	0.002	<2e-16 *
log eGFR	-0.194	0.006	<2e-16 *
.log CRP	0.0502	0.002	<2e-16 *

The majority of patients (55.8 and 22.2 percent) had moderate to severe vitamin D deficiency. Based on vitamin D levels, groups were divided into vitamin D levels >30 µg/L, 15-30 µg/L and <15 µg/L (Table 3)

Pearson's correlation coefficient (R) was calculated after logarithmic transformation of PSA and vitamin D levels to obtain a near-normal distribution in each dimension. The linear regression line for the logarithm of the data points is shown in red. The two-dimensional distribution and the correlation were also calculated for the whole patient population (Figure 3 left) and for patients with PSA above 4 µg/L (Figure 3 right).

The Pearson correlation coefficients determined were close to 0, suggesting that there is no linear relationship between PSA and vitamin D levels.

Our results from the analysis of the relationship between TSH and PSA are summarised in Table 4. A significant relationship was found between lnPSA levels and age, and between lnPSA and lnTSH levels; a 10% decrease in TSH levels leads to a 0.42% increase in PSA levels. [Tóth Z et al, 2019]

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Table 3 Distribution of patients (total number: 5136) by age and vitamin D level

Vitamin D levels	<50 years	50 - 59 years	60 - 69 years	at least 70 years
Number of patients				
<15 µg/L	280	226	309	327
15-30 µg/L	806	677	755	629
30< µg/L	351	229	300	247
PSA 95th percentile values (µg/L) in different age groups at different vitamin D levels				
<15 µg/L	2,0	3,4	7,7	11,0
15-30 µg/L	2,1	3,8	7,0	9,4
30< µg/L	2,4	4,7	8,2	8,7
Percentage of patients with PSA >4 µg/L				
<15 µg/L	1.0%	3.9%	14.9%	22.3%
15-30 µg/L	0.7%	5.3%	12.4%	18.2%
30< µg/L	1.4%	6.5%	16.3%	23.4%

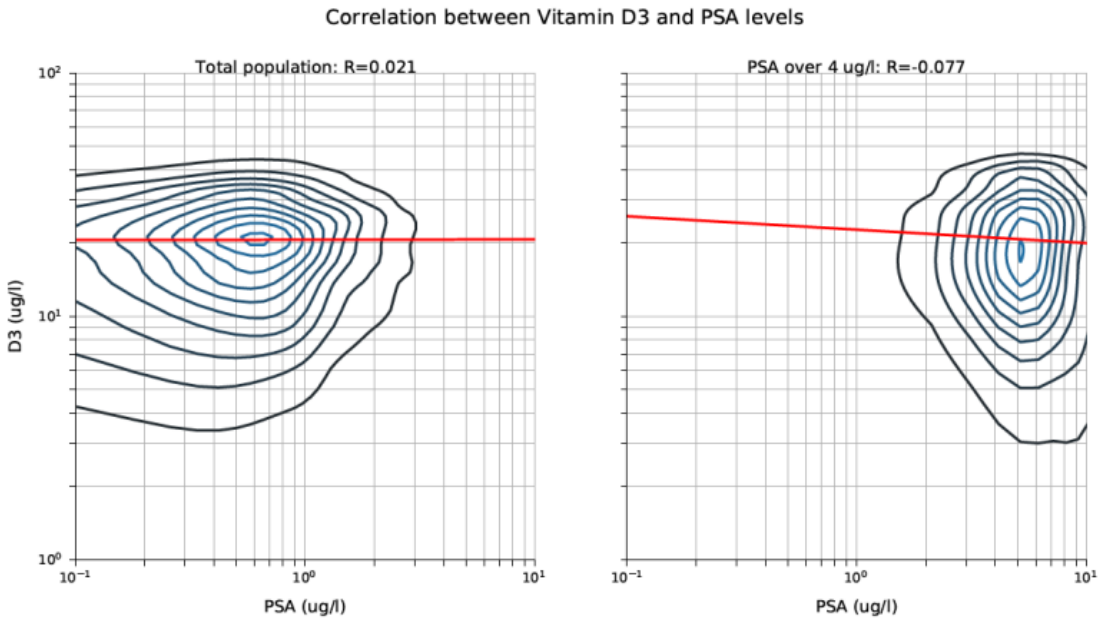

Figure 3. Bivariate kernel density estimates of PSA and vitamin D values measured in our laboratory between October 2007 and June 2018. Solid lines represent coordinates with the same data density in two-dimensional space, smoothed by a Gaussian function. Because of the characteristics of the PSA test, all values below 0.1 µg/L are reported here as 0.1 µg/L; bias was corrected for by kernel density estimation.

The PSA levels that would (theoretically) be associated with different TSH values in the same patient are demonstrated in Table 5. In a direct comparison of hyperthyroid and euthyroid patients, PSA levels were significantly higher in hyperthyroid patients (n = 405) compared to euthyroid patients (n = 6698) (median, interquartile range) (PSA level: 1.118 [0.639-2.338] vs. 0.920 [0.508-1.826] μg/L, p = 0.016).

Table 4 Relationship between TSH and PSA levels in men aged 40 to 75 years (n = 7279). Descriptive statistics and results of multiple logistic regression. IQR = interquartile range; PSA = prostate-specific antigen; TSH = thyroid-stimulating hormone

Age, year (mean, standard deviation)	58.9 ± 9.22
PSA (median, IQR) μg/L	0.931 (0.511; 1.86)
TSH (median, IQR) mU/l	1.265 (0.828; 1.89)
Results of multiple regression analysis (effect on PSA levels)	
lnTSH (estimate (error of estimate), p-value)	-0.042 (0.014). 0.003
Age (estimate (error of estimate), p-value)	0.028 (0.001). <0.001
The r-value of the correlation	
InTSH	0.297
Age	0.472

Table 5 Hypothetical effect of reduced TSH levels on measured PSA levels within a patient. The analysis suggests that in hyperthyroidism, measured PSA levels can rise to levels close to or well above the cut-off of 4 μg/L in euthyroidism in patients with euthyroid PSA levels of 2 and 4 μg/L, respectively

Measured TSH level (mU/L)	PSA level difference compared to hypothetical 2 mU/ml TSH	PSA levels (μg/L)		
2	0	1	2	4
1	4.20%	1.04	2.08	4.16
0.5	8.40%	1.08	2.16	4.32
0.2	42%	1.42	2.84	5.68
0.1	84%	1.84	3.68	7.36

PSA = prostate-specific antigen; TSH = thyroid-stimulating hormone

4.2. Impact of antibiotic prophylaxis in TRUS-guided biopsies on the microbiome

The relative prevalence before and after antibacterial therapeutic intervention at the strain, genus and order level is shown in Figure 4. Table 6 shows the mean values. [Tóth Z et al., 2022]

At strain level, 4 strains were detected at all time points with a median frequency of at least 1.0%. The abundance of Actinobacteria and Firmicutes decreased dramatically, while the abundance of Bacteroides and Proteobacteria increased following antibiotic therapy. The Firmicutes:Bacteroides ratio was reversed (from 2.81 to 0.74, p 0.035).

