

## GDF15 and Hepcidin as Prognostic Factors in Patients with Prostate Cancer

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### Abstract

**Background:** Prostate Cancer (PCa) is a frequent malignancy worldwide. Prognosis of prostate cancer (PCa) is diverse with 80% not life-threatening even if untreated. In contrast, 20% are aggressive with short course of the disease. Hence the need for prognostic markers to predict aggressiveness, patients' outcome, and efficacy of treatment is increasing.

**Methods:** We retrospectively analyzed serum and demographic data from 38 PCa-patients. Serum growth differentiation factor 15 (GDF15), hepcidin and interleukin-6 (IL-6) were measured.

**Results:** Serum GDF15 levels were higher in metastatic castration-resistant PCa patients with rapid disease progression than in metastatic PCa patients with slow disease progression. There was a trend to higher serum hepcidin - but not IL-6 - serum levels in patients with rapid disease progression than in slow disease progression. Prostate-specific antigen (PSA) correlated with serum GDF15 and hepcidin levels. Serum GDF15 levels correlated with serum hepcidin levels. The height of serum GDF15 and hepcidin levels correlated with survival of patients in months. Increase of 25 ng/ml serum hepcidin predicted an increase in mortality by 10%.

**Conclusion:** In conclusion, the current manuscript supports the use of serum GDF15 and serum hepcidin measurements as prognostic markers in PCa.

**Keywords:** GDF15; Hepcidin; Prostate cancer; Prognostic marker

### Abbreviations:

ACD: Anemia of Chronic Disease; AI: Anemia of Inflammation; CRPC: Castration-Resistant Prostate Cancer; GDF15: Growth Differentiation Factor 15; IL-6: Interleukin-6; iPSA: initial PSA; PCa: Prostate Cancer; PSA: Prostate-Specific Antigen; RPX: Radical Prostatectomy

### Introduction

Prostate Cancer (PCa) is a leading malignancy worldwide, especially in aging populations. In Europe an estimate of around 417.000 men were newly diagnosed with PCa in 2012 [1]. Severity of PCa is diverse: On the one hand, many PCas are indolent with a slow disease progression. These will in most cases not become life-threatening, even if not treated at all. Between the years 1996 and 2003, 91% of men diagnosed with PCa had stage 1 or 2 of disease and 5-year survival rates approached 100% [2].

On the other hand, about 92.000 men died in 2012 due to PCa [1]. In these cases, an anti-hormonal PCa therapy was not successful due to a change to androgen-independent PCa that was refractory to available therapies (castration-resistant prostate cancer (CRPC)).

State of the art in screening for and monitoring of PCa is measurement of prostate specific antigen (PSA), which is widely available. An increase of PSA occurs early in the disease. PSA levels of 2-10 ng/ml do not provide information on the progress and aggressiveness of PCa in a variety of scenarios. Therefore the PSA-screening leads to a marked increase of new cases [3-5]. Up to 80% of PCas are over-diagnosed, indicating that these PCas would never have caused relevant problems [6].

Diagnosis of cancer in many cases leads to treatment of patients. Current state of the art therapies like radical prostatectomy, radiation and hormonal therapy have side effects that might surpass the benefit of treatment: even more so if the cancer is not aggressive per se [7]. The question arises whether patients with early stage PCa benefit from radical therapy, and how to distinguish between non-aggressive, intermediate, and aggressive PCa.

PSA screening has reduced PCa specific mortality, but aggressive forms of PCa are in many cases not cured by radical prostatectomy and radiation therapy [8,9]. The quality of life could potentially be better without therapy and therefore fewer side effects within the remaining life-time.

Currently, active surveillance is performed: PSA is regularly checked and prostate biopsies are taken. In case of PCa progression, radical treatment is offered [10].

Active surveillance is time-consuming and not always satisfactory for patients.

Because of the diversity of PCa, there is a substantial and emerging need for new biomarkers that indicate prognosis, severity of cancer, effectiveness of treatment and patients' outcome [11]. Additionally, in advanced PCa that has evolved into CRPC after primary anti-hormonal therapy, biomarkers are equally needed in order to predict progression and aggressiveness of tumor growth in relation to different potentially life-prolonging therapies.

Growth differentiation factor 15 (GDF15) and hepcidin might serve as novel prognostic markers for PCa disease progression and patients' prognosis.

