ORIGINAL ARTICLE

Matrix Metalloproteases and Cathepsin D in Human Serum do not Cleave Prolactin to Generate Vasoinhibin

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SUMMARY

Background: **Vasoinhibin is generated in the pituitary gland and in multiple target tissues by proteolytic cleavage of prolactin by matrix metalloproteinases and cathepsin D. A dysregulation of vasoinhibin generation appears to contribute to diabetic retinopathy and diabetic macular edema, retinopathy of prematurity, peripartum cardiomyopathy, and preeclampsia. Here, we investigate whether vasoinhibin is generated by matrix metalloproteinases and cathepsin D in human serum.**
Methods: **The abundance of matrix metalloproteinases 1, 2, 3, 8, 9, 10, 13, tissue inhibitors of metalloproteinases 1, 2, 4, and the activity of cathepsin D in serum samples were determined. Samples from healthy male (n = 3) and female (n = 2) subjects, pregnant subjects (n = 2), and patients with type 2 diabetes mellitus (n = 2) were investigated. The samples were incubated with recombinant prolactin at 37°C, under different pH, time, and buffer conditions. Prolactin and cleaved prolactin products were investigated by SDS-PAGE and western blotting.**
Results: **Matrix metalloproteases-1, -2, -3, -8, -9, -10, -13, TIMP-1, -2, and -4, and the activity of cathepsin D were detected in all sera. Full-length prolactin incubated with human sera, containing endogenous matrix metalloproteinases and cathepsin D, remained intact at neutral pH during a time frame from 1 to 24 hours. Partial enzymatic cleavage of prolactin resulting in the generation of a vasoinhibin-like 17 kDa peptide was observed in samples incubated at pH 3.4. Heat inactivation of the serum and the addition of an MMP inhibitor suppressed the generation of the 17 kDa peptide, indicating that its generation was MMP-mediated.**
Conclusions: **Vasoinhibin generation by enzymatic cleavage of prolactin by matrix metalloproteases or cathepsin D does not occur in human serum at physiological pH. A limited proteolysis of prolactin, resulting in the generation of a vasoinhibin-like peptide with an apparent molecular weight of 17 kDa occurs in serum at acidic pH. The generation of vasoinhibin may require the cellular and tissue microenvironments.**
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KEY WORDS

INTRODUCTION

The human anterior pituitary hormone prolactin (PRL), fundamental for lactation and colloquially referred to as the "nursing hormone", is a 199 amino acid protein with

a molecular mass of 23 kDa. Proteolytic cleavage of PRL can generate vasoinhibin which demonstrates a wide range of endocrine, paracrine, and autocrine effects not shared with its precursor PRL, including the regulation of blood vessel growth, vasopermeability, and vasodilation [1-3], and non-vascular effects such as stimulation of vasopressin release [4], thrombolytic effects [5], and the stimulation of anxiety- and depression-related behaviors [6-10]. Vasoinhibin signals through a still-unidentified receptor on endothelial cells distinct from the PRL-receptor and has multiple other binding partners to mediate its effects [2,5,11]. The regulation of vasoinhibin generation occurs at the hypothalamic, the pituitary, and the target tissue levels and this organizational principle is described as the prolactin/vasoinhibin axis [1]. The questions of where (location/anatomical compartment), how (which enzyme), how much (PRL to vasoinhibin ratio), and which vasoinhibin isoforms (cleavage site and molecular mass) are being generated are relevant for a number of diseases [10]. Recent studies have focused on retinal disorders [12, 13], joint diseases [14], and pregnancy associated syndromes, for example diabetic retinopathy [12,15], rheumatoid arthritis [14,16], peripartum-cardiomyopathy [17-19] and (pre-)eclampsia [20,21]. Of note, two clinical trials in which vasoinhibin levels are the target of pharmacological interventions were initiated, one for the treatment of diabetic retinopathy and diabetic macular edema, and another for the treatment of peripartum cardiomyopathy (ClinicalTrials.gov Identifier: NCT031 61652 and NCT00998556, respectively) [22,23]. The principle and rational behind these clinical trials were recently reviewed [24].