At the order level, 7 orders occurred with a median abundance of at least 1% within the total microbiome. Of these, Bacteroides and Clostridia together accounted for 75% and 91% of the total microbiome before and after treatment, respectively. The abundance of Bacteroidales and Veillonellales increased, while that of Clostridiales and Coriobacteriales decreased after antibiotic therapy.

At the genus level, we detected 15 genera with a relative frequency of at least 1% median at any time point and in at least 3 patients. Together, these were responsible for 61.87% and 79.89% of the total microbiome, respectively. Four genera showed significant changes in abundance; of these, Bacteroides increased, while Roseburia, Faecalibacterium and Collinsella decreased dramatically.

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Table 6 Relative abundance of the main bacterial strains, orders and genera detected in stool samples before and after ciprofloxacin-clindamycin combination therapy for the prevention of TRUS biopsy. Patients received ciprofloxacin 500 mg twice daily and clindamycin 150 mg twice daily. Combination therapy was started on the day before biopsy; then ciprofloxacin and clindamycin were continued on days 5 and 8 after TRUS; sampling was performed on the day before biopsy and 14 days after biopsy. The "n" indicates the number of patients with a detectable high (0.1%) prevalence of the genus/strain/strain. Q1 and Q3 denote the percentile values of 25 and 75 percentiles, respectively.

Phylum	BASELINE (before antibiotic therapy), relative abundance (per cent)				14 days after biopsy, relative abundance (per cent)				p value
	patients with >0.1 abundance of, n =	MEDIAN	Q1	Q3	patients with >0.1 abundance of, n =	MEDIAN	Q1	Q3	
Firmicutes	*9*	*64.51*	*52.76*	*73.4*	*9*	*41.18*	*36.44*	*42.51*	*0.007*
Bacteroidetes	*9*	*21.73*	*17.52*	*31.25*	*9*	*55.37*	*36.02*	*57.09*	*0.000*
Proteobacteria	9	3.2	2.22	4.38	9	4.32	2.56	5.83	0.314
Actinobacteria	*9*	*2.91*	*1.72*	*4.46*	*9*	*0.99*	*0.38*	*1.71*	*0.017*
Firmicutes/Bacteroidetes ratio	*9*	*2.81*	*2.06*	*4.01*	*9*	*0.74*	*0.65*	*1.18*	*0.035*

Order	BASELINE (before antibiotic therapy), relative abundance (per cent)				14 days after biopsy, relative abundance (per cent)				p value
	patients with >0.1 abundance of	MEDIAN	Q1	Q3	patients with >0.1 abundance of	MEDIAN	Q1	Q3	
Clostridiales	*9*	*53.67*	*46.27*	*61.57*	*9*	*35.41*	*31.72*	*37.18*	*0.013*
Bacteroidales	*9*	*21.73*	*17.11*	*31.25*	*9*	*55.37*	*36.02*	*57.07*	*0.003*
Veillonellales	*9*	*1.82*	*0.06*	*2.54*	*7*	*2.74*	*0.05*	*3.73*	*0.014*
Burkholderiales	7	1.47	0.55	2.10	6	2.42	0.00	5.04	0.114
Coriobacteriales	*8*	*1.41*	*0.23*	*2.44*	*7*	*0.03*	*0.01*	*0.51*	*0.009*
Lactobacillales	9	1.15	0.89	1.67	9	1.15	0.78	2.61	0.322
Erysipelotrichales	9	0.62	0.6	1.1	9	1.35	1.3	1.42	0.118

Genus	BASELINE (before antibiotic therapy), relative abundance (per cent)				14 days after biopsy, relative abundance (per cent)				p value
	patients with >0.1 abundance of	MEDIAN	Q1	Q3	patients with >0.1 abundance of	MEDIAN	Q1	Q3	
Faecalibacterium	*9*	*15.28*	*13.30*	*17.81*	*8*	*1.16*	*0.01*	*3.45*	*0.013*
Bacteroides	*9*	*11.340*	*9.76*	*23.32*	*9*	*45.81*	*34.52*	*50.66*	*0.002*
Blautia	9	8.020	7.05	11.68	9	9.750	3.06	11.68	0.409
Roseburia	*9*	*6.770*	*2.60*	*11.23*	*8*	*1.99*	*0.53*	*3.21*	*0.004*
Subdoligranulum	9	3.850	1.99	5.30	8	0.77	0.29	1.92	0.171
Pseudobutyri-vibrio	9	3.240	2.11	3.45	8	0.97	0.11	2.86	0.270
Alistipes	*9*	*2.170*	*1.34*	*2.82*	*7*	*0.53*	*0.43*	*1.15*	*0.010*
Sarcina	8	1.60	0.69	3.23	7	0.11	0.01	0.96	0.084
Dialister	7	1.85	1.41	2.16	5	0.01	0.00	2.72	0.927
Collinsella	*9*	*1.380*	*0.230*	*2.31*	*6*	*0.03*	*0.00*	*0.46*	*0.007*
Barnesiella	7	1.14	0.10	1.29	3	0.00	0.00	0.21	0.050
Fusicatenibacter	9	1.11	0.61	2.60	7	1.53	0.06	4.32	0.399
Dorea	9	1.08	0.88	1.80	8	3.16	1.28	4.94	0.177

