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Haemostaseological Changes of VWF and FVIII during Pregnancy and the Oestrus Cycle in a Porcine Model of Von Willebrand Disease

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Abstract: Pregnancy and the oestrus cycle are challenging for female patients suffering from von Willebrand disease (VWD). Therefore, our study aimed to investigate the changes in von Willebrand factor (VWF) and factor VIII (FVIII) during pregnancy and the oestrus cycle in our porcine model of von Willebrand disease compared with the wild-type. Plasma analyses regarding primary hemostasis, secondary hemostasis, and VWF multimers, as well as immunohistochemistry analyses of VWF in the uterus and ovary, were performed. For levels of VWF and FVIII activities, significant elevations were seen in the last trimester. Primary hemostasis improved towards the end of pregnancy. In the oestrus cycle, significantly lower VWF values can be seen in the immunohistochemistry of the ovaries during the oestrus, while values were highest in the metoestrus. VWF multimer patterns in pigs were similar to the ones in human VWD patients. In summary, the course of VWF and FVIII during pregnancy and the oestrus cycle in porcine VWD were investigated for the first time. The porcine model seems to be suitable for haemostaseological studies on VWD. This provides an advantage for investigating reproduction-related bleeding and understanding the underlying mechanisms of post-partum hemorrhage or miscarriage in women with VWD.

Keywords: von Willebrand disease; pregnancy; oestrus cycle; haemostasis; animal model; sus scrofa

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1. Introduction

Von Willebrand disease (VWD) is the most common inherited bleeding disorder worldwide, affecting up to 1% of the population. It can be classified into different types and subtypes depending on the underlying change in the von Willebrand factor (VWF). Patients affected by VWD type 1 (VWD T1) and VWD type 3 (VWD T3) show a quantitative loss up to complete absence of VWF, while type 2 comprises qualitative aberrations of VWF. Correct classification, as described in the guidelines for VWD of the American Society of Hematology (ASH), the International Society on Thrombosis and Haemostasis (ISTH), the National Hemophilia Foundation (NHF), and the World Federation of Hemophilia (WFH), requires laboratory testing in combination with a careful history and assessment of the phenotype [1]. Correct classification can be challenging, necessitating a comparative analysis of both primary and secondary hemostasis, which can be aided by multimer analysis.

VWF is a monomeric protein after synthesis and is converted into various multimeric forms, leading to a molecular weight of 800 to 20,000 kDa [2]. The main function of VWF is to allow platelets to bind to a site of injury. VWF also binds FVIII, protecting it from

degradation. Further VWF binds collagen, which is important for the immobilisation of platelets on sites of injury. In blood, VWF is normally formed as a dimer, which can also form multimers through interdimeric disulfide bonds, consisting of up to 40 dimers [3]. The collagen binding and platelet aggregation potential of VWF increase with a higher degree of multimerisation [3].

Pregnancy is associated with changes in coagulation and fibrinolysis in healthy women, reflecting the physiological and necessary adaptation of endothelial cells [4,5]. Even in pregnant women as well as female dogs with VWD T1, some procoagulant changes have been detected in VWF and FVIII [6,7]. However, as no corresponding studies have been conducted in pigs, we studied haemostaseological parameters during pregnancy and the oestrus cycle in our porcine model of VWD T1. It is widely discussed that women with VWD have a higher risk of bleeding before or after pregnancy. Performing a study in a large animal model similar to human VWD patients will shed light on this discussion and help to investigate the changes pronouncing or reducing the risk of bleeding in detail. We hypothesise to find analogic procoagulant changes in pigs with VWD as reported in women and dogs during pregnancy and cycle.

Our VWD pig model shows quantitative defects in plasma VWF. This is initially caused by a large tandem duplication within the VWF gene, including the exons 17 and 18, causing a frameshift and a premature termination codon [8]. In humans, VWD T1 usually follows an autosomal dominant inheritance, and VWD T3 is usually autosomal recessive [9]. Some mutations cause VWD T3 when homozygous; however, individuals heterozygous for the same mutation are phenotypically classified as T1 [10], which corresponds to a semidominant mode of inheritance. In our pig model, VWD is also inherited semi-dominantly, with pigs heterozygous for the causal mutation showing VWD T1 and homozygous pigs showing VWD T3 [11]. Phenotypically, the pigs used in our model are most similar to human VWD T1 patients [12].

Since our pig model has proven to be suitable for the genetic and histological investigation of VWD [11,13,14] and includes three different genotypes (WT, VWD T1, and VWD T3) in a pig strain with an otherwise similar genetic background, it brings a large benefit to analysing VWF and other factors related to hemostasis at regular intervals during pregnancy and the oestrus cycle. This may also provide deeper knowledge of haemostaseological changes during this sensitive period.

2. Materials and Methods

Blood samples were collected from a total of eleven wild-type (WT) and ten VWD T1 sows regularly during pregnancy and/or oestrus cycle. The jugular vein was chosen for sampling using a 2.00 × 10 mm cannula and blood tubes containing the anticoagulant citrate (3.2%). For further investigation of primary hemostasis during pregnancy, blood was additionally collected in 3.8% citrate tubes (Table 1). The collection was approved by the Lower Saxony State Office for Consumer Protection and Food Safety, Oldenburg, Germany (AZ33.19-42502-04-182940). All blood collections were performed in accordance with national and international guidelines for animal welfare and in accordance with the ARRIVE guidelines.

Blood sampling was performed in two WT and three VWD T1 animals during a complete pregnancy. Between day 0 and day 35 of pregnancy (o. p.), counting from the day of insemination, samples were taken every 7 days. From day 35 o. p., sampling intervals were increased to 14 days, with the last sampling on day 105 o. p.

In addition, blood samples were taken from five WT and ten VWD T1 sows throughout the oestrus cycle, including oestrus (days 1–2), metoestrus (days 3–6), dioestrus (days 7–17), and prooestrus (days 18–21). The differing numbers of animals in pregnancy or reproductive cycle were due to loss during pregnancy and individual differences in cycle irrespectively. Only animals that were definitely pregnant were included in the experiments.