The generation of vasoinhibin occurs in the pituitary gland [25] and in numerous peripheral tissues and locations, including the human endothelium [26], placenta [27,28], cartilage [7], amniotic [21,29] and subretinal fluid [30], and the rodent hypothalamus, the neuro- and adenohypophysis [6,31], the retina [32], the mammary gland, the liver [33], and the heart [19]. Prolactin-cleaving enzymes, capable of generating vasoinhibin, are matrix metalloproteinases (MMP) [7], cathepsin D [34], and bone morphogenic protein-1 (BMP-1) [35]. Cleavage of full-length PRL by MMP can result in the generation of vasoinhibin isoforms with molecular masses of 12.5, 14.1, 16.8, and 17.7 kDa, [7]. Fifteen, 16.5, and 17 kDa isoforms are generated by cathepsin D [36], whereas cleavage by BMP-1 generates a 17 kDa vasoinhibin isoform [35].

Matrix metalloproteinases and cathepsin D are present in serum [19,37,38], but it is not known whether they are able to cleave PRL while circulating in blood, or whether the generation of vasoinhibin by these enzymes requires the contact of PRL to components of the cellular and tissue microenvironment. Here, we examine if the circulation is a compartment of vasoinhibin production. This question is relevant for clinical studies in the aforementioned diseases. There is evidence for a direct correlation between the activity of cathepsin D in serum and the circulating levels of vasoinhibin in postpartum cardiomyopathy and preeclampsia [19,39]. These findings may imply that vasoinhibin is produced while circulating in blood. Alternatively, the correlation of circulating enzyme activity and vasoinhibin levels may be the result of enzymes and vasoinhibin entering the circulation from their production site, and not reflect that vasoinhibin is being generated in the circulation [40]. Therefore, investigating the role of circulating enzymes in the generation of vasoinhibin in serum will contribute to locate the anatomical sites and compartments of vasoinhibin generation.

MATERIALS AND METHODS

Matrix metalloproteinases array assay
A human MMP antibody array - membrane was used according to the instructions of the manufacturer to determine the abundance of MMP and TIMP in human sera (Abcam, Cambridge, England, product code: ab13 4004]. Briefly, 200 µL serum samples were diluted 1:5 and incubated with the array membrane. After washing and secondary antibody incubation, the chemiluminescent HRP signal was detected with the Chemi Doc MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA; cat. no. 170-8280] and densitometric analyses were performed using the Image Lab Software, Version 5.2 build 14 (Bio-Rad).

Cathepsin D activity assay
The activity of cathepsin D in the serum samples was assessed with an assay kit (Abcam, product code: ab 65302). The assay was performed according to the instructions of the manufacturer using 50 µL serum and the acid reaction buffer (pH 3.5) supplied in the kit, and the replacement of the acid reaction buffer by PBS (pH 7.4). The kit was a fluorescence-based assay that utilized a cathepsin D substrate sequence (GKPILFFRLK (Dnp)-D-R-NH_2) labeled with MCA. Samples that contain cathepsin D were expected to cleave the synthetic substrate and to generate a fluorescent signal. The signal was quantified using a fluorescence plate reader (Synergy 2, BioTek Instruments GmbH, Bad Friedrichshall, Germany) at Ex/Em 328/460 nm, normalized against blank wells, and the mean of duplicate wells was calculated. Black 96-well microplates with a flat, transparent bottom were used (Brand GmbH + Co KG, Wertheim, Germany).

Antibodies, PRL, and human sera
A rabbit polyclonal anti-PRL primary antibody was used for detecting PRL and cleaved PRL products (Antibodies-online GmbH, Aachen, Germany, code ABIN1 538445, dilution 1:3,000). This antibody was produced with a synthetic peptide for immunization, comprising a sequence of 28 amino acids from the central region of human PRL. A horseradish peroxidase-conjugated secondary antibody (HRP-goat anti-rabbit IgG (1:3,000),

code 111-035-045) from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA) was used to detect and visualize the primary antibody. Human sera (refer to Table 1 for details) for research purposes were obtained from BBI Solutions (Cardiff, UK), as was recombinant human PRL (rhPRL), expressed in a *Pichia pastoris* culture (cat. no. P700-1). A human PRL ELISA was performed to determine PRL levels in serum samples according to manufacturer instructions (Abcam, ab108655).

Incubations
One microgram rhPRL was incubated alone and in combination with 0.5 µL human serum, corresponding to 22 - 54 µg total protein, and 5 µL buffer solution at 37°C and 600 rpm using a thermo-mixer (Thermo Mixer C, Eppendorf, Hamburg, Germany, cat. no.: 5382000015). Periods ranging from 1 to 24 hours were employed as indicated. A citrate-phosphate buffer at a variety of pH values [41] was used as indicated. Heat inactivation of serum samples was done by heating the samples at 95°C for 5 minutes. A vial of the MMP inhibitor Marimastat (Abcam, product code: ab141276) was dissolved to make a 100 mM solution, and 0.5 µL of this solution was added to serum samples for MMP inhibition.