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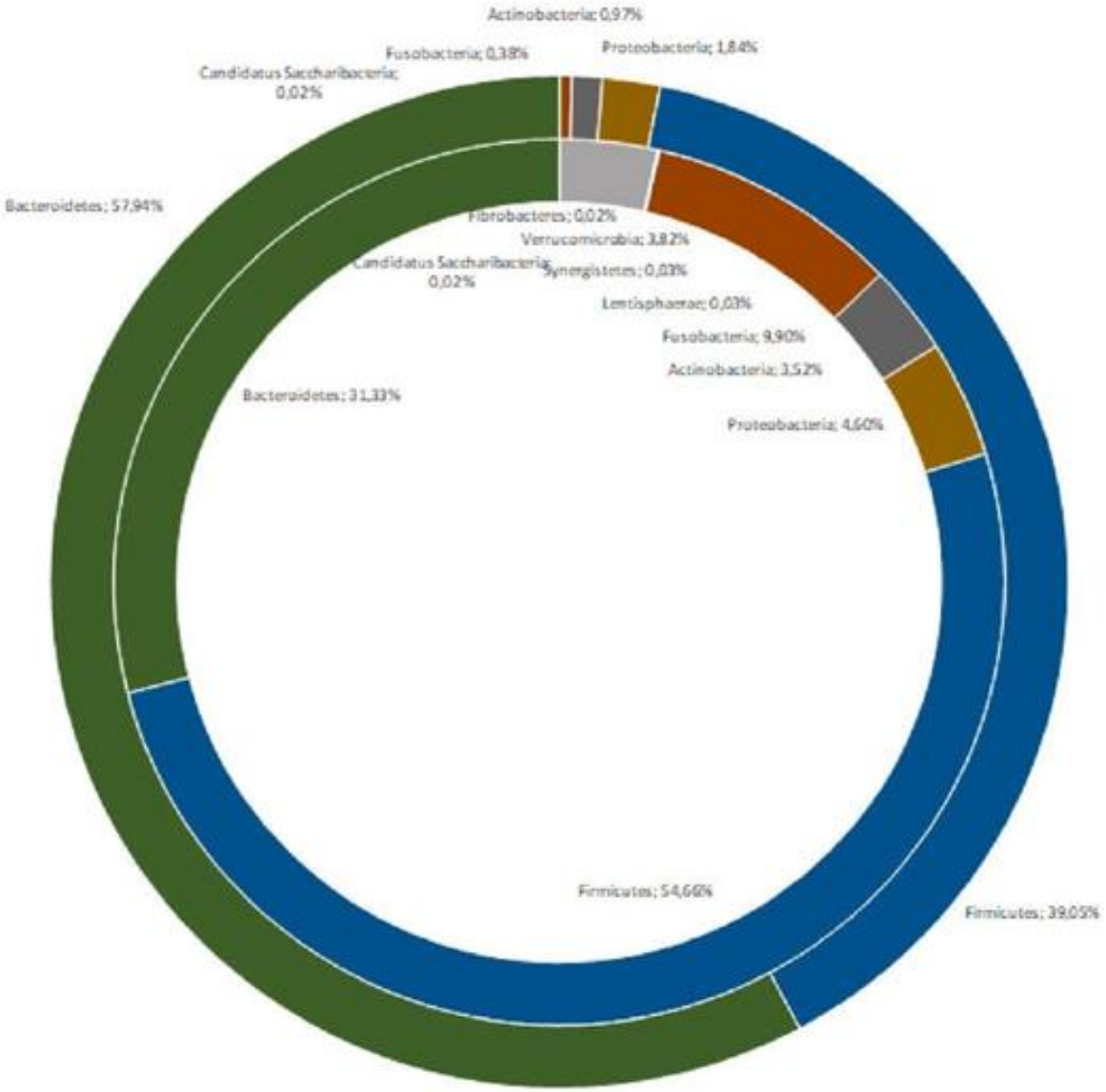

Figure 4a.

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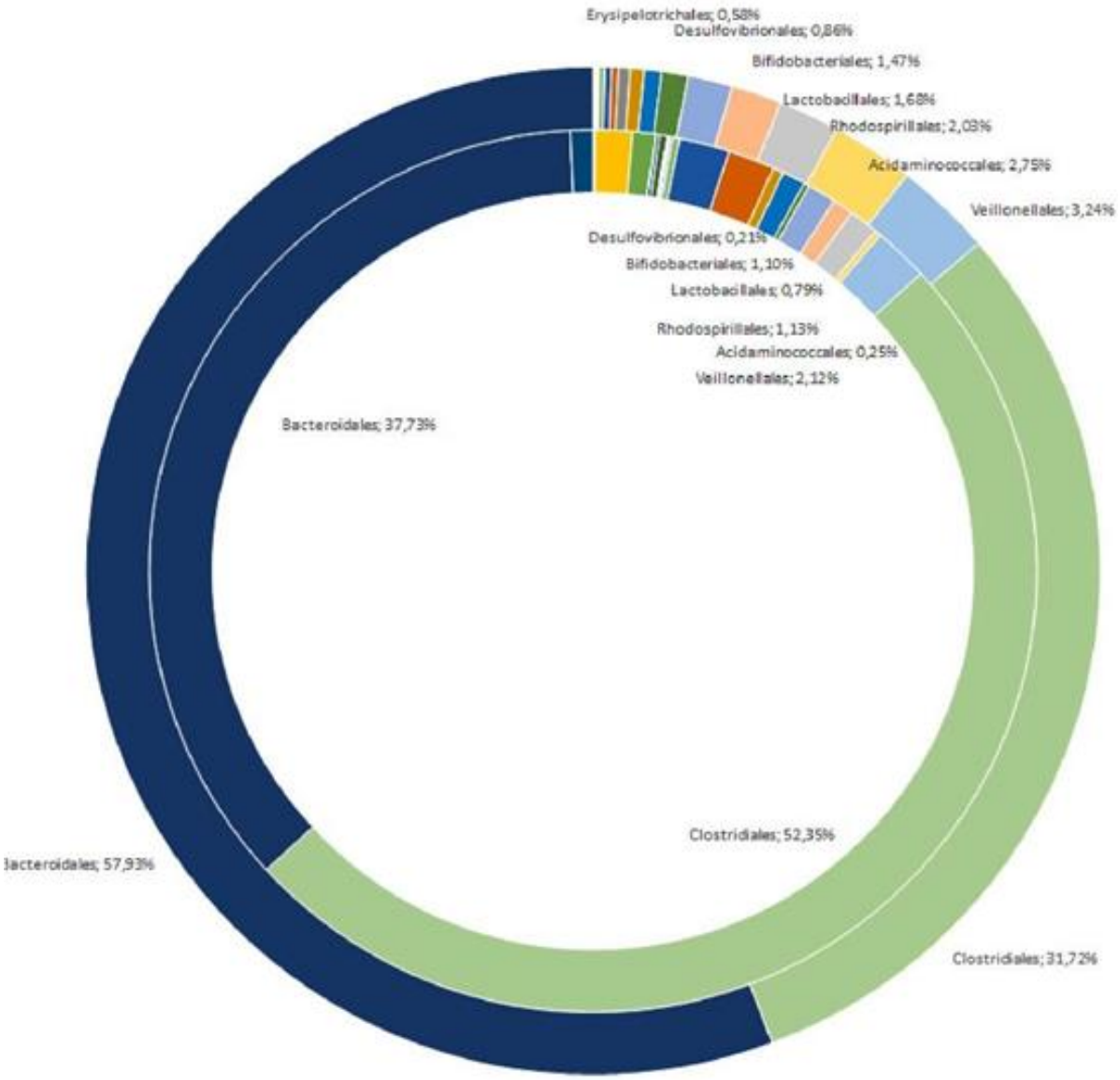