The method of blood collection was specifically undertaken using operators trained to work with the animals to reduce stress and stress-mediated platelet activation and VWF

release as a consequence. Moreover, to reduce the stress for individual animals, we also conditioned the pigs to receive a treat after blood collection. Thus, we were able to reduce restraint in many pigs during sampling in the course of pregnancy.

Table 1. Details of the used detection/quantification systems.

Equipment, Manufacturer	Factor	Test Set	Additional Reagents	Human Reference Range
ACL TOP 750 BASE, Werfen GmbH, Kirchheim near Munich	VWF Act	Siemens INNOVANCE VWF Ac	HemosIL factor diluent (Werfen GmbH, Kirchheim, Germany)	48–173%
	F8 cSL	HemosIL ELECTRACHROME Factor VIII	HemosIL factor diluent (Werfen GmbH, Kirchheim, Germany)	70–210%
PFA-200™, Siemens Healthcare, Erlangen, Germany	C/EPI			<165 s
	C/ADP			<121 s
	P2Y			<107 s
KABE LABORTECHNIK GmbH	Blood tubes for primary coagulation	Primavette® S PHC/PFA		
	Blood tubes for secondary coagulation factors and multimers	Primavette® S Coagulation		
Hydrasys 2 Scan Focusing, SEBIA GmbH, Mainz, Germany		Hydragel 11 Von WILLEBRAND MULTIMERS Kit		

VWF Act: von Willebrand factor activity; F8 cSL: chromogen FVIII.

2.1. Tissue Samples

Uterine and ovarian tissue from 36 WT sows was collected directly after death at the slaughterhouse (Leine Fleisch GmbH, Gleidingen, Germany) and fixed in neutral buffered formalin for 48 h. The samples were cut to a size of 1 square cm, dehydrated in alcohol, and embedded in paraffin according to a standard protocol. Sections of 3–5 µm thickness were cut using a Leica 1512 rotary microtome (Leica Biosystems, Wetzlar, Germany) and transferred to glass slides. To analyse each state of the oestrus cycle, ovary and uterus samples of the same individual were kept together and the cycle status was categorised by macroscopic observation of the ovary. Ovaries with follicles less than 3 mm in diameter or with only some larger follicles were categorised as prooestrus. Oestrus was indicated by a high number of follicles over 3.5 mm in diameter. Corpora haemorrhagica are typically observed after ovulation and thus indicate the metoestrus state. More than 2 large corpora lutea over 10 mm indicated dioestrus state.

2.2. Primary and Secondary Haemostasis

After collection, samples intended for platelet function analysis were measured directly within 30 min. Citrate samples for all other analyses were centrifuged twice at 4000× *g* for 15 min. Plasma was aliquoted and frozen at −80 °C or measured directly within 4 h after sampling.

The measurement of closure times (CT) using the Platelet Function Analyzer 200 (PFA 200, Siemens Healthcare GmbH, Erlangen, Germany) was performed directly within the maximum interval of 30 min after blood collection. In the PFA analysis, 800 µL of blood is soaked through differently coated membranes. We used membranes coated with collagen and epinephrine

(C/EPI), adenosine 5′-diphosphate (C/ADP), prostaglandin E1, adenosine 5′-diphosphate, and calcium (P2Y). These platelet agonists enable the secretion of the platelet storage granules [15,16] or simulate the P2Y12 receptor [17]. High shear stress in the capillary triggers platelet adhesion, activation, and aggregation of platelets into the most stable plug possible, which seals the membrane and is referred to as closure time [17,18].

Standard human haemostaseological tests were carried out to investigate the coagulation factors of the pigs. For this purpose, human standards were used. The factors VWF:GPIbM and FVIII:C were measured with assays of Siemens Healthcare GmbH (Erlangen, Germany) adapted for use on the ACL Top 750 BASE (Werfen GmbH, Kirchheim, Germany). Samples were pre-diluted 1:2 or 1:4 with HemosIL factor diluent (Werfen GmbH, Kirchheim, Germany) before measurement, as needed.

2.3. Assessment of Pregnancy

For the evaluation, pregnancy was separated into four periods. During the period before implantation (BI), samples were taken on days 0, 7, and 14. Early pregnancy (EP) comprises the period from implantation until the completion of placentation at the end of the first trimester. Samples were obtained on days 21, 28, and 35. Samples from days 49 and 63 were assigned to mid-pregnancy (MP) and corresponded to the second trimester. Samples taken on days 77, 91, and 105 were assigned to late-pregnancy (LP), thus representing the third trimester.

2.4. VWF-Multimers

The VWF multimerisation tests were performed semi-automatically. The blood samples were taken as citrated plasma and prepared according to standard methods. Two different types of VWF multimer analyses were used.

As the first of these methods, SDS-agarose gel electrophoresis was used. Samples were diluted in sample buffer containing disodium EDTA salt, tris(hydroxymethyl)aminomethane (TRIS), and sodium dodecyl sulphate (SDS). The gel wells were loaded, and electrophoresis was performed according to standard laboratory procedures. VWF separations were then blotted onto nitrocellulose membranes. For visualisation of the multimers, they were blocked and incubated with two different antibodies. For luminographic visualisation, luminol iodophenol was used in combination with a translucent polyethylene film, and the results were recorded on X-ray films.

In the second method, samples were measured semi-automatically with the Hydrasys 2 Scan Focusing (SEBIA GmbH, Mainz, Germany) using the Hydragel-11 multimer kit (SEBIA GmbH, Mainz, Germany) according to the manufacturer's instructions. This was a two-step assay in which electrophoresis was performed first to separate VWF multimers according to their molecular weight. This was followed by visualisation by immunofixation with a specific anti-VWF antiserum for qualitative visual and densitometric evaluation [19]. The gels were scanned, and densitometric graphs and percentages of low-, intermediate-, and high-molecular-weight multimers were generated using SEBIAs Phoresis software. The densitometric graphs were evaluated according to the manufacturer's instructions, with the peaks representing low molecular weight multimers (LMWM), intermediate molecular weight multimers (IMWM), and high molecular weight multimers (HMWM) corresponding to the bands on the gel. The first three bands represented LMWM, the next four IMWM, and the last HMWM.

2.5. Immunohistochemistry (IHC)

The staining and analysis of slides included three different sites of the uterus and ovary for each individual animal. Sections containing all specific layers for the respective tissue on HE-staining were selected for evaluation by the IHC. Standard IHC protocols were used, and the dilution of VWF antibody yielding the best staining was chosen (1:3000, DAKO A0082).