GDF15 is a member of the transforming growth factor beta superfamily (TGF- $\beta$ ) [12]. Activation of the TGF- $\beta$ /bone morphogenetic protein (BMP) signaling pathway induces the iron regulatory hormone hepcidin. GDF15 expression is associated with tissue damage, plays a role in regeneration and has anti-apoptotic and tissue-protective functions [13]. In patients with thalassemic anemia, GDF15 can regulate the hepatic hormone hepcidin [14].

Hepcidin controls systemic iron homeostasis. Iron [15] and inflammation [16] induce hepcidin expression, while iron deficiency and hypoxia [17] reduce hepcidin expression. Hormones differently regulate hepcidin. Induction of hepcidin induces internalization and degradation of ferroportin, the only known iron exporter in vertebrates. Ferroportin is expressed in enterocytes, macrophages and hepatocytes. Hepcidin therefore restricts systemically available iron by regulation of dietary iron absorption as well as recycling of iron from senescent erythrocytes and mobilization of iron from stores [16,18].

In intact regulation, availability and full iron stores activate the inhibitory circuitry of iron absorption and release by hepcidin induction. In acute or chronic inflammatory states as well as neoplastic diseases, IL-6 and other pro-inflammatory cytokines such as GDF15 induce the expression of hepcidin - independently of the current iron status [19]. Therefore, hepcidin might be induced albeit iron deficiency anemia is present. This leads to a reduction of iron available for erythropoiesis and thus causes anemia of chronic disease (ACD, also called anemia of cancer or anemia of inflammation (AI)) [16,18].

In a variety of neoplastic diseases, serum hepcidin levels are elevated, for example in renal cell carcinoma [20], myeloid leukemia [21] and multiple myeloma [22]. In breast cancer, there was a trend towards use of tumor hepcidin expression as a prognostic marker. In the presence of high ferroportin, high tumor hepcidin expression was related to a shorter 5-year disease- and metastasis-free survival than lower rate of hepcidin expression with a survival >95% [23].

Breast cancer and PCa both are hormone-dependent cancers. GDF15 and hepcidin might serve as prognostic markers in PCa, as Tanno et al. reported that metastatic PCa patients had higher hepcidin, IL-6 and a trend towards higher GDF15 levels than patients with non-metastatic PCa [24].

Therefore the goal of the current study was to determine if serum GDF15 and serum hepcidin may serve as prognostic markers in PCa with slow or rapid disease progression compared to organ-confined PCa.

Our data indicates that GDF15 and hepcidin, but not IL-6, levels are higher in metastasized patients with rapid disease progression than in

metastasized patients with slow disease progression. Organ-confined controls had the lowest GDF15, hepcidin, and IL-6 serum levels.

The currently most frequently used marker PSA correlated with serum GDF15 and serum hepcidin, but not with serum IL-6 levels. GDF15 serum levels correlated with serum hepcidin levels. Impressively, height of GDF15 and serum hepcidin levels indicated survival of patients and risk of death increased with each unit:

An induction of GDF15 by 1000 pg/ml increased the risk of mortality by 20.6%. An induction of 25 ng/ml serum hepcidin raised risk of death by 10%. In conclusion, the current manuscript supports the use of GDF15 and serum hepcidin measurements as prognostic markers in PCa.

## Materials and Methods

### Study design

The study was approved by the institutional review board of the University of Muenster. Enrolled subjects provided written informed consent in accordance with the Declaration of Helsinki. PCa patients treated in the Department of Urology at Muenster University Hospital provided serum samples. We measured serum GDF15, serum hepcidin and serum IL-6 in the patients' sera and retrospectively analyzed demographic data and patients' outcome.

### Patient cohort

We chose representative PCa-patients from a larger collection of samples according to disease progression and availability of patients' data: of n=38 total patients, n=26 patients survived at least ten years after PCa diagnosis. We refer to this group of patients as "slow progression". n=6 patients died within 3.5 years after diagnosis of PCa. This group of patients is named "rapid progression" below. All patients with slow progression and rapid disease progression presented with metastases. Each patient received various therapies before sample collection, including prostatectomy (RPX), irradiation, chemotherapy and diverse anti-cancer drugs. In all slow and rapid progression patients, cancer had already changed from hormone-dependent to castration-resistant (CRPC).

Of the n=32 CRPC patients, in n=17 RPX was performed at some point during their treatment. In these cases, the serum samples were collected between 8 and 20 years after RPX.

A third group of n=6 patients is entitled "organ-confined". These patients received RPX in 2009. After RPX, these patients were relapse-free until their last follow-up in the out-patient clinic in the Department of Urology at Muenster University Hospital in 2011 or 2012. Samples of n=4 patients were taken within two months before surgery; n=1 sample was collected six months and n=1 sample 21 months prior to RPX.