Figure 4b

Figure 4. Graphical representation of the change in median values of relative abundance of the main strains (Figure 4a) and orders (Figure 4b) in relation to antibiotic therapy (see text for details). The outer and inner circles indicate the distributions before and after ciprofloxacin-clindamycin combination therapy, respectively.

4.3. Predictive value of blood count for mortality

In our analysis, we processed data from 97 patients. The clinical characteristics of patients who died (n=82) and did not die (n=15) are summarised in Table 7. It can be seen that none of the elements of the blood counts determined at the time of diagnosis of PCA were significantly different between the two groups; in contrast, Gleason score, age of patients at the time of diagnosis, higher PSA level, palliative treatment used were more frequent in the group of patients who died.

Table 7 Baseline clinical data of patients at the time of diagnosis of PCA. p value: based on 2-sample t-test (except for palliative care and PSA levels, where a nonparametric test was used)

	Patients who died (n=82)	Live patients (n=15)	p value
Age at diagnosis (years)	72,4±9,3	63,14±7,44	0,0004
Age at death (years)	78,8±8,8		
Survival time in case of death (years)	6,4±5,7		
Number of comorbidities	1,50±0,82	1,13±0,35	NS
Gleason point value	6,37±1,78	5,27±1,75	<0,0001
Palliative treatment	38/82	1/15	0,001
PSA level (ng/mL) [median, min-max]	42,11 (4 - >150)	12,47 (2 - 40)	0,001
Blood count parameters			
White blood cell count (G/L)	9,92±8,73	8,77±3,65	NS
Red blood cell count (T/L)	4,30±0,55	4,45±0,61	NS
Haemoglobin level (g/L)	131±18,3	133,07±16,81	NS
Average red blood cell volume (fL)	91±4,7	89,46±2,89	NS
Platelet count (G/L)	237±84,2	202,80±63,16	NS

In the group of patients who died, multivariate regression analysis indicated a significant effect of age (p=0.004) and PSA and GLEASON score (both p=0.033) on overall survival (Table 8). Overall survival was not affected by any of the blood parameters tested.

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Table 8 Cox regression analysis of the relationship between the clinical parameters assessed at the time of diagnosis of PCA and the overall survival of patients.

	B	SE	Forest	Sig.	Exp(B)
Age	0,052	0,018	8,320	0,004	1,054
Co-morbidity	0,281	0,173	2,641	0,104	1,325
Palliative care	-0,238	0,275	0,751	0,386	0,788
Gleason score	0,183	0,085	4,569	0,033	1,200
PSA level	0,006	0,003	4,526	0,033	1,006
Blood count parameters					
Red blood cell count	0,066	0,261	0,065	0,799	1,069
White blood cell count	0,004	0,038	0,011	0,916	1,004
Platelet count	-0,004	0,004	0,832	0,362	0,996
Neutrophil granulocytes, %	-0,045	0,035	1,693	0,193	0,956
Lymphocytes (%)	0,006	0,042	0,023	0,879	1,006
Neutrophil/lymphocyte	-0,036	0,134	0,074	0,786	0,964
Platelets/lymphocytes	0,055	0,051	1,201	0,273	1,057

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5. Discussion

5.1 Distribution of PSA levels as a function of other laboratory parameters

In our retrospective data analysis, we tested the hypothesis that PSA reference ranges should be adjusted for some common laboratory abnormalities indicating impaired liver function, the presence of systemic inflammation, thyroid dysfunction or vitamin D deficiency. [Tóth Z. et al, 2020 (a and b)][Tóth Z. et al, 2019]

For nearly 3 decades, measuring PSA levels has been an essential part of the laboratory armamentarium for early detection of PCA. With tens of millions of tests performed worldwide every year, the importance of properly determining PSA levels for clinical decision-making cannot be overestimated. The use of an inadequately low cut-off value would lead to an increase in the number of false positive results, resulting in a dramatic increase in the number of unfounded tests including biopsies, while an inadequately high cut-off value would lead to a high rate of false negative results and an increase in the number of undetected cases of PSA.

PSA levels are strongly influenced by the age of the patient, as PSA levels themselves can rise in ageing men, even without a clear change in prostate status. It is therefore recommended to use age-specific reference ranges to increase the specificity of screening [Partin et al., 1996]. However, the risk of liver and kidney damage is increased in the ageing population. In addition, vitamin D deficiency and impaired thyroid function are common. The incidence of asymptomatic or very asymptomatic prostatitis is not negligible. All these disorders may be associated with alterations in the synthesis, release, composition (relative proportion of PSA bound to proteins) and circulating half-life of PSA. Indeed, a number of studies suggest that these conditions may have a significant impact on PSA levels.

Vicentini and colleagues [Vicentini et al., 2009] analysed PSA levels in patients with liver cirrhosis. This study prospectively evaluated patients with severe liver failure on the liver transplant waiting list and reported an inverse association between fibrosis severity and PSA levels. Inci and colleagues [Inci et al., 2013] reported similar observations in cirrhotic patients grouped by Child-Pugh score. These data on a relatively small number of subjects (n=112 and 82, respectively) are consistent with data from two large epidemiological studies of patients with varying degrees of liver fibrosis associated with

non-alcoholic fatty liver disease [Yoon et al., 2015][Wang et al., 2019]. Both studies showed that patients with advanced liver disease have a significantly lower likelihood of abnormal PSA levels, limiting the utility of PSA levels in screening for PCA in this patient population.