SDS-PAGE and western blotting
Samples were subjected to SDS-PAGE using a BioRad Mini-Protean Cell with 4 - 20% SDS stain-free precast gels (Bio-Rad, cat. no. 456-8093). Laemmli sample buffer containing β-mercaptoethanol was added and all samples were denatured at 95°C for 5 minutes before loading onto the SDS-PAGE. The molecular weight standard Precision Plus Protein Western C Standards (Bio-Rad, cat. no. 161-0376) was used. Electrophoresis was performed at 200 V for approximately 30 minutes. The proteins were transferred onto a PVDF membrane, pore size 0.4 µm (Bio-Rad, cat. no. 100-26918) using the Bio-Rad Trans-Blot Turbo Transfer System (cat. no. 170-4155) at 25 V, 1.4 A for 7 minutes. The membrane was blocked by incubation with 5% dry milk in TBST at RT for 45 minutes on an orbital shaker. The membrane was incubated with a primary antibody at RT for 1.5 hours under rotation, washed, and incubated with a secondary antibody at RT for 1.5 hours under rotation. After repeated washing, the membrane was incubated with 6 mL of a development solution (Pierce ECL Western blotting Substrate, Waltham, MA, USA, cat. no. 32209) for 1 minute. Detection of total protein (UV activation of stain-free gel), fluorescent molecular weight markers, and chemiluminescence images were performed using the Chemi Doc MP Imaging System.

Statistical analyses
All statistical analyses were performed using GraphPad Prism 6.02 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Detection of matrix metalloproteinases
Matrix metalloproteases-1, -2, -3, -8, -9, -10, -13, and TIMP-1, -2, and -4 were detectable in all serum samples. Figure 1A shows the position of the detection areas on the array membrane, while Figure 1B demonstrates two representative membranes generated by performing the assay with the serum samples. Figure 1C shows a graphical representation of the optical density measured from each spot on the MMP array membrane (normalized and mean of two spots). Figure 2 shows scatter plots of the densitometric data for MMP or TIMP. MMP-1, -2, -10, -13, and TIMP-4 show relatively low abundance (Figure 2A). In contrast, MMP-3, -8, -9, TIMP-1 and TIMP-2 appear of high abundance (Figure 2B). Noteworthy, MMP-8, the MMP which cleaves PRL to generate vasoinhibins with the highest relative potency among MMPs [7], appeared the as most abundant MMP (Figure 2B). All MMP and TIMP levels showed a normal distribution (Table 2). The MMP-13 group required the removal of an outlier to show a normal distribution (sample 7 showed an exceptionally high level of MMP-13; the outlier was statistically identified and removed for the normality test).

Determination of cathepsin D activity
The activity of cathepsin D was detected in serum samples of all 9 subjects, as demonstrated by the increase in relative fluorescence units (RFU) against the blank wells containing only buffer (Figure 3). The activity was detectable at pH 7.4 and pH 3.5. The activity values at pH 7.4 showed a normal distribution, whereas the values at pH 3.5 did not. No outlier elimination could be performed due to the small sample size. With the exception of sample 7, the activity appeared lower at acidic pH compared to pH 7 (Figure 3). Significantly lower activity levels at acidic pH were confirmed with the Wilcoxon matched-pairs signed rank test (p = 0.0195, Table 2).

Incubation of PRL with serum samples
Incubation of rhPRL with serum from 8 subjects at pH 7.4 for 3 hours demonstrated a relatively high stability of the amino acid chain of rhPRL, as no PRL-fragments could be detected (Figure 4A, Supplemental Figure 1). When the samples were incubated at pH 3.4, western blotting analysis of sample 8 revealed a 17 kDa PRL-fragment (Figure 4B, Supplemental Figure 2). The generation of this fragment was suppressed by heat inactivation of the serum prior to incubation and by addition of the MMP-inhibitor marimastat (Figure 4C). The mean molecular weight of the PRL-fragment evaluated in this sample from twelve independent blots was 17.7 ± 0.4 kDa. The appearance of a 21 kDa species of PRL was observed at pH 3.4 (Figure 4B). This PRL species also appeared when PRL is incubated with acid buffer in the absence of serum (Figure 4D). The 17 kDa PRL band also appeared in blots of sample 9, however, ex-

Table 1. Clinical and demographic information, and PRL levels of patients from whom serum samples were investigated.