In each case, we chose per definition the most recent serum sample if multiple serum samples were stored.

### Patients' data

Patients' data were collected retrospectively by screening of patients' electronic data files as follows: date of birth; date of PCa diagnosis; date of first visit at Department of Urology, University Hospital Muenster; initial tumor stage; initial tumor grade and Gleason score; initial PSA (iPSA); current and previous treatment;

beginning of treatment; PSA changes during therapy; hemoglobin (Hb), transferrin, transferrin saturation, ferritin and serum iron. If applicable, occurrence of metastasis and date of death was captured.

### Changes in PSA

Changes in PSA were calculated as follows: the lowest PSA level (nadir) in patients responding to therapy or the highest levels (non-responder) are indicated as in relative changes to PSA values before treatment.

### Measurement of serum hepcidin, GDF15 and IL-6

Serum hepcidin was measured in triplicates using an extraction-free competitive ELISA kit from Peninsula Laboratories (BACHEM GROUP, San Carlos, CA, USA).

GDF15 and IL-6 were measured in duplicates with ELISA kits from R&D Systems (R&D SYSTEMS INC., Minneapolis, MN, USA/ R&D Systems Europe, Ltd., Abingdon, UK). Absorption was measured using a BioTek photometer (EL808, BIOTEK® INSTRUMENTS INC.,

Vermont, USA) and the accompanying software Gen5 (Gen5 Data Analysis Software, BIOTEK® INSTRUMENTS INC., Vermont, USA).

### Statistical analysis

Data was analyzed using GraphPad Prism 6® (La Jolla, CA, USA) and IBM SPSS® Statistics 22 for Windows (IBM Corporation, Somers, NY, USA).

As results did not follow normal distribution, median and interquartile ranges (IQR) for values of GDF15, hepcidin and IL-6 between the three progression groups (organ-confined, slow and rapid progression) were tested using Kruskal-Wallis-tests followed by Dunn's test for pairwise comparisons.

Differences in serum hepcidin, serum GDF15 and serum IL-6 between metastatic and non-metastatic patients were compared using Mann-Whitney-U-tests. The metastatic group included the patients from the rapid and slow progression group (n=32), while non-metastatic patients were identical with the organ-confined group (n=6 patients).

	Organ-confined	Rapid progression	Slow progression	Differences between groups
Number of patients	6	6	26	-
Age at sample collection	70.1 ± 4.6	68.8 ± 10.4	74.2 ± 6.5	n.s.
Hemoglobin (g/dl) at sample collection	n.a.	10.7 ± 2.2	11.5 ± 2	n.s.
Initial TNM	T2-3 N0 M0	T4 N0-2 M0-1	T1-4 N0-2 M0	-
iPSA	10.9 ± 5.0	215.1 ± 261.5	30.9 ± 37.6	Kruskal-Wallis *p=0.02; *p=0.024 (1 vs. 2)
Gleason-Score	6.8 ± 0.4	8.8 ± 1.3	7.1 ± 1.3	Kruskal-Wallis *p=0.03; *p=0.03 (1 vs. 2)
Development of metastasis	No	Yes	Yes	-

**Table 1:** Patients' characteristics

Correlations by Spearman (rS) and Pearson (rP) of serum hepcidin, GDF15 and IL-6 levels with each other as well as with hemoglobin, ferritin, transferrin, transferrin saturation, iron, PSA and PSA-reduction during treatment were determined.

For Kaplan-Meier survival analysis, patients were grouped according to their serum GDF15 levels in three groups: 1) Serum GDF15<1300 pg/ml (n=10), 2) 1300-3000 pg/ml (n=9) and 3) Serum GDF15>3000 pg/ml (n=19). A study by Wollert et al. determined GDF15 reference values in presumably healthy subjects [25]. Normal values ranged from 0-1200 pg/ml. Therefore, GDF15 serum levels below 1300 pg/ml represent normal values and mild elevation.

For a second Kaplan-Meier survival analysis patients, were grouped according to their serum hepcidin levels: 1) serum hepcidin levels<50 ng/ml (n=13), 2) 50-100 ng/ml (n=6) and 3) serum hepcidin>100 ng/ml (n=16). This categorization was chosen according to hepcidin reference values obtained in the general population in the Netherlands [26]. Normal reference values were below 37.8 ng/ml. Serum hepcidin levels below 50 ng/ml therefore represent normal values and mild elevation.