Several groups have investigated PSA levels in dialysed and non-dialysed end-stage renal disease [Sasagawa et al., 1998; Danişman et al., 2000; Douville et al., 1998] and in patients with varying degrees of renal impairment [Bruun et al., 2009]. The results show that PSA levels are not significantly affected by renal function alone, and that renal function has no effect on PSA levels. Therefore, reference ranges for total PSA levels do not need to be adjusted for the severity of renal impairment [Amiri, 2016][Coppolino et al., 2014].

CRP is a widely accepted marker of systemic inflammation. Several studies suggest that it can also be used as a prognostic factor for PCA [Lehrer et al, 2005; Beer et al, 2008; Kim et al, 2013; Prins et al, 2012]. However, two studies in healthy men have indicated that while neutrophil cell count and lymphocyte to lymphocyte ratio and fibrinogen levels in blood count are directly related to PSA, CRP levels themselves are not related to PSA [Yun et al., 2017; McDonald et al., 2014].

These studies have been conducted on populations with well-characterised health status and/or patients classified according to specific characteristics. However, the situation may not be clear in everyday clinical practice, where clinicians have to treat a mixed, ageing patient population with different chronic and acute conditions. The value of data collected from such populations is that they reflect 'real-life' clinical practice.

These results were consistent with published reports of lower than average or higher than average PSA levels in patients with liver or kidney failure (as defined by laboratory abnormalities). However, the correlations disappeared when the age of the patients was taken into account, suggesting that the effect of these conditions alone on PSA levels is negligible. (Our analysis also showed that age alone is associated with high ALT and low eGFR levels.) Therefore, the cut-off values for PSA levels should not be adjusted for ALT or eGFR, used as surrogate markers of hepatic and renal impairment, respectively, in this study. However, it should be stressed that we were not aware of the diagnosis and general clinical status of the patients. We cannot exclude the possibility that there may be specific

liver or kidney diseases hallmarked by markers other than ALT and eGFR, respectively, that have a significant impact on PSA metabolism or lifetime.

No relationship was found between PSA and vitamin D levels. Epidemiological observations and, more recently, an intervention study have suggested a possible role for vitamin D3 and vitamin D3 supplementation in the prevention of PCA. The results of the recent VITAL trial support that vitamin D3 at a dose of 2000 IU/day reduces mortality from PCA by up to 12%. [Manson et al., 2019]. Daily high-dose vitamin D3 supplementation and adequate levels of active vitamin D inhibit the transition of early, low-risk PCA to more aggressive forms. [Hollis et al., 2013],[Ramakrishnan et al., 2019]. However, the benefits of vitamin D in the therapy of advanced PCA are less convincing. [Shahvazi et al., 2019]

The prostate-protective mechanism of action of vitamin D is widely studied. Vitamin D receptor, as a nuclear receptor, has a significant effect on gene regulation involved in prostate cell differentiation and metabolism and is a generally recognised antitumour agent. [Krishnan et al., 2003; Shiota et al., 2019] Recent data suggest that non-genomic effects, in particular those affecting mitochondrial respiration, may also play a role. [Blajszczak et al., 2019] Another study showed that at appropriate levels, active vitamin D metabolite inhibits the intracrine conversion of dehydroepiandrosterone to prostate growth-stimulating androgens such as testosterone and dihydrotestosterone. [Smith et al, 2019]

Several studies have reported an inverse association between vitamin D levels and risk and aggressiveness of PCA [Grant, 2020; Manson et al., 2019; Hollis et al., 2013]. Thus, we hypothesized that the putative effect of vitamin D on prostate health would be reflected by PSA, a surrogate marker of prostate pathology. However, the results of our analysis in a hospitalized patient population do not support this hypothesis. These results are consistent with the results of several small studies that have looked for an association between vitamin D levels and PSA levels. A prospective study involving 105 healthy men with no baseline PSA change documented that, although blood levels of vitamin D increased, PSA levels did not change on vitamin D administration. [Chandler et al, 2014] No association between vitamin D and PSA was found in 71 patients on peritoneal dialysis. [Passadakis et al, 2004] Another study involving 1705 subjects found no direct

association between PSA and vitamin D levels in non-PCA patients. [Nair-Shalliker et al., 2014]

Unlike ALT or eGFR or vitamin D, CRP levels are directly associated with higher PSA levels. Median and 75% PSA levels in patients with systemic inflammation (defined as CRP levels >50 mg/L) were more than 20% and 50% higher, respectively, than in those with low CRP levels (CRP levels <5 mg/L). This association remained significant after adjusting for patient age. Based on this finding, it is therefore recommended that clinical decisions based on PSA should be postponed until CRP normalisation.

For the first time in the literature, we found evidence that PSA levels can be significantly influenced by thyroid function, with lower PSA levels in hypothyroidism and higher PSA levels in hyperthyroidism. The effect is quantifiable: a 10% change in TSH levels results in a 0.42% change in PSA levels in the opposite direction to TSH levels. Although at first glance this seems negligible (a change of 0.42% is well below the measurement uncertainty of 5-10% for PSA determination), this is not the case when the fluctuation in TSH levels is acknowledged.

The healthy reference range for TSH levels is generally given as a range between 0.3 and 4.3 mU/l. TSH responds exponentially to variations in thyroid hormone levels. A small increase in thyroid hormone levels triggers a large decrease in TSH levels [Baloch et al., 2003]. As a result, for example, TSH in mild hyperthyroidism shows a 10-fold or 1000% change compared to the euthyroid state (0.2 vs. 2.0 mU/l). In this case, this can be accompanied by a roughly 40% increase in PSA levels, which can be particularly troublesome for near-threshold PSA levels. On the one hand, this raises the possibility that PSA cut-off values calculated in a predominantly euthyroid population may need to be adjusted in hyperthyroid (or hypothyroid) populations; and on the other hand, that in the case of abnormally high PSA levels, it is worthwhile to check thyroid function and, if abnormalities are detected, to treat the PSA level with greater caution.