Sample no.	Gender	Age	Diagnosis	PRL-level (ng/mL)
1	female	55	none, healthy control	14.3
2	female	31	none, healthy control	8.1
3	male	25	none, healthy control	10.4
4	male	55	none, healthy control	2.4
5	female	40	pregnant, no disease	210
6	female	32	pregnant, no disease	189
7	male	81	Diabetes Mellitus, type 2	25
8	female	55	Diabetes Mellitus, type 2	5.2
9	male	34	none, healthy control	12

The present study comprised 9 serum samples from male and female patients, two of whom were pregnant and demonstrated high PRL levels. The other samples demonstrated normal PRL levels. Prolactin levels between 1 and 25 ng/mL are considered normal.

Table 2. Statistical assessment of serum proteases measurements.

Matrix metalloproteases and tissue inhibitors of metalloproteases in serum										
	MMP-1	MMP-2	MMP-3	MMP-8	MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-4
Number of samples	9	9	9	9	9	9	9	9	9	9
Mean (abundance by optical	15,219	13,167	101,744	351,854	240,722	16,757	17,937	447,180	278,689	30,729
Std. Deviation	5,593	6,067	86,573	215,800	136,068	13,467	13,530	132,358	181,405	18,676
Lower 95% CI of mean	10,920	8,504	35,199	185,976	136,131	6,405	7,537	345,441	139,249	16,373
Upper 95% CI of mean	19,519	17,830	168,290	517,733	345,312	27108	28,336	548,919	418,129	45,085
Passed normality test? (Shapiro-Wilk)	yes	yes	yes	yes	yes	yes	yes *	yes	yes	yes
Cathepsin D activity in serum										
Sample no.	1	2	3	4	5	6	7	8	9	normality test
pH 7.4, RFU	172	174	165	183	228	226	210	191	230	yes
pH 3.5, RFU	126	152	142	99	170	154	249	140	140	no
activity at pH 3.4 significantly lower (p = 0.0195)										

The statistical assessment of the MMP and TIMP measurements showed a normal distribution of all parameters within each group, except of MMP-13, in which normal distribution was only present after removal of an outlier. Cathepsin D activity showed a normal distribution at pH 7.4, whereas the values at pH 3.5 did not. No outlier elimination could be performed due to the small sample size. A significantly lower activity of cathepsin D activity at acidic pH was present (Wilcoxon matched-pairs signed rank test).

clusively at pH 3.4 (Figure 4E). In this sample, the band was also visible at pH 3.4 at incubation times of 6, 12, 18, and 24 hours; however, it did not increase in intensity with longer incubation times (Figure 4F, G, H, and I). PRL proteolytic processing found in samples 8 and 9 do not appear to be associated to the physiological condi-