The slides were deparaffinised in xylene for 2 × 10 min and rehydrated in a series of graded alcohols (isopropanol for 2 min, absolute ethanol, a mixture of 80% ethanol and hydrogen peroxide for 30 min, and 70% ethanol for 2 min). The slides were then washed three times for 5 min in TBS-T. Unmasking of the epitopes was conducted by heating at 96–99 °C for 20 min in TEC buffer solution. Then, the slides were washed again three times for 5 min in TBS-T. Incubation with primary antibodies diluted with phosphate buffer solution (PBS) containing 1% bovine serum albumin (BSA) was conducted overnight at 4 °C in a humid chamber. The next day, the slides were incubated with secondary antibodies for 30 min, and the staining was visualised with DAB (3′3-diaminobenzene) treatment for 5 min. The slides were counterstained with Delafield's hematoxylin and mounted with Eukitt®. Negative controls were performed by replacing the primary antibody with a phosphate buffer solution containing 3% bovine serum albumin and performing IgG controls. Primary antibody specificity is demonstrated by identical staining patterns as previously seen in pigs with this antibody [11].

2.6. Statistics and Software

IHC slides were digitalised with the AxioScan.Z1 automatic microscope (Carl Zeiss AG, Oberkochen, Germany). For evaluation, the QuPath software version 0.3.2 was used [20]. All scans were set as image type "brightfield (H-DAB)". A full image annotation was then created, and cells were detected via "Cell detection". The counted cells were classified as positive and negative based on their staining intensity. The standard settings for "Cell detection" were applied, except for minor changes (detection image: optical density sum; desired pixel size: 0.2).

Statistics for coagulation factors, PFA, and IHC were performed using the Mann-Whitney test (https://astatsa.com, accessed on 30 September 2023). A p-value < 0.0166 was considered significant after Bonferroni correction for multiple testing [21] for the three tests of 3.8% citrate tubes (PFA C/EPI, C/ADP, P2Y) and the three tests of 3.2% citrate tubes (VWF:GPIbM, FVIII:C, multimers), respectively. For the IHC of tissue samples, a p-value < 0.05 was considered significant. In the first step, the analysis was performed on pregnant pigs with regard to the different stages and phenotypes. Subsequently, the pigs in the cycle were compared regarding the different stages and phenotypes.

3. Results

3.1. Primary and Secondary Haemostasis

3.1.1. Collagen and Epinephrine (C/EPI) (Figure 1A)

The results regarding the CT for the C/EPI cartridge were at or above the upper end of the human reference range. In WT animals, an increase in CT was measured from before implantation (BI, 170 s) to mid-pregnancy (MP, 231 s), followed by a decrease towards late-pregnancy (LP, 199 s). VWD T1 animals showed a similar pattern, although the CT was significantly higher during complete pregnancy, mainly in BI (263 s, $p = 0.012$) and EP (270 s, $p = 0.016$) compared with the WT animals, while the values of both phenotype groups converged somewhat at later stages of gestation.