Patients' survival after collection of serum samples was determined. Groups were compared using the log-rank test. All inferential statistics are intended to be exploratory (hypotheses generating), not confirmatory, and are interpreted accordingly. To determine the effect on serum GDF15 and serum hepcidin levels on patients' survival, univariate Cox-regressions were performed. Hazard Ratios (HR) and corresponding 95% confidence intervals (95% CI) were calculated. The local significance level was set to 0.05. No adjustment for multiple testing was performed.

## Results

### Patients' characteristics

Patients' characteristics are shown in Table 1. The organ-confined group and rapid progression group consisted of n=6 patients each, the slow progression group of n=26 patients. There was no relevant difference in patients' age (Mean 70.1 ± SD4.6; 68.8 ± 10.4 and 74.2 ± 6.5 years). Hb levels at time of serum collection were determined in

patients of the slow and rapid progression group and did not differ (Hb= 10.7 ± 2.2; 11.5 ± 2.0 g/dl).

Initial TNM stages were T2-3N0M0 in the organ-confined group, T4N0-2M0-1 in the rapid progression group and T1-4N0-2M0 in the slow progression group.

Median baseline initial PSA (iPSA) was 10.6; IQR 9.5 ng/ml in the organ-confined group, compared to 142.8; IQR 340.3 ng/ml in the rapid progression and 18.4; IQR 31.6 ng/ml in patients with slow progression (Kruskal-Wallis \* $p=0.02$ ; \* $p=0.024$  organ-confined vs. rapid progression patients).

The median initial Gleason-Score was 7.0 in organ-confined patients, 9.0 in rapid progression, and 7.0 in patients with slow progression (Kruskal-Wallis \* $p=0.03$ , \* $p=0.03$  organ-confined vs. rapid progression patients).

The patients in the organ-confined group were relapse-free in all follow-ups until 2011 or 2012, whereas all patients with rapid and slow progression presented with metastases.

### **GDF15, hepcidin and IL-6 levels in patients with organ-confined, slow and rapid PCa disease progression**

In order to determine if serum GDF15 levels differed in patients with organ-confined, slow progression and rapid progression PCa disease, we determined serum GDF15 levels in these patients (Figure 1A). There was a difference in serum GDF15 levels between groups (Kruskal-Wallis \* $p<0.001$ ). Compared with organ-confined controls, serum GDF15 levels were higher in both rapid (\* $p<0.001$ ) and slow progression (# $p=0.004$ ).

As a second probable prognostic marker we measured serum hepcidin (Figure 1B).

There was a trend towards higher hepcidin in rapid compared with slow progression (Kruskal-Wallis  $p=0.143$ , rapid vs. slow progression  $p=0.073$ ).

As a third probable prognostic marker, we measured serum IL-6 (Figure 1C). There were no differences between groups (Kruskal-Wallis  $p=0.365$ ).

These findings indicate that serum GDF15 and serum hepcidin might serve as prognostic markers.

### **GDF15, hepcidin and IL-6 serum levels in patients with and without metastasis**

As patients with metastases generally have a worse outcome compared to non-metastatic patients, we compared hepcidin, GDF15 and IL-6 serum levels of metastatic and non-metastatic patients.

Serum GDF15 levels were higher in metastatic than in non-metastatic patients (\* $p<0.001$ ; Supplemental Figure 1A). There were no significant differences between metastatic and non-metastatic patients in either serum hepcidin (Supplemental Figure 1B) or serum IL-6 (Supplemental Figure 1C).

Our results indicate that higher serum GDF15 levels are associated with advanced stages of PCa.

### **Correlations of hepcidin, GDF15 and IL-6 with PSA**

We correlated serum GDF15, hepcidin and IL-6 levels with the established prognostic marker PSA (Figure 2A-C). GDF15 correlated with PSA remaining after treatment (Spearman Correlation= $r_s$ , Pearson Correlation= $p_s$ ) ( $r_s=0.5$ ;  $p_s=0.02$ ;  $r_p=0.14$ ;  $R^2=0.02$ ; Figure 2A) and PSA at time point of sample collection (Supplemental Table I).

Hepcidin correlated with elevated PSA after definitive treatment ( $r_s=0.49$ ;  $p_s=0.02$ ,  $r_p=0.04$ ;  $R^2=0.001$ ; Figure 2B), but not with PSA at time point of sample collection (Supplemental Table I).