tion of subjects (Table 1). The endogenous PRL levels present in the serum samples are shown in Table 1. The two samples from pregnant women demonstrated, as expected, elevated PRL levels. The levels of the other samples were within a normal PRL reference range (between 1 and 25 ng/mL).
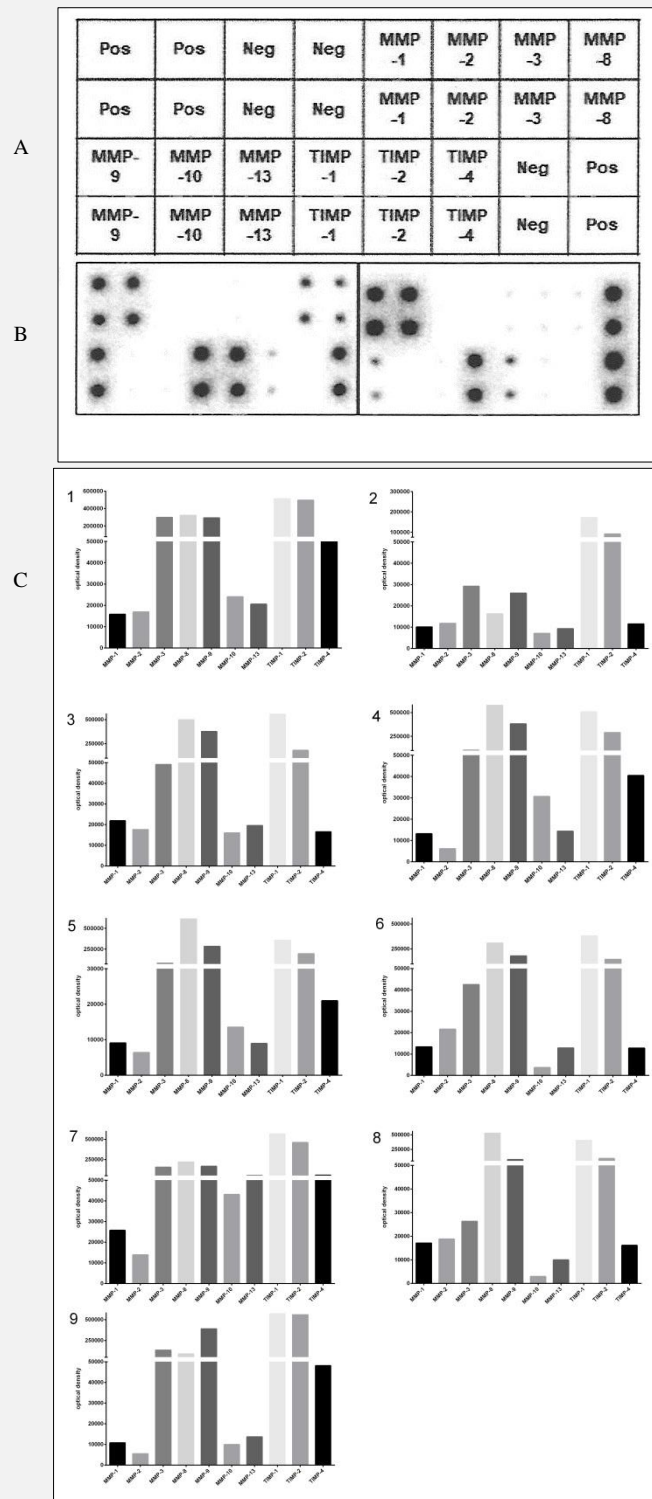
Figure 1. Evaluation of matrix metalloproteinases in human sera.

A membrane-based matrix metalloproteinases (MMP) array assay was performed to evaluate the presence and relative abundance of matrix metalloproteinase-1, -2, -3, -8, -9, -10, -13, and tissue inhibitors of matrix metalloproteinase-1, -2 and -4 (TIMP) in sera from 9 human subjects. A. The positions for each analyte on the membrane. B. Two representative array membranes (subject 1 and 8) are shown. C. Densitometric values presented in bar graphs for each membrane. The y-axes were separated into two segments. Values were normalized and represent the mean of two spots for each parameter.
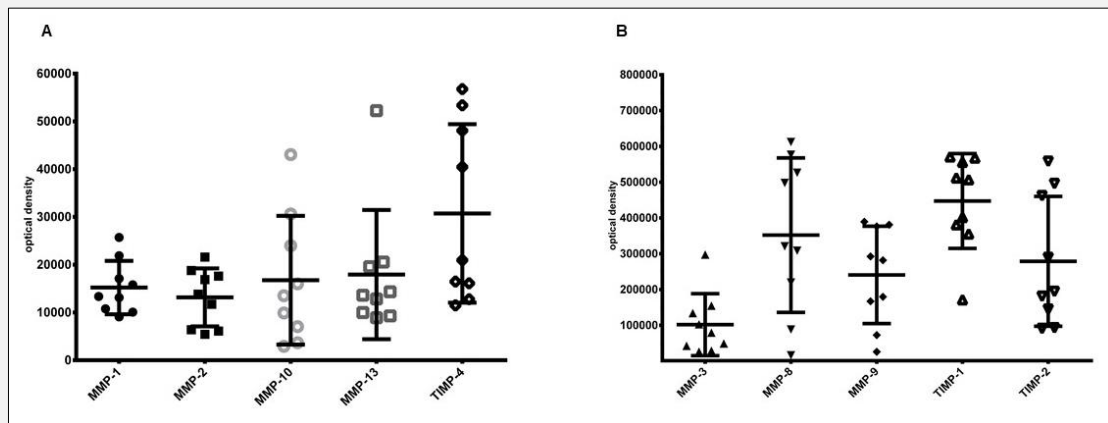
Figure 2. Scatter plots of densitometric data for MMP and TIMP detected in human sera.