In everyday urological practice, the relationship with TSH level may be of particular importance in establishing the indication for repeat prostate biopsy, as it may reduce the number of unnecessary biopsy procedures. However, based on our cross-sectional study, it is not possible to say what role the underlying disease, medication or even time of day plays in the TSH level decline. (Moreover, in our work we measured total TSH levels, and the test we used differentiates between TSHs produced in the pars tuberalis and pars

distalis of the pituitary, which may have different biological characteristics [Drees et al., 2014]. Regardless, our observation is consistent with the previous clinical observation that hyperthyroid individuals have a higher prevalence of PCA compared to euthyroid individuals [Mondul et al., 2012], and that high thyroid hormone levels enhance prostate cell proliferation [Pinto et al., 2011].

The advantage of our retrospective data collection on the relationship between PSA and laboratory parameters is that we had the opportunity to evaluate a statistically large number of data from tens of thousands of patients. A limitation of the results is that the patients included in the database were enrolled at level 3 of health care (Semmelweis University clinics); thus, the conclusions can only be applied to this relatively specific population. Unfortunately, the database does not include the prescribing diagnosis; exactly what disease the patient had, whether they had received any medication that might have affected PSA levels. Although the database may indeed be distorted by the data on patients with PSA and those treated in different specialised outpatient clinics, the extent of this distortion seems limited given the nearly half a hundred clinics served by the Institute of Laboratory Medicine and the large number of patients treated there. In addition, each patient was included in the database only once (through a first-time sample). In our opinion, our analysis suggests that the PSA reference range depends not only on the age of the patients but also on their inflammatory status (CRP level) and thyroid function (TSH level). When assessing PSA levels, patients' liver and kidney function and vitamin D levels should not be taken into account.

5.2. Impact of antibiotic prophylaxis in prostate biopsy on the gut microbiome

The role of the microbiome in the development of prostate disorders is supported by animal data and clinical observations. In mice, modulation of the microbiome has been shown to induce intraepithelial neoplasia and microinvasive cancer of the prostate [Poutahidis et al., 2013]. Clinical data also indicate the influence of the microbiome on prostate health. In a study of 128 patients, patients with benign prostatic hypertrophy had a higher Firmicutes/Bacteriodetes ratio compared to patients without prostatic hypertrophy [Takezawa et al., 2021]. Also, in a study involving 8 patients with benign prostatic hyperplasia and 12 patients with PCA, biologically significant differences were observed: the Bacteriodes massiliensis strain was more frequent, while Faecalibacterium

prausnitzii and Eubacterium rectalie strains were present in lower proportions compared to controls [Golombos et al., 2018].

In another study, the faecal microbiome was assessed in patients undergoing prostate biopsy. The histological results showed that 64 patients were diagnosed with cancer and 41 patients with benign enlargement. The microbiome tests showed a correlation between microbiome composition and cancer/non-cancer status. Pathway analysis identified several aberrant metabolic pathways that predispose to cancer development [Liss et al., 2018].

A further study included 96 patients with PCA and 56 patients without PCA. It was found that the abundance of Rikenellaceae, Alistipes and Lachnospira strains increased in the high-risk and cancer groups. Based on the characteristics of the microbiome, the authors developed a specific index that could predict the outcome of histopathological examination (the accuracy of the index, incidentally, significantly exceeded that of PSA levels) [Matsushita et al., 2021]. Interestingly, other studies have failed to confirm these changes. Based on stool samples from 30 patients, the gut microbiome was not strikingly different between PCA and prostate enlargement [Alanee et al., 2019].

In our study, we included patients who had a biopsy for suspected PCA, which was later confirmed by histopathology. [Tóth Z et al, 2019] Our results showed that a short course of ciprofloxacin - clindamycin combination therapy to prevent biopsy-associated bacterial infections radically alters the composition of the gut microbiome and can be detected 2 weeks after biopsy. As a sign of this, the relative abundance of the dominant strains (i.e. Firmicutes, Bacteroidetes and Actinobacteria) is significantly altered. This result is in agreement with another study on a similar number of subjects [Li et al., 2022]. Participants received a single 1 g oral dose of an amoxicillin-clavulanic acid combination immediately before biopsy. Significant differences were identified for the stool microbiome determined before the procedure and 5 weeks later. The relative abundance of Firmicutes and Bacteroidetes varied in opposite directions, with a significant decrease in the latter.

In our practice, patients are subjected to a much longer course of antibiotic therapy, which includes ciprofloxacin and clindamycin. Although this therapeutic regimen is effective in reducing the post-biopsy infection rate, its impact on the composition of the microbiome must also be considered.

Regardless of the therapeutic indication, the impact of antibiotic therapy on the gut microbiome is generally recognised [Patangia et al., 2022]. Complications of antibiotic therapy include dysbiosis (leading to Clostridium difficile overgrowth and pseudomonal colitis in extreme cases) and, in the longer term, the development of resistant strains. Our results show that, even without these clinically obvious situations, short-term combination therapy - which many urologists consider harmless and use routinely - can still have profound effects on the gut microbiome.

It should be noted that, theoretically, topical povidone-iodine applied to patients prior to biopsy may also affect the rectal microbiome. However, as sampling was performed the day before and 14 days after povidone-iodine application, the impact of this on our results was considered negligible.

The microbiome is a complex community of microorganisms with a very large number of components. Their contribution to health and disease depends on individual characteristics and relative abundance; the precise role of the majority is less well understood. The challenge is that many of the smaller strains are only transiently present, and at the level of detection in faecal samples. We have therefore focused our work on the major components only. The Firmicutes phylum includes Gram-positive bacteria belonging to nearly 250 genera.

In our study, Firmicutes strain members were responsible for about two-thirds of the faecal microbiome; this proportion decreased significantly with antibiotic administration. Firmicutes bacteria are involved in energy homeostasis and have been associated with metabolic disorders. The prevalence of Firmicutes has been reported to increase in obesity [Crovesy et al., 2020]. At the genus level, Faecalibacterium (also a member of the Firmicutes phylum) was the most common bacterium; it accounted for 15% of the total microbiome of patients before antibiotic treatment. Remarkably, this bacterium produces anti-inflammatory substances that alleviate the course of intestinal diseases [Maioli et al., 2021]. After antibiotic exposure, the proportion of both Firmicutes and Faecalibacterium strains decreased dramatically in our patients.

The second most common strain in our patients' microbiome was Bacteroidotes. These bacteria have a very broad metabolic potential. They have multiple roles; they protect the intestinal tract against pathogens, maintain the intestinal mucosa, and provide vitamins and essential substances to the elements of the microbiome community. They play a

central role in maintaining gut integrity and a healthy immune system [Zafar et al., 2021]. However, there are specific strains that promote malignancies by producing toxins. Dysbiosis (which occurs during antibiotic therapy in conjunction with antibiotic therapy) can lead to the emergence of these pathological strains. In our patients, the relative abundance of Bacteroidetes strain has increased many-fold.