IL-6 correlated with PSA remaining after treatment in Pearson, but not in Spearman correlation ( $r_s=0.18$ ;  $p_s=0.42$ ;  $r_p=0.7$ ;  $p<0.0001$ ;  $R^2=0.48$ ; Figure 2C) (Supplemental Table I).

The correlation of GDF15 and hepcidin with PSA remaining after treatment indicates that both parameters follow the same trend (Spearman).

### **Correlation of serum hepcidin levels with serum GDF15 levels**

Cytokines such as IL-6 are known to induce hepcidin expression. In contrast hormones differently regulate hepcidin. As GDF15 inhibits hepcidin expression, we expected a correlation between serum hepcidin levels and serum GDF15 levels in PCa patients. Hepcidin levels correlated with serum GDF15 levels ( $r_s=0.41$ ;  $p_s=0.01$ ,  $r_p=0.4$ ;  $R^2=0.16$ , Figure 2D). The result indicates that serum GDF15 and serum hepcidin levels follow the same trend and that there is a connection between serum GDF15 and hepcidin levels.

### **Systemic iron homeostasis**

Hepcidin is the systemic iron regulator in the human body. Therefore, we were interested in the correlation of serum hepcidin and GDF15 with the iron status.

There was a trend towards correlation of serum GDF15 levels with transferrin ( $r_p=-0.63$ ,  $p_p=0.053$ ,  $R^2=0.39$ ; Supplemental Figure 2A). Serum GDF15 levels correlated with ferritin ( $r_p=0.71$ ;  $p_p=0.02$ ;  $R^2=0.51$ ; Supplemental Figure 2B). Serum hepcidin levels correlated with transferrin levels ( $r_p=-0.68$ ;  $p_p=0.03$ ;  $R^2=0.46$ ; Supplemental Figure 2C). There was a trend towards correlation of serum hepcidin with ferritin ( $r_p=0.62$ ;  $p_p=0.058$ ;  $R^2=0.38$ ; Supplemental Figure 2D). Correlations can also be found in the Supplemental Table 1.

IL-6 did not correlate with the patients' iron status. The data indicates that there is a linear dependence of GDF15 and hepcidin with iron homeostasis. The results suggest that not only hepcidin but also GDF15 are involved in iron homeostasis.

### **GDF15 and hepcidin as a predictor of patients' survival**

A requirement for a prognostic marker is the prediction of patients' survival.

We analyzed serum GDF15 levels in relation to patients' survival. Patients were categorized according to their serum GDF15 levels. We depicted GDF15 serum levels and patients' survival in months (Figure 3A). Length of survival was different between groups (Log-Rank \* $p<0.001$ ) and survival was longest in patients with GDF15 levels <1300 pg/ml and shortest in patients with GDF15 levels >3000 pg/ml (median 4 months). Cox-regression revealed that each elevation of



GDF15 by 1000 pg/ml increased the risk of mortality by 20.6% (HR=1.206, 95% CI= [1.115, 1.306],  $p<0.001$ ).

In a next step, we categorized all patients according to their serum hepcidin levels and depicted the serum hepcidin levels with patients' survival (Figure 3B). Survival in months is different between the three groups (Log-Rank  $*p=0.007$ ). Of  $n=13$  patients with normal to mildly elevated serum hepcidin levels of  $<50$  ng/ml  $n=3$  patients died, while the patients with serum hepcidin levels over 100 ng/ml had the shortest survival (median 4 months). Survival of the patients with serum hepcidin levels of 50-100 ng/ml was higher than in patients with serum hepcidin levels  $>100$  ng/ml, and lower than in organ-confined patients. Univariate Cox regression analysis revealed that each hepcidin elevation by 25 ng/ml raised the risk of mortality by 9.8% (HR=1.098, 95% CI=[1.034, 1.165],  $p=0.002$ ).

All organ-confined patients survived until time of the analysis while all patients with rapid progression died. Of the patients who progressed slowly, 12 (46.2%) died until May 2014.

These results indicate that GDF15 and hepcidin serum levels cannot only be used as predictor of patients' survival: additionally, the height of GDF15 and hepcidin indicate mortality and length of survival.

## Discussion

PCa is a frequent malignancy in patients. It is characterized by different grades of severity and disease progression. On the one hand, PCa progression may stagnate for years, even without treatment. On the other hand, a variety of PCas is highly aggressive and requires therapy in order to decrease mortality. Current state of the art is surveillance with PSA. PSA increases early and has a low specificity so that PCa is an over-diagnosed disorder. Therefore, it is desirable to find prognostic markers to predict patients' outcome and tumor aggressiveness in order to decide on PCa treatment.