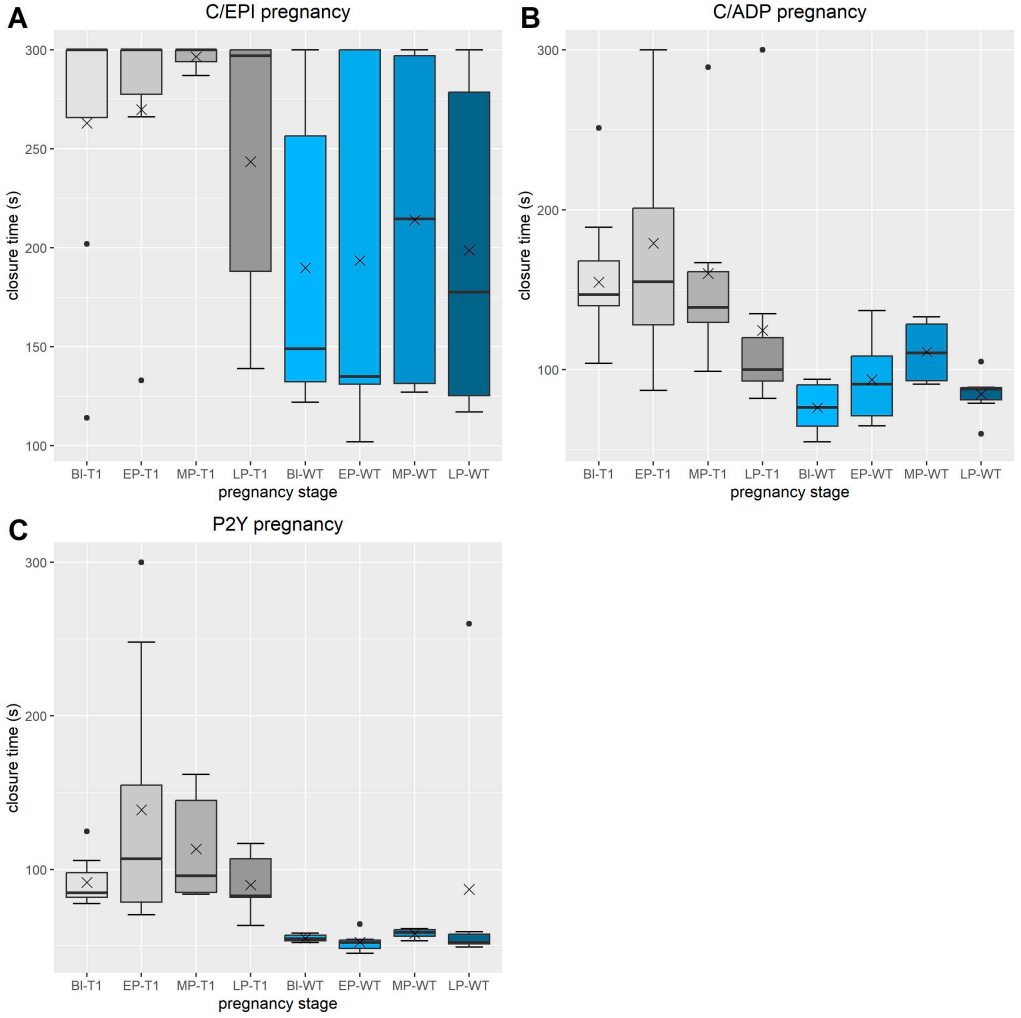

Figure 1. Boxplots of the measurements of PFA 200 using the collagen and epinephrin (C/EPI) (**A**), collagen and adenosine 5′-diphosphate (C/ADP) (**B**), and the prostaglandin E1, adenosine 5′-diphosphate, and calcium (P2Y) (**C**) cartridge over the course of pregnancy. (**A**) Comparing VWD T1 (T1) to the wild-type (WT), noticeably longer closure times were seen before implantation (BI) and in early pregnancy (EP). In mid-pregnancy (MP) and late pregnancy (LP), no significant differences were seen between the genotypes. (**B**) Comparing stages of pregnancy by phenotype, an increase from BI to MP and a decrease towards LP were seen in WT. Even in VWD T1, a significant decrease was seen from BI to LP ($p = 0.005$). Comparing the genotypes VWD T1 (T1) and wild-type (WT), before implantation (BI, $p = 0.0001$) and in early pregnancy (EP, $p = 0.016$), significant longer closure times were seen in VWD T1. In mid-pregnancy (MP) and late pregnancy (LP), no significant differences between genotypes were seen. (**C**) Comparing stages of pregnancy by phenotype, no significant difference was seen. Comparing the genotypes VWD T1 (T1) and wild-type (WT), before implantation (BI, $p = 0.00004$), in early pregnancy (EP, $p = 0.001$), in mid-pregnancy (MP, $p = 0.014$), and late pregnancy (LP, $p = 0.003$), significant longer closure times were seen in VWD T1. The mean is indicated by x, the median by the black line, and the bars show the lower and upper percentiles. Outliers are presented as black dots.

3.1.2. Collagen and Adenosine 5′-Diphosphate (C/ADP) (Figure 1B)

When using the C/ADP cartridge, all WT animals remained within the range of the human reference values for CT. The VWD T1 animals, on the other hand, were constantly above the human reference values of <121 s. When comparing stages of pregnancy by phenotype, WT animals showed an increase in CT from BI to MP (78 s to 111 s, $p = 0.04$)

and a decrease towards the end of pregnancy (85 s, $p = 0.04$). VWD T1 animals showed a significant decrease in CT from a mean of 155 s in BI to 104 s in LP ($p = 0.005$).

In addition, we compared WT with VWD T1 during pregnancy. At baseline BI and in EP, VWD T1 animals showed a significantly longer mean CT than WT animals (BI: $p = 0.0001$, EP: $p = 0.016$). In the middle and at the end of pregnancy, however, no significant difference was observed.

3.1.3. Prostaglandin E1, Adenosine 5′-Diphosphate, and Calcium (P2Y) (Figure 1C)

Comparing the P2Y measurements of WT and VWD T1 animals during pregnancy, the consistently higher CT values of VWD T1 animals throughout pregnancy were statistically significant. The CT values of WT animals remained constant at 52–58 s on average. The mean values of VWD T1 animals varied between 90 s and 114 s. BI mean values were measured with a significant difference of 92 s for VWD T1 and 52 s for WT animals ($p = 0.00004$). Similarly, a significant difference in CT was found for EP ($p = 0.001$), MP ($p = 0.01$), and LP ($p = 0.003$).

When comparing the mean CT within genotypes, no significant differences were found between the stages of pregnancy. All animals remained within the range of the human reference values or slightly above.

3.1.4. VWF Activity (Figure 2)

All measured values for the WT and VWD T1 animals regarding VWF:GPIbM were in the range of the human reference values. During pregnancy, WT animals remained constantly at significantly higher VWF:GPIbM values than VWD T1 animals (BI/EP/MP $p = 0.0001$; LP $p = 0.0005$). A slight increase from BI to LP was observed in WT (BI VWF:GPIbM = 134%, LP VWF:GPIbM = 144%), showing much variation. In VWD T1, an increase in VWF:GPIbM from BI to LP, especially after MP, was also seen, but at a generally lower level. It showed less variation and was significant (BI VWF:GPIbM = 71%, LP VWF:GPIbM = 84%, $p = 0.006$).

In the reproductive cycle, the values remained almost the same. The difference between WT and VWD T1 was significant in oestrus (WT VWF:GPIbM = 109%, VWD T1 VWF:GPIbM = 78%, $p = 0.002$), metoestrus (WT VWF:GPIbM = 105%, VWD T1 VWF:GPIbM = 79%, $p = 0.0001$), and dioestrus (WT VWF:GPIbM = 122%, VWD T1 VWF:GPIbM = 76%, $p = 0.004$), but not significantly different in prooestrus (WT VWF:GPIbM = 99%, VWD T1 VWF:GPIbM = 83%).

3.1.5. FVIII Chromogen (Figure 3)

The measured FVIII:C values were at the upper end or above the human reference values. In pregnant WT animals, the values stayed above the respective mean values of VWD T1 animals. A not-significant increase in FVIII:C was detected in WT from BI (FVIII:C = 247%) to LP (FVIII:C = 272%, $p = 0.0497$). Comparing pregnant WT with pregnant VWD T1 animals, FVIII:C levels visibly diverged in MP (WT FVIII:C = 251%, VWD T1 FVIII:C = 199%, $p = 0.0885$), and this trend continued to a significant difference in LP (WT FVIII:C = 272%, VWD T1 FVIII:C = 226%, $p = 0.005$).

In the reproductive cycle, WT animals also showed consistently higher values than VWD T1 animals. This difference was noticeable but not significant after correction for multiple testing in oestrus (WT FVIII:C = 265%, VWD T1 FVIII:C = 189%, $p = 0.03$), metoestrus (WT FVIII:C = 224%, VWD T1 FVIII:C = 155%, $p = 0.02$), and dioestrus (WT FVIII:C = 216%, VWD T1 FVIII:C = 162%, $p = 0.02$).