A. Relatively low abundant MMP and TIMP were MMP-1, -2, -10, and -13, and TIMP-4. B. Relatively high abundant MMP and TIMP were MMP-3, -8, -9, and TIMP-1 and -2. Note different scale of the y-axis between Figure 2A and 2B. In addition to the individual data points, the mean and the standard deviation are shown.
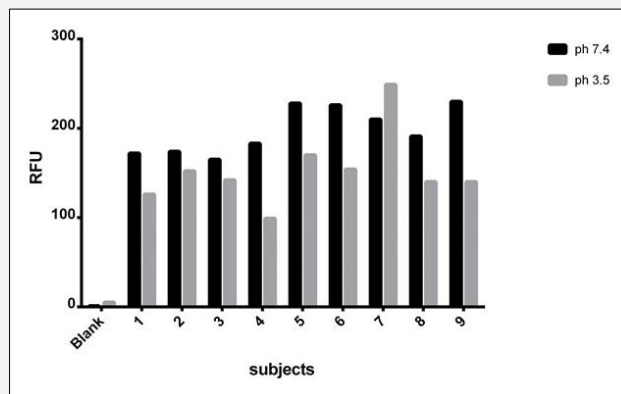

Figure 3. Activity of cathepsin D in human sera.

Human sera from 9 subjects demonstrated activity of cathepsin D, indicated by the increase in relative fluorescence units (RFU) compared to the negative control containing only buffer. Each sample was tested at neutral pH (7.4, black bars) and acidic pH (3.5, grey bars).

DISCUSSION

This study investigates whether matrix metalloproteinases and cathepsin D in human serum cleave PRL to generate vasoinhibin *in vitro*. This is a relevant question, as studies investigating peripheral vasoinhibin levels in vasoinhibin-related diseases frequently report serum vasoinhibin levels along with systemic concentrations of PRL-cleaving proteases [19,39], implying thereby that vasoinhibin may be generated from PRL while in the circulation. Direct proof of vasoinhibin generation in the circulation, however, is not available. As an alternate to being cleaved in the circulatory compartment, systemic vasoinhibin may enter the circula-
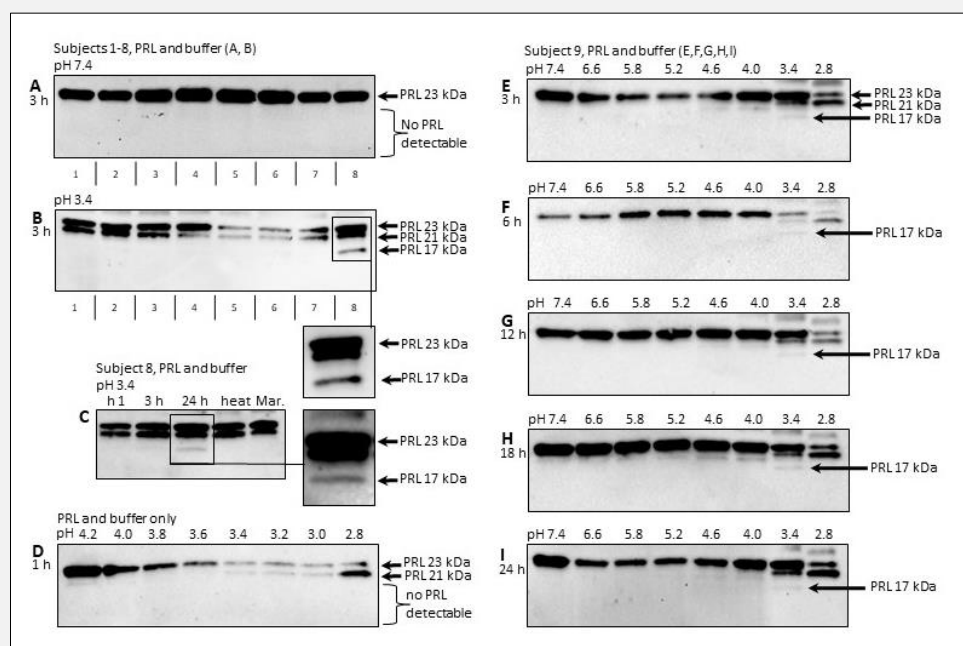
Figure 4. Incubation of human serum samples with recombinant human prolactin.