GDF15 and the iron regulator hepcidin are two hormones that might serve as prognostic markers, as differences in serum GDF15 and serum hepcidin levels have been reported for metastasized patients compared to healthy controls.

The goal of the current retrospective analysis of  $n=38$  patients with PCa was to evaluate the use of serum GDF15, hepcidin and IL-6 as a prognostic marker in the three groups of patients with organ-confined PCa, slow disease progression and rapid disease progression. The latter two groups are characterized by presence of metastases.

The study data indicates that serum GDF15 levels are higher in patients with rapid PCa disease progression than in patients with slow disease progression. The lowest GDF15 serum levels were measured in organ-confined controls.

Comparing metastasized patients to non-metastasized patients revealed that GDF15 serum levels were higher in metastasized than in non-metastasized patients.

For serum hepcidin levels, there was a trend in the same direction. Mean values of patients with rapid disease progression were higher than in slow disease progression. Organ-confined controls had the lowest hepcidin levels.

In order to evaluate the use of GDF15 and hepcidin serum levels in PCa patients, we analyzed the correlations of GDF15 and hepcidin with common practice PSA. Serum hepcidin and GDF15 levels correlated with PSA remaining after treatment. We conclude that

serum GDF15 and serum hepcidin levels were higher when PSA was not reduced by treatment.

Serum GDF15 and serum hepcidin levels depend on each other. In addition, hepcidin and GDF15 correlate with the patients' iron status. Therefore, not only hepcidin, but also GDF15 influence iron homeostasis.

Patients with high GDF15 or high serum hepcidin levels had a marked impairment of survival. In contrast, low serum hepcidin levels were associated with high survival rates.

Our findings support our hypothesis that serum levels of GDF15 and hepcidin may be useful markers for PCa disease progression and predictors of patients' survival.

Of course, the number of patients included in this retrospective analysis is small. Still, it is impressive that the results are concise and clear. Prospective studies and larger cohorts are required to evaluate the use of serum GDF15 and hepcidin levels as prognostic markers for PCa disease progression. In addition, future studies will have to examine serum GDF15 and hepcidin levels in regard to certain PCa therapies (e.g. sequential analysis from baseline to progression on a particular drug in order to potentially guide therapy).

Hepatic hepcidin expression regulates systemic iron homeostasis. Interestingly, not only hepcidin, but also serum GDF15 levels correlated with iron parameters. GDF15 belongs to the TGF- $\beta$ /BMP superfamily. Activation of BMP signaling induces hepcidin. The detailed signaling mechanisms of GDF15 remain to be elucidated. As reviewed by Vanhara et al., GDF15 is induced in certain disorders (such as ischemic heart damage, diabetes mellitus and rheumatoid arthritis) [13]. Tanno et al. reported that GDF15 can suppress hepcidin in thalassemic anemia [14]. In our study, patients with rapid disease progression had an induction of serum GDF15 levels compared to organ-confined controls and also compared to slow disease progression. Serum GDF15 levels might be induced as a compensatory mechanism of hepcidin induction. An alternative explanation for the induction of both serum GDF15 and hepcidin levels is that aggressive PCa might induce both simultaneously. Future studies, especially after different treatments of PCa, will have to reveal whether both mediators are induced by PCa. Alternatively, hepcidin or GDF15 might be induced first, and cause induction of the other.

We did not analyze GDF15 in prostatic cancer tissue. Other investigators reported that prostate cancer cell lines behave heterogeneously, both in terms of GDF15 expression, as well as in terms of cell adhesion and apoptosis [27]. GDF15 production by prostate cells was significantly elevated in malignant LNCaP and PC-3 cell lines, but not in malignant DU-145 and nonmalignant PZ-HPV-7 cell lines. In DU-145 cells, treatment with GDF15 led to morphological changes, reduced cell adhesion and detachment, which was followed by apoptosis.

Our results are in line with Tanno and colleagues, who reported that serum hepcidin levels differ in metastatic compared with non-metastatic patients [24]. In our study we saw a difference in serum GDF15 levels while in the study of Tanno et al. there was no significant difference in GDF15 levels between groups. Tanno et al. analyzed  $n=14$  non-metastatic vs.  $n=15$  metastatic PCa patients. We examined  $n=6$  organ-confined controls vs.  $n=32$  metastasized PCa patients.