The Firmicutes/Bacteroidetes ratio is increasingly used as a surrogate biomarker for dysbiosis. Firmicutes/Bacteroidetes ratios are more often elevated in obesity and decreased in some inflammatory conditions [Stojanov et al., 2020]. However, current data are rather controversial and there is no clear consensus on a "healthy" Firmicutes/Bacteroidetes ratio [Magne et al., 2020].

The results of our study have several important clinical implications.

1. Preventive antibiotic administration can clearly bias any microbiome analysis. Since antibiotics are widely used in medicine and can be present as contaminants in food (antibiotics are commonly given in high doses in animal husbandry), this can affect the results for many patients. In other words, it can bias the results of all tests that investigate the contribution of the microbiome to disease and that aim to identify diagnostic changes in the composition of the microbiome.

2. In our patients, the composition of the gut microbiome has changed after antibiotic treatment. Based on our current knowledge, it is not possible to say whether this change is detrimental and whether it has long-term effects on diseases, including prostate abnormalities.

3. In our work, we compared only those types of microorganisms that are present in relatively high numbers in the gut. Bacteria present in smaller quantities were not considered. It is not possible to say whether relative variation in these smaller components is a risk factor for disease.

4. Although changes in the microbiome other than the gut microbiome (e.g. the prostate and other urogenital microbiomes) in relation to antibiotic therapy have not been tested, it is likely that antibiotic therapy may also alter their composition. Therefore, there is a possibility that antibiotic administration associated with biopsy may also have an indirect and currently unpredictable effect on prostate status. This potential undesirable effect should be taken into account when indicating transrectal biopsy with antibiotic protection. (Note: Data similar to our results have led to the conclusion that the histological sampling

method, which is essential in the diagnosis of PCA, has been undergoing a transformation in recent years. Instead of the procedures previously followed, transperineal biopsy sampling is nowadays increasingly used, with a significantly lower risk of infection.)

5.3. Predictive value of general blood count parameters for overall survival in prostate cancer

As medical diagnostics have evolved, PCA is being detected at an earlier and earlier stage. The disease is suspected by biomarkers, which have become more widely used in recent decades, particularly elevated PSA levels, and physical examination, while increasingly accurate histological and imaging tests are helping to make the diagnosis. Based on the literature, it is clear that Gleason score and PSA level (and the rate of its rise, i.e. PSA velocity) predict mortality in patients with PCA [Buhmeida et al., 2006]

In addition to the above, the predictive value of routine laboratory parameters at the time of diagnosis, especially of certain elements of the blood count, has been raised. Their use for predictive purposes seemed promising because they are readily available, inexpensive, and easily incorporated into routine patient care.

Gleason score, tumour volume, surgical margin involvement and Ki-67 index have been shown to be prognostic factors for survival in prostate cancer. Also of prognostic significance are p53 status, tumour stage (TNM classification) and degree of aneuploidy. It is a general observation that pre-treatment PSA levels may also be of predictive significance, especially if the tumour has spread outside the prostate. [Buhmeida et al., 2006] Post-operative PSA level nadir can also help to predict prostate-specific and all-cause mortality. [Tseng et al, 2012]

In addition to the above, clinical decision making and the intensity of patient care would be greatly assisted by the use of other markers that are available for daily practice. Such markers could include certain blood parameters that characterise the inflammatory status of the body. White blood cell count, white blood cell subtypes, platelet count and their ratios appear to be particularly promising in a number of tumour types. In addition to the very large number of observations abroad, there are also Hungarian data on this. A marked increase in the neutrophil-to-lymphocyte ratio is associated with shorter survival in malignancies, regardless of histological diagnosis. [Deme, 2022] Thrombocytosis is an

unfavourable prognostic marker in breast cancer [Somogyi et al., 2019], colorectal cancer [Herold et al., 2018] and head and neck cancer [Szilasi et al., 2021].

In prostate cancer, there is also evidence that blood tests can help predict the risk and outcome of the disease. A review paper analysing pooled data from 7228 patients in 18 trials showed that overall survival is worse with elevated neutrophil/lymphocyte cell ratios and that the length of time without relapse based on biochemical markers is reduced. [Guo et al., 2018] Elevated platelet count is also an unfavourable prognostic sign in this disease. The results of this analysis were confirmed by the UK Biobank data. [Watts et al., 2020] The study included over 200,000 cancer-free men, of whom 5,723 were diagnosed with prostate cancer during an average follow-up of 6.8 years. It was found that there is an association between higher platelet count and prostate cancer risk, and that higher white blood cell and neutrophil counts predict prostate cancer mortality.

If these clinical observations can be replicated in a non-selected group of Hungarian prostate cancer patients, it may influence clinical decision making and the choice of therapy. Therefore, in our work, we analysed the ability of the blood count to predict overall survival almost 20 years after the diagnosis of PCA.

Our results suggest that the elements of the blood count do not predict which patients will survive an average of 20 years after PCA diagnosis. Our multiple regression analysis also suggests that survival in the group of patients who died was not associated with any of the blood count parameters previously thought to be significant in this regard. (In contrast to the blood count, Gleason score, PSA level, which is proportional to disease severity, was highly associated with mortality and survival, in line with the results of others [Buhmeida et al., 2006]. Likewise, the number of comorbidities and, of course, the age at diagnosis of the patients were strongly associated with these outcomes.) On this basis, blood count elements are not suitable to estimate mortality in a non-selected group of patients with PCA.

Several factors may explain the discrepancy between previous observations and our present results.

(1) Several publications have used a cut-off value for blood parameters according to some principle, dichotomising the population under study and comparing disease progression. The data suggest that the cut-off value is highly population-

dependent, and that it is unrealistic for each urology department to set its own cut-off value.

(2) In our case, the course of the disease, e.g. progression-free survival, recurrence rate, PCA-specific mortality, could not be assessed. The only endpoint available to us was overall survival, which was profoundly influenced by a number of factors, mainly age, stage of underlying disease, and the justification for palliative treatment.