Recombinant human prolactin (PRL) was incubated with human serum samples with a variation in pH and time. A. No proteolytic cleavage of PRL was observed at neutral pH. B. At pH 3.4, sample 8 revealed a PRL-fragment of 17 kDa. C. The generation of this fragment was suppressible by heat inactivation of the serum prior to incubation (heat) and by the MMP-inhibitor marimastat (Mar.). D. A 21 kDa species of PRL was observed at acidic buffer conditions (B) and also appears in acidic buffer without serum. E. A 17 kDa band also appeared in blots of sample 9, however, only at pH 3.4. F, G, H, and I. In sample 9, the band was also visible at pH 3.4 and incubation times of 6, 12, 18, and 24 h. It did not increase in intensity with longer incubation times.

circulation after being generated at the pituitary [25] and target tissue level [40]. Investigating whether PRL-cleaving enzymes in serum contribute to vasoinhibin generation will help identify the anatomical sites and compartments of vasoinhibin generation, which are not completely known. In the present study, no cleavage of PRL was observed after incubating the hormone with serum samples from 9 subjects at neutral pH. The generation of a 17.7 kDa PRL band at acidic pH, which appears as a vasoinhibin-like peptide, was observed in the samples from two subjects, regardless of their different physiopathological state and PRL serum values.

Methodological considerations and limitations
An *in-vitro* model was used, incubating recombinant PRL with serum in a buffer at different pH values. A relevant limitation in this set-up is whether an adequate enzyme-substrate ratio can be reached with this method, especially because PRL has to be added in excess to allow subsequent detection of its proteolytic products. The PRL amount used (1 µg per 0.5 µL serum) is by far higher than the physiological levels expected in the cir-

culation (Table 1), but was done due to the limited sensitivity of the western blotting system. However, even in the presence of a substrate (PRL) excess, the cleavage of a fraction of PRL was detected using samples 8 and 9. The lack of detection with other samples may imply that, if occurring, the cleaved fraction accounted for less than the detection limit (approximately 1 ng) of the assay. Hence, a possible vasoinhibin generation below this level can neither be confirmed nor excluded. MMP-8, the MMP which cleaves PRL with the highest relative potency of all MMP [7], is expected to be present at a level of approximately 3 - 4 ng/mL in normal human sera [37]. This means the amount of MMP-8 incubated with the recombinant PRL was only 1 - 2 pg. Also, when detecting MMP and cathepsin D, far more volume of serum was used (200 µL and 50 µL, respectively) than what was tested in the incubations (0.5 µL). Despite the non-physiological enzyme-substrate ratio, which was due to technical reasons, the small amount of MMP expected in the serum samples possessed the capacity to cleave recombinant PRL but required the condition of an acid pH (Figure 4B). Therefore, it can be assumed that the methodology used in this study was

adequate to address the question of vasoinhibin generation by serum proteases; however, the limitation that using a system with higher sensitivity and a more physiological enzyme-substrate ratio may produce different results needs to be acknowledged. The activity of cathepsin D in the sera was surprising, as the pH optimum appeared to be at a neutral and not at an acidic value. However, cathepsin D is known to be active in human serum, as demonstrated by various studies [42,43]. Also, a model of kidney explant maintained at physiological pH, secreted cathepsin D able to cleave PRL and generated vasoinhibin [40]. Therefore, the generation of vasoinhibin by cathepsin D in human serum is a possible scenario.

Generation of a vasoinhibin-like, 17 kDa PRL at acidic pH

The molecular mass of the 17.7 kDa PRL species is consistent with a cleavage of full-length PRL between the amino acids serine at position 155 and leucine at position 156, which is a known PRL cleavage site of matrix metalloproteinases for the generation of a 17 kDa vasoinhibin-isoform [7]. The generation of the 17.7 kDa PRL species was suppressed by heat inactivation and by the MMP inhibitor marimastat, implying that the 17.7 kDa PRL-fragment corresponds to the known 17.7 kDa MMP-cleaved vasoinhibin-isoform. However, due to a lack of sequencing or mass spectrometric data, and possible inaccuracies of molecular mass determination in our western blotting system, we cannot provide full confirmation that the 17.7 kDa PRL observed in this study is derived from a cleavage at this site. This limitation is important because other known PRL cleavage sites are next to the one between SER 155 and LEU 156. Noteworthy, the generation of the 17.7 kDa PRL was observed only at pH 3.4. As this is very different from the physiological pH of 7.4 in the circulation, which, even under pathological circumstances does not reach pH 3.4, the generation of the 17.7 kDa PRL is rather unlikely to occur in the circulation *in vivo*. However, the generation of the 17.7 kDa vasoinhibin-like fragment at acidic pH by MMP is generally consistent with what is reported in the literature, as MMP have been shown to be active at pH 4.5 digesting cartilage proteoglycan [44]. It is unclear why only the 17.7 kDa PRL is generated and no 12.5 kDa, 14.1 kDa, and 16.8 kDa species can be observed, which are also expected to be generated by MMP. This indicates that the cleavage site for the 17.7 kDa PRL may be the preferred cleavage site of serum MMP.