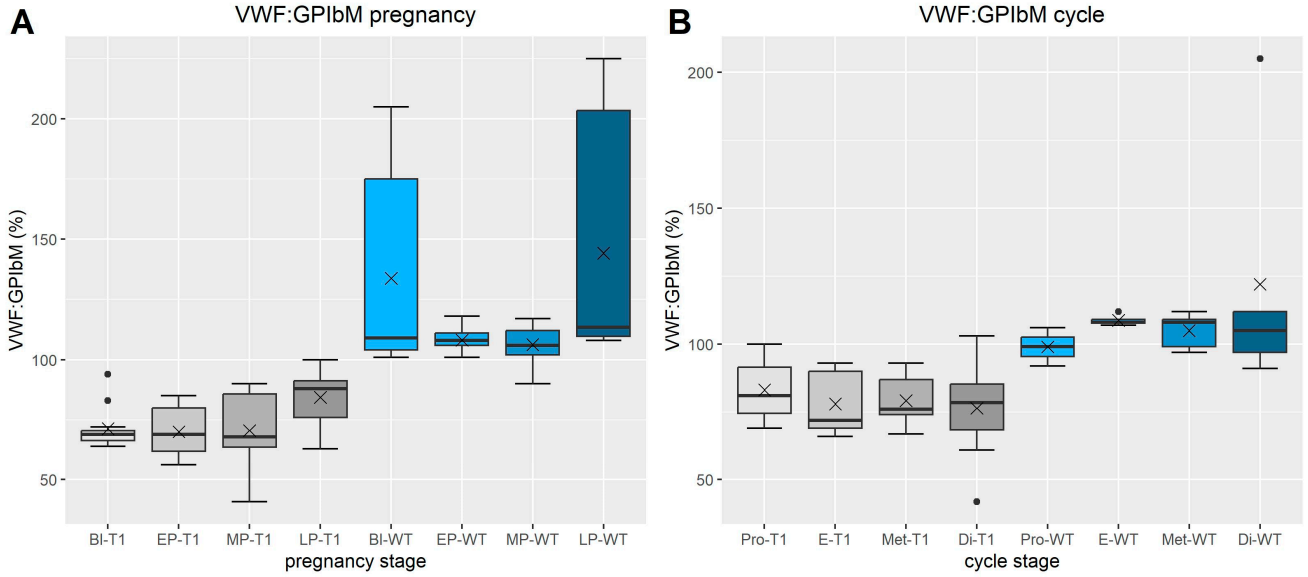

Figure 2. Boxplots for von Willebrand factor activity (VWF:GPIbM) over the course of pregnancy (**A**) and the oestrus cycle (**B**). (**A**): Comparing stages of pregnancy by phenotype, a significant increase in VWD T1 was seen from before implantation (BI) to late pregnancy (LP, $p = 0.0059$). Comparing the genotypes VWD T1 (T1) and wild-type (WT), before implantation (BI, $p = 0.0001$), in early pregnancy (EP, $p = 0.0001$), in mid-pregnancy (MP, $p = 0.0001$), and in late pregnancy (LP, $p = 0.0005$), significant higher factor activity was seen in WT. (**B**): Comparing cycle stages by phenotype, no significant differences were seen. Comparing the genotypes VWD T1 (T1) and wild-type (WT), in oestrus (E, $p = 0.002$), in metoestrus (Met, $p = 0.0001$), and in dioestrus (Di, $p = 0.004$), higher factor activity was seen in WT. The mean is indicated by x, the median by the black line, and the bars show 25 to 75 percentiles. Outliers are presented as black dots.

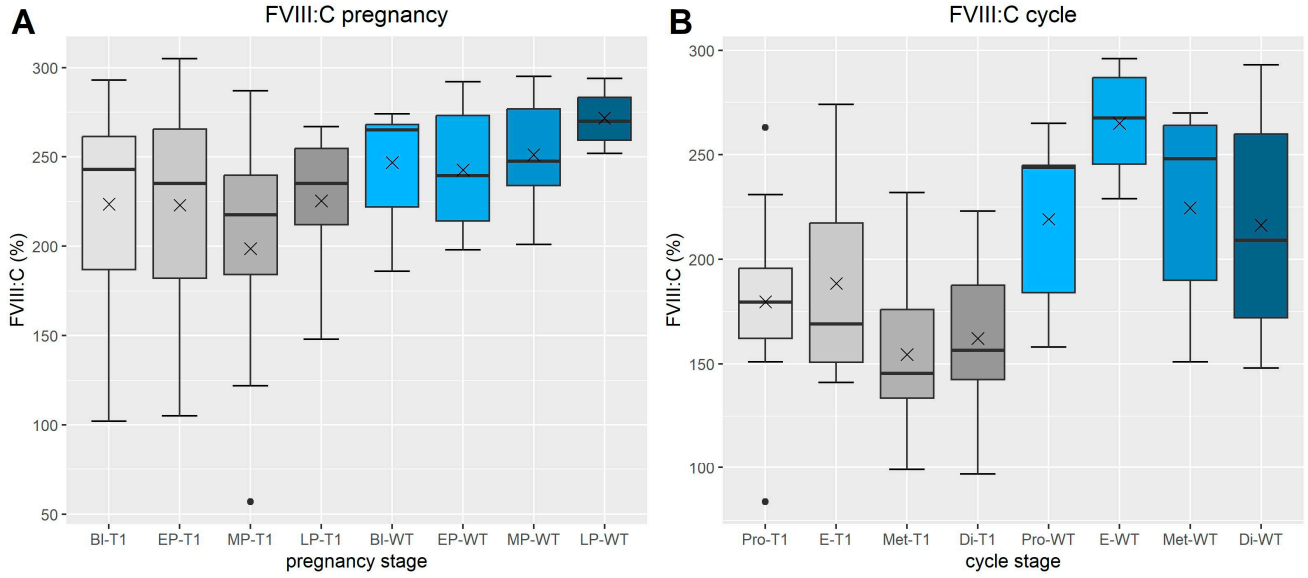

Figure 3. Boxplots for chromogen FVIII (FVIII:C) over the course of pregnancy (**A**) and oestrus cycle (**B**). (**A**) Comparing stages of pregnancy by phenotype, no significant differences were seen. Comparing the genotypes VWD T1 (T1) and wild-type (WT) in late pregnancy (LP, $p = 0.0048$), significantly higher FVIII levels were seen in WT. (**B**) Comparing stages of the cycle by the genotypes VWD T1 (T1) and wild-type (WT), no significant differences were seen. The mean is indicated by x, the median by the black line, and the bars show 25 to 75 percentiles. Outliers are presented as black dots.