(3) Deaths due to comorbidities are likely to have been a significant contributor to deaths unrelated to the prostate. However, due to the nature of the data collection - the time of death was determined by the date of inactivation of the social security number - it was not possible to analyse prostate-specific or cardiovascular mortality separately. It cannot be excluded that some blood parameters may still be of predictive significance for some specific mortality.

(4) The long-term outcome of the disease may depend in part on the composition of the care team. The advantage of our study is that the patients evaluated were all treated solely by me, so that it was not necessary to adjust for heterogeneity between treating physicians in the analysis.

This lack of association does not exclude the possibility that patients with the same clinical stage and clinical status could still be differentiated from patients at higher risk on the basis of blood counts. This would require an assessment of the predictive value of the blood count in a significantly larger population. This is made more difficult by the fact that previous study results were often obtained on a much simpler technical platform (in our case, a 3-part diffuse haematology machine, whereas today blood counting is generally performed on 5-part diffuse haematology machines that capture much more detail). Furthermore, the results of long-term survival studies may be modified by the fact that treatment algorithms (which also modify progression) may change during the follow-up period.

There are some limitations to our research.

(1) The outcome (survival or length of survival) was determined by the inactivation of the social security number in the NEAK database. It cannot be excluded that the patients classified as alive with an uncancelled social security number may have included patients who moved abroad and died there. Although a large

number of Hungarian citizens have moved abroad in recent decades, the patients examined – predominantly elderly men who also had PCA – were probably under-represented in this population.

(2) The number of patients of around 100 did not allow to include more elements (e.g. TNM stage, treatment modalities used) in the multiple regression study. These factors can also influence survival, independently of blood counts.

(3) Based on the 3 part diffuse hematological analyser, we conventionally consider 'large' cells as neutrophil granulocytes and 'small' cells as lymphocytes. No data were available for e.g. monocyte count, nucleated red blood cells or immature granulocytes.

In conclusion, we found that conventional blood parameters measured or derived from these parameters at the time of PCA diagnosis do not help in estimating 20-years all cause mortality of patients.

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6. Conclusions

My work has led me to the following main findings:

1. In the evaluation of the reference range of prostate-specific antigen
    a. In the case of general laboratory parameters suggestive of liver disease, kidney disease or vitamin D deficiency, no modification of the reference range is justified.
    b. PSA levels rise in inflammation. It is therefore recommended to measure PSA levels when CRP levels are low.
    c. In hyperthyroidism, PSA levels rise. In such a case, clinical judgement should be made only on the basis of the PSA level, with increased caution.
2. The combination of ciprofloxacin - clindamycin, routinely used before prostate biopsy, has a marked and potentially detrimental effect on the gut microbiome; increasing the proportion of Bacteroides strains and decreasing the proportion of Firmicutes strains.
3. When diagnosing PCA, conventional blood count parameters do not predict mortality from any cause in patients.

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7. Summary

Prostate cancer (PCA) is the second most commonly diagnosed cancer in men. The pathology of the disease is determined by the stage at which it is detected. In my PhD work, I analysed the association of prostate-specific antigen (PSA) levels used in PCA screening with other pathological conditions in a database of tens of thousands of data.

The definitive diagnosis of PCA is based on the results of histopathological examination of a biopsy specimen. The biopsy is performed under antibiotic protection. In the second part of my work, I evaluated how the combination of ciprofloxacin-clindamycin administered for a short term affects the composition of the colonic microbiome.

The treatment of diagnosed PCA and the aggressiveness of the treatment will affect the prognosis of patients. In a small cohort of patients (n=97), I analysed whether blood count elements measured at the time of PCA diagnosis predict 20 years all cause mortality.

I have shown that in the case of general laboratory parameters suggestive of liver disease, kidney disease or vitamin D deficiency, a change in the PSA reference range is not justified. In contrast, PSA levels are elevated in inflammation; therefore, PSA measurement is recommended at low CRP levels. PSA levels are elevated in hyperthyroidism. Therefore, in the case of low TSH levels suggestive of hyperthyroidism, clinical judgement should be made with increased caution on the basis of PSA levels alone.

The combination of ciprofloxacin - clindamycin, routinely used in urological practice, has a marked and potentially detrimental effect on the gut microbiome; increasing the proportion of Bacteroides strains and decreasing the proportion of Firmicutes strains. At the same time, the diversity of the microbiome is markedly reduced. If these changes also occur in the microbiome of the prostate, it can have a negative impact on prostate health.

In contrast to some of the literature, conventional blood parameters do not predict mortality from any cause in patients diagnosed with PCA.

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9. Own communications

9.1 Publications on which the dissertation is based

1. Tóth Z, Szalay B, Gyarmati B, Jalal DA, Vásárhelyi B, Szabó T. Vitamin D Deficiency has no Impact on PSA Reference Ranges in a General University Hospital - A Retrospective Analysis. EJIFCC. 2020 Sep 29;31(3):225-230. PMID: 33061877; PMCID: PMC7545131.
2. Tóth Z, Gyarmati B, Szabó T, Vásárhelyi B. An inverse significant association between thyroid stimulatory hormone (TSH) and prostate specific antigen (PSA) blood levels in males 40-75 years of age 40-75 years of age. Orv Hetil. 2019 Sep;160(35):1376-1379. doi: 10.1556/650.2019.31340. PMID: 31448641.
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4. Toth Z, Szalay B, Gyarmati B, Jalal DA, Vasarhelyi B, Szabo T. Prostate specific antigen serum levels in patients with different levels of hepatic or renal impairment and in those with systemic inflammation in a university hospital. A retrospective analysis of 10 years of laboratory data. Open Access J Urol Nephrol 2020, 5(3): 000184. doi: 10.23880/OAJUN-16000184

9.2. Own publications not on the subject of the thesis

1. Jalal DA, Vásárhelyi B, Blaha B, Tóth Z, Szabó TG, Gyarmati B. Interrelationship of hemoglobin A1c level lipid profile, uric acid, C-reactive protein levels and age in a large hospital database. Mol Cell Probes. 2023 Dec;72:101933. doi: 10.1016/j.mcp.2023.101933. epub 2023 Sep 20. PMID: 37722548.
2. Vásárhelyi B, Dlovan AJ, Blaha B, Tóth Z, Szabó GT, Gyarmati B. Relationship between red blood cell parameters and hemoglobin A1c levels based on a retrospective evaluation of 10 years of data. Orv Hetil. 2024 Feb 18;165(7):243-248. doi: 10.1556/650.2024.32982. PMID: 38368578.
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