Impact on future clinical and experimental investigations

A dysregulation of the generation of vasoinhibin appears to occur in several diseases, such as vasoproliferative retinopathies and pregnancy-associated diseases. In diabetic retinopathy and diabetic macular edema, as well as in peripartum cardiomyopathy, the emerging role of vasoinhibin has prompted the initiation of clinical trials in which the therapeutic strategy consists in the pharmacological stimulation and inhibition of vasoinhibin generation. In diabetic retinopathy and diabetic macular edema, a dopamine D2-receptor antagonist is evaluated with the intention to increase pituitary PRL-secretion and a subsequent elevation of retinal vasoinhibin, which may have beneficial effects [23,24]. In peripartum cardiomyopathy, a dopamine D2-receptor agonist is used to inhibit pituitary PRL-secretion with the intention of inhibiting vasoinhibin generation in the heart [45]. This intervention at the systemic level has unintended side-effects beyond its desired effect in the retina and the heart, as PRL is elevated or reduced in the circulation with the consequence of increasing possible effects of hyperprolactinemia (in case of PRL-elevation) or the elimination of all endocrine effects of PRL (in case of PRL-inhibition). Alternative therapeutic strategies, however, are currently not available. The main reason is an incomplete understanding of vasoinhibin-generating sites and factors controlling their generation. Our findings suggest that serum proteases do not cleave PRL while it circulates in blood. This indicates that the vasoinhibin generation is likely restricted to the pituitary gland and peripheral target tissue microenvironment. It is possible, however, that under pathophysiological circumstances, a higher activity of circulating proteases could indeed result in vasoinhibin generation from PRL at the blood compartment level, as implied by the studies linking cathepsin D and vasoinhibin values in the circulation of patients with PPCM and preeclampsia [19,39]. It will be relevant to produce evidence showing that the sera of these patients cleave PRL. However, the present study shows no apparent differences in the protease activity nor in the cleavage of PRL in serum samples from healthy men and women, pregnant or not, nor in individuals with type 2 diabetes. Although an increased number of samples merits further research, our present findings suggest that circulating vasoinhibin levels likely originate from the cleavage of PRL at the pituitary gland, the heart, the placenta, and perhaps in association with the endothelium, a main target tissue of vasoinhibins [26]. This is remarkable, as secretion from the pituitary gland indicates endocrine regulation, whereas local cleavage suggests paracrine/autocrine regulation of vasoinhibin levels and effects. Differentiating between these two possibilities, or understanding their interplay, will help to improve monitoring and interpreting PRL and vasoinhibin levels in vasoinhibin-related diseases, particularly during the establishment of diagnoses and pharmacological intervention. Also, a more precise understanding of vasoinhibin generation sites and controlling mechanisms will facilitate the development of tailor-made, targeted therapies of vasoinhibin-related diseases, replacing the relatively unspecific intervention at the systemic level.

Author Contributions:
Conception and design of research: JT, CC, TB; performed experiments: JT, NS, AIMV, MZ; analyzed data: JT, NS, AIMV, MZ; interpreted results of experiments: JT, NS, AIMV, MZ, GME, CC, TB; prepared figures: JT; wrote manuscript: JT; edited and revised manuscript: all authors, approved final version of manuscript: all authors.

Ethical Statement:
The work described in the present article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The human serum samples were purchased from BBI Solutions, Cardiff, UK, where they were collected under IRB/IEC Ethics approval and Informed Consent, Ethics Declaration codes SG375-2 and S112-1, including the approval to publish demographic and clinical data.

Prior Presentation:
Parts of this study were presented at the 18th European Congress of Endocrinology, Munich, Germany, May 28 - 31, 2016, Poster ECE2016-EP868.

Data Availability:
A dataset containing high-resolution and whole-membrane images of western blots shown in Figure 4B, and supplemental Figures 1 and 2 was deposited at the Harvard Dataverse and can be accessed by its URL: https://doi.org/10.7910/DVN/R0DEMC

Declaration of Interest:
The authors declare that they have no conflict of interest to disclose.

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