3.2. VWF-Multimers (Figures 4 and 5)

The multimeric structure of VWF was analysed in terms of the distribution and the presence, loss, or partial loss of specific multimeric sizes during the different stages of gestation. Analysis of the general structure of densitometric graphs revealed a lower amount of multimers of each size compared to the human control. The peaks of the individual multimer sizes were not as clearly separated in pigs compared with humans. The bands in the gel were also not well delineated. The multimer pattern in VWD T1 appeared to be similar to that of the WT animals but with a lower intensity of bands. In addition, the bands of the pig samples seemed to be slightly shifted downward compared to the human samples (Figure 4). The percentages of LMWM, IMWM, and high HMWM are shown in Figure 5 and Supplementary Table S2. In the last trimester of pregnancy, VWD T1 animals appeared to show a slight relative decrease in HMWM with a shift towards IMWM. No clear trends could be described in WT animals. Due to the inhomogeneity of the data, no statistical analysis were performed. The additionally performed multimer analysis with SDS agarose gel electrophoresis showed the same shift and pattern (Figure 4) for WT and VWD T1 animals as with the semi-automatic method.

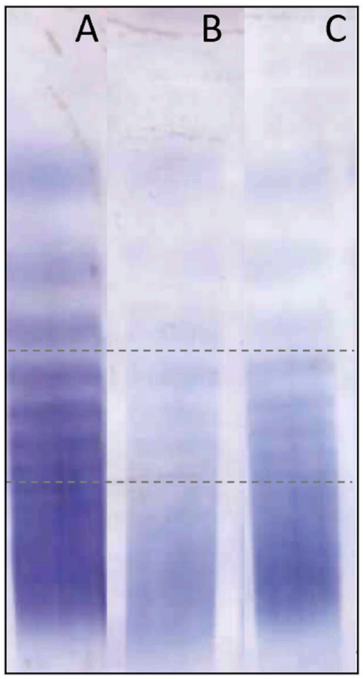

Figure 4. Representative results of the detection of multimers in porcine plasma in pregnant pigs. Comparison of the human control (**A**) with the genotypes VWD type 1 (**B**) and wild-type (**C**). Bands in pigs were not as clearly delineated as in human control. They seemed to be slightly shifted downward. The intensity of bands generally appeared lower in pigs and even lower in VWD T1 than in WT animals. Dashed lines indicate the transitions between high-molecular-weight, intermediate-molecular-weight, and low-molecular-weight multimers. Therefore, the upper three bands indicate low-molecular-weight multimers. Bands 4–7 represent intermediate-molecular-weight-multimers, and all bands beyond the second line indicate high-molecular-weight-multimers.

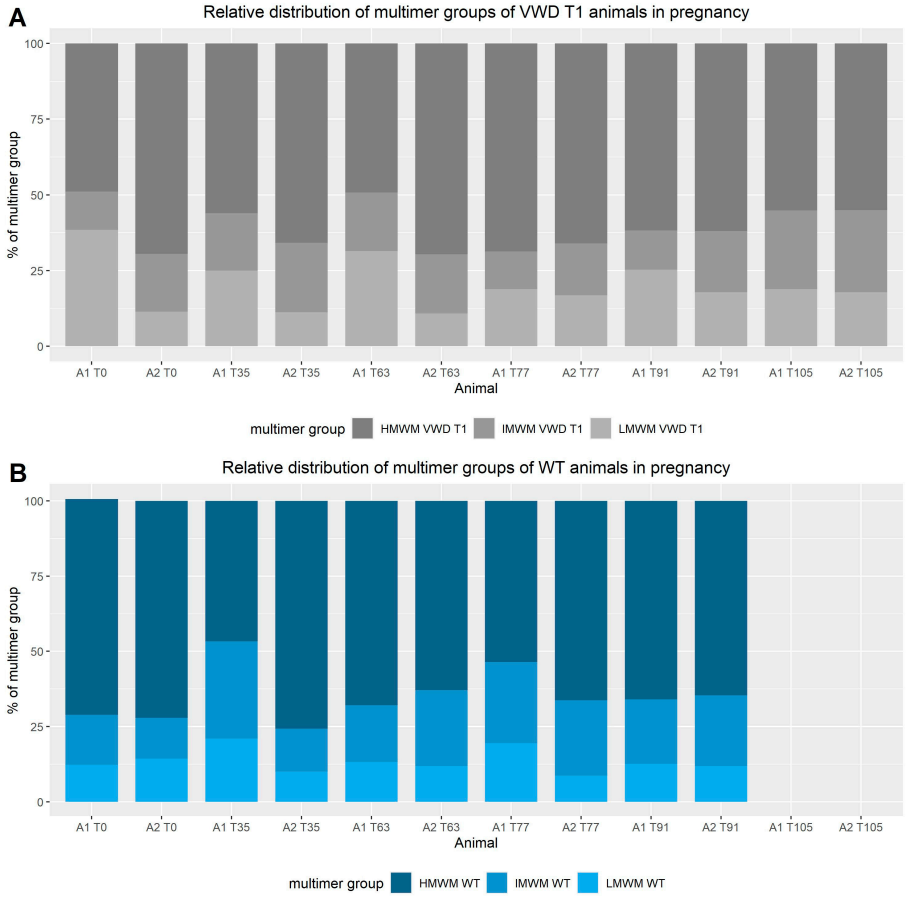

Figure 5. Relative distribution of multimers in porcine plasma in pregnant pigs. Barplots of the genotypes VWD type 1 (**A**) and wild-type (**B**) for two animals tested (A1 or A2) on different days of pregnancy (Tn). There was no clear tendency visible during the course of pregnancy for both genotypes and large intra-genotype differences were present until the end of pregnancy. For values, see Supplementary Table S2. For WT T105, no valid values could be measured.