Patients with PCa present different PCa disease progressions. In addition to the analysis between metastatic and non-metastatic PCa

patients, we therefore divided metastatic PCa patients into rapid and slow disease progression.

Drachenberg et al. measured elevated IL-6 serum levels in castration-resistant patients compared to healthy controls and mild disease stages [28]. Tanno et al. reported higher IL-6 levels in metastatic than in non metastatic patients [24]. In our study, neither comparison between rapid and slow disease progression nor metastatic and non-metastatic PCa patients showed differences. Therefore IL-6 does not appear to serve as a reliable prognostic marker in PCa patients.

While there was a trend towards higher hepcidin in rapid compared to slow disease progression, GDF15 differed between progression groups.

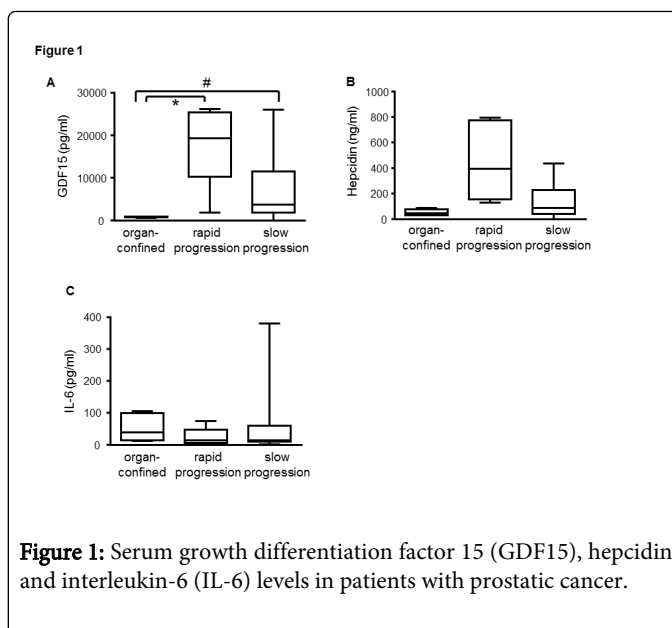
Welsh et al. reported that serum GDF15 levels were elevated in metastatic prostatic cancer patients compared with normal controls [29]. This data is in line with the present study. We compared rapid and slow disease progression to organ-confined PCa patients. GDF15 serum levels differed between organ-confined controls compared with rapid and slow progression in metastatic PCa patients. GDF15 therefore might serve as a prognostic marker in PCa that allows prognosis of disease progression.

## Conclusion

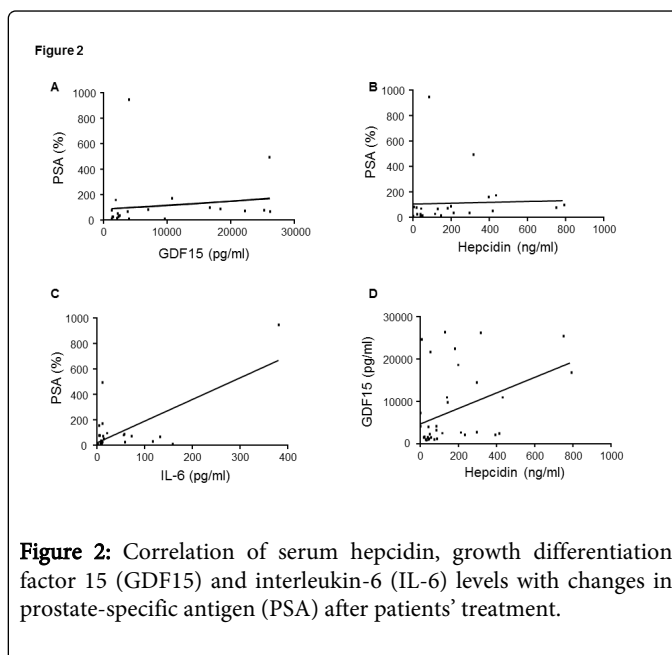
In conclusion, prognostic markers for the frequent malignancy PCa with variable disease progression are highly relevant to monitor PCa therapy, to predict efficacy of treatment and thereby guide therapeutic decisions and to predict patients' survival.

The presented data indicated that serum GDF15 and serum hepcidin levels, but not IL-6 levels, were higher in metastasized patients with rapid disease progression than in slow disease progression than in organ-confined controls. GDF15 serum levels correlated with serum hepcidin levels. Impressively, height of GDF15 and serum hepcidin levels indicated survival of patients and risk of death: An induction of GDF15 by 1000 pg/ml increased the risk of mortality by 20.6%, an induction of 25 ng/ml serum hepcidin induction increased risk of death by 10%. In conclusion, the current manuscript demonstrates that serum GDF15 and serum hepcidin may be useful prognostic markers to predict patients' disease progression and survival.