3.3. Evaluation of the Immunohistochemistry (IHC) (Figures 6 and 7)

The uterus and ovary of non-pregnant juvenile or cycling wild-type sows were examined for VWF protein expression. The same staining patterns as previously seen in pigs with this antibody demonstrate the specificity of the primary antibody. Negative and IgG controls were performed to ensure specific binding of the secondary antibody; both were negative in uterine and ovarian tissues. The cycle stages of prooestrus, oestrus, metoestrus, and dioestrus were evaluated.

When comparing juvenile and postpubertal animals, there were no remarkable differences in the uterus or ovary. There were also no significant differences in protein expression in the uterus of the postpubertal animals. However, significant changes were found in the ovaries of the postpubertal animals. In oestrus, the mean percentage of positively stained cells was significantly lower (23.8%) than in the preceding prooestrus (28.3%, $p = 0.01$) and subsequent metoestrus (33.2%, $p = 0.001$). The difference between the mean values of metoestrus and dioestrus (28.1%) was not significant, nor was the comparison of dioestrus and subsequent prooestrus.

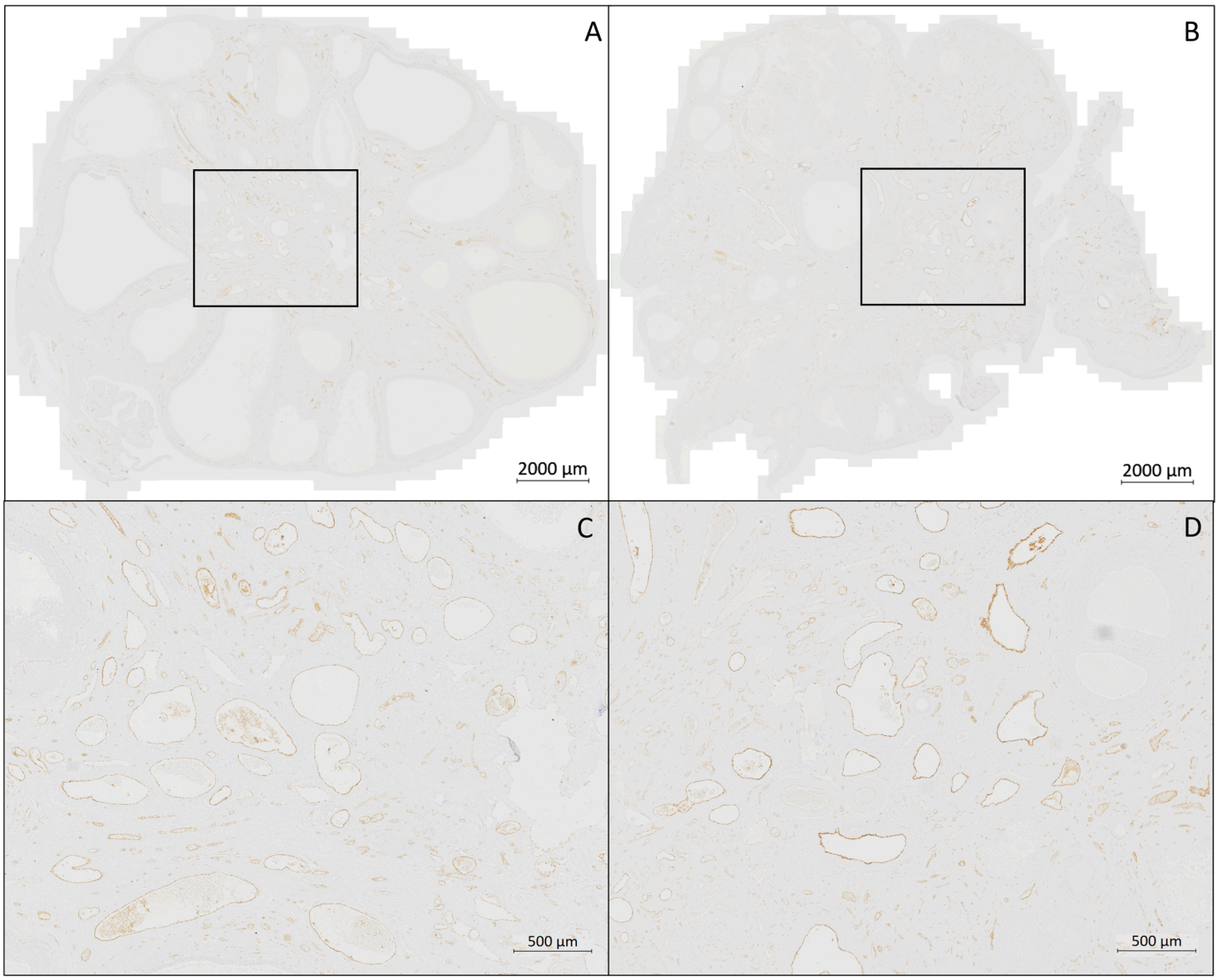

Figure 6. Representative results of FVIII staining of ovarian tissue sections in non-pregnant wild-type pigs. Sections of ovarian tissue in the different cycle stages of oestrus (**A**) and metoestrus (**B**). Details are shown below for oestrus (**C**) and metoestrus (**D**). The relative amount of positively stained cells from oestrus (23.8%, $p = 0.01$) towards metoestrus increased significantly (33.2%, $p = 0.001$). Results with p-values ≤ 0.05 were regarded as significant. Scale bars (**A**,**B**) = 2000 µm. Scale bars (**C**,**D**) = 500 µm.