Serum GDF15 (A), hepcidin (B) and IL-6 (C) levels were measured in organ-confined (n=6), rapid progression (n=5) and slow progression prostatic cancer patients (PCa) (n=24) with ELISA kits. (A) There was a difference in serum GDF15 levels between groups (Kruskal-Wallis  $*p<0.001$ ). Compared to organ-confined controls, serum GDF15 levels were higher in both rapid ( $*p<0.001$ ) and slow progression ( $\#p=0.004$ ). (B) There was a trend towards higher hepcidin in rapid compared with slow progression ( $p=0.073$ ). (C) There was no difference in serum IL-6 levels between the three groups.

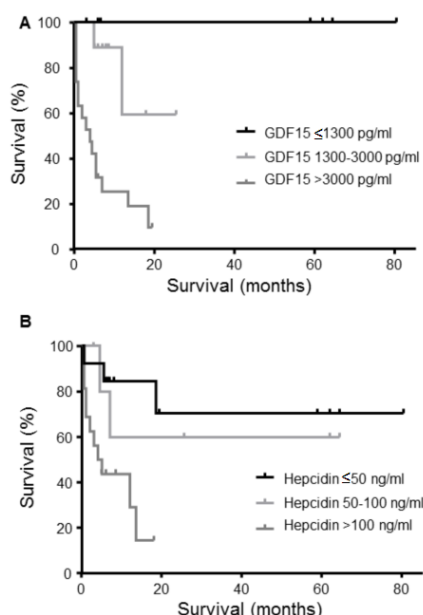


**Figure 1:** Serum growth differentiation factor 15 (GDF15), hepcidin and interleukin-6 (IL-6) levels in patients with prostatic cancer.



**Figure 2:** Correlation of serum hepcidin, growth differentiation factor 15 (GDF15) and interleukin-6 (IL-6) levels with changes in prostate-specific antigen (PSA) after patients' treatment.

We measured hepcidin, GDF15 and IL-6 with ELISA, and correlated to PSA values obtained from the patients' data files. PSA values measured before treatment and PSA values after PCa treatment were obtained and calculated as %PSA change after treatment. (A) Serum hepcidin levels correlated with PSA remaining after treatment (n=22, rS=0.49,  $p=0.02$ ). (B) Serum GDF15 levels correlated with PSA remaining after treatment (n=22, rS=0.5,  $p=0.02$ ). (C) Serum IL-6 levels did not correlate with PSA remaining after treatment. (D) Hepcidin and GDF15 correlated with each other (n=35, rP=0.40\*;  $p=0.02$ ). Asterisks behind correlation coefficients denote statistical significance:  $p<0.05$ .



**Figure 3:** Patients' survival correlates with serum growth differentiation factor 15 (GDF15) and serum hepcidin levels.

(A) Patients were assigned to groups according to their serum GDF15 levels: a)  $<1300\text{pg/ml}$  ( $n=10$ ), b)  $1300\text{-}3000\text{ pg/ml}$  ( $n=9$ ), c)  $>3000\text{pg/ml}$  ( $n=19$ ). Kaplan-Meier survival analysis revealed that survival differed between groups (Log-Rank  $*p<0.001$ ). It was longest in patients with levels  $<1300\text{pg/ml}$  and shortest in patients with levels  $>3000\text{pg/ml}$  (median survival 4 months). (B) Patients were assigned to groups according to their serum hepcidin levels: a)  $<50\text{ng/ml}$  ( $n=13$ ), b)  $50\text{-}100\text{ng/ml}$  ( $n=6$ ), c)  $>100\text{ng/ml}$  ( $n=16$ ). Survival differed between the three groups (Log-Rank  $*p=0.007$ ). Of the patients with normal to mildly elevated serum hepcidin levels of  $<50\text{ ng/ml}$  ( $n=13$ ),  $n=3$  patients died, while the patients with serum hepcidin levels over  $100\text{ ng/ml}$  had the shortest survival (median 4 months). The survival of the patients with serum hepcidin levels of  $50\text{-}100\text{ ng/ml}$  was in between the survival of the two other groups. Ticks indicate censored patients; survival (months) depicts the length of survival after withdrawal of the serum sample.

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