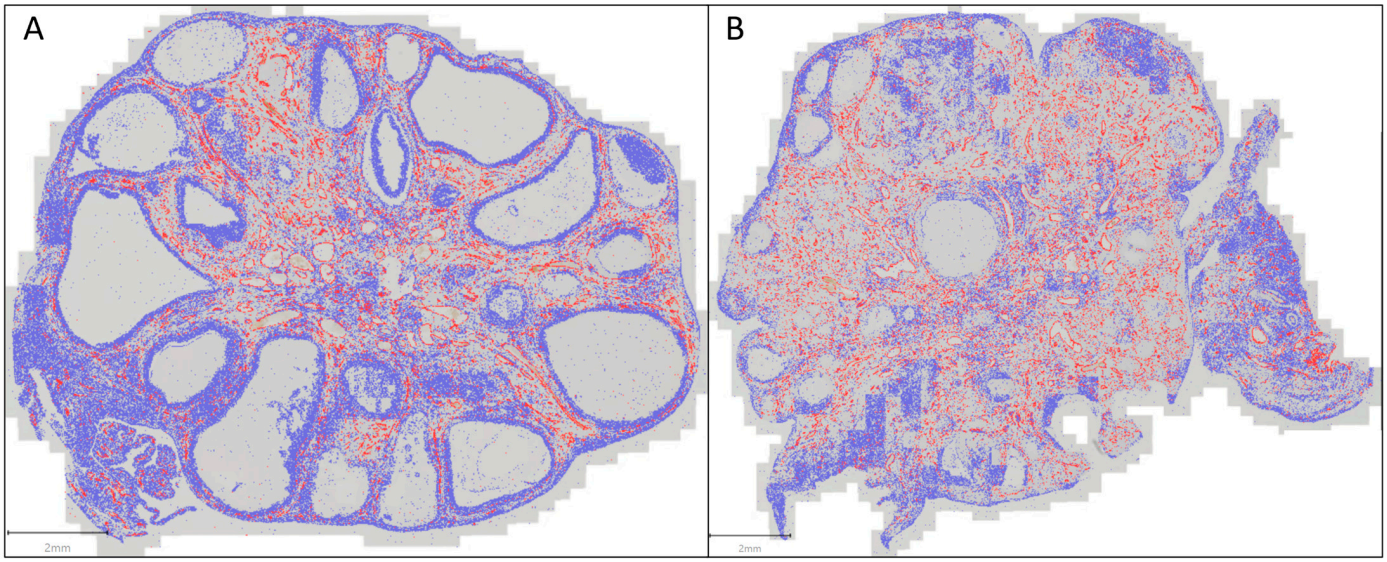

Figure 7. Representative results of the evaluation method for IHC using QuPath. Sections of ovarian tissue in the different cycle stages of oestrus (**A**) and metoestrus (**B**). Positive cells are coloured red, and negative cells are shown in blue. Scale bars = 2000 µm.

4. Discussion

4.1. Pregnancy and Haemostaseological Changes

Pregnancy is a major physiological challenge for all mammals. In healthy women, it represents a "hypercoagulable state", as several haemostaseological factors increase during pregnancy to lower the risk of complications during pregnancy and childbirth for both mother and child [22]. For women affected by VWD, a potentially increased risk in pregnancy loss is discussed controversially. However, there is consensus that these women are more likely to experience antepartum as well as postpartum bleeding [23]. Interestingly, no similar effects were observed in female dogs affected by VWD [24].

The PFA 200 test (Siemens Healthcare, Erlangen, Germany) is used to determine the ability of platelets to aggregate. It is well known that both commonly performed cartridges, collagen and epinephrine (C/EPI) and collagen and adenosine 5′-diphosphate (C/ADP), are suitable diagnostic tools for VWD [25]. Sensitivity is high but associated with low specificity, and there are many factors that may influence the result, such as blood group, platelet count, or haematocrit [25,26]. For quantitative types of VWD, PFA tests show a sensitivity of 89% in human patients [25]. Generally, the results of VWD patients do not reach the reference range of healthy individuals [26]. In our study, compared with the human reference range, we observed prolonged closure times (CT) for C/EPI in pigs of both genotypes. This excess might be due to the high sensitivity of the C/EPI cartridge for human blood. Nevertheless, significantly higher CT were seen in VWD T1 compared to WT pigs, except for LP, where the values of VWD T1 and WT converged.

Concerning C/ADP, a normal CT value in terms of human standards was measured in WT animals, while in VWD T1 animals, the values most of the time were above the human reference values. As also seen for C/EPI, graphs of C/ADP for WT and VWD T1 pigs constantly converged during the progression of pregnancy. From MP to LP, a decrease in CT was observed in VWD T1 as well as WT animals. In pregnant humans, shorter CTs compared to non-pregnant females are also seen [26]. The described changes in CT mean faster platelet activation and aggregation, which is advantageous for avoiding major bleeding during pregnancy and delivery. Even if VWD T1 animals did not achieve the PFA values of the WT animals, any approximation to healthy individuals can be regarded as an improvement. As the PFA system is very sensitive to changes in VWF plasma levels, the described changes might be directly related to a higher VWF:Act during

pregnancy [27]. Additionally, the PFA measurement and coagulation in general may be affected by oestrogen and/or progesterone levels, which increase during pregnancy [28].

The abnormal C/EPI results in WT pigs compared to the human reference might be a peculiarity of porcine platelets, which were reported to show ADP-mediated activation, while epinephrine-mediated activation seems to be impeded [29–31]. Regarding our results, we suppose that epinephrine-mediated activation indeed takes place in pigs but is not as efficacious as in humans.

The CT is primarily influenced by collagen, with EPI and ADP demonstrating comparatively weaker agonistic activity (ADP acting more strongly than EPI). Consequently, in both analyses, collagen is the main factor. A high similarity between porcine and human propolypeptide VWF was shown, but also the porcine platelet von Willebrand Antigen II was found to inhibit collagen-induced aggregation of platelets [32]. This might have the largest impact on CT in pigs. It could also be the reason why the CT does not shorten, as expected, but fluctuates. Possibly, the cartridges developed for the evaluation of human blood are not completely compatible with porcine blood. Nevertheless, it is possible to compare the pigs between the genotypes during the course of pregnancy. The VWD T1 animals show a generally prolonged CT compared to WT animals, with an improvement in CT towards the end of pregnancy.

An additional cartridge was utilised to assess drug inhibition of the ADP P2Y12 receptor, which plays a crucial role in primary haemostasis by triggering granule secretion, aggregation, and mediating ADP binding [33,34]. In our study on pigs, we observed significant differences between genotypes at each stage of pregnancy. WT animals consistently maintained levels within the reference range of <107 s. VWD T1 animals also generally fell within this range, with some exceptions in one specific pig. The correlation with the two standard cassettes is low. Considering all three cassettes combined, a VWD detection rate of 96% was reported [35]. Consequently, incorporating the P2Y cartridge appears to be a valuable addition to VWD diagnostics in pigs.

In summary, the PFA 200 system (Siemens Healthcare, Erlangen, Germany) seems to be suitable for usage in pig model studies. The changes known to occur in pregnant women were seen in a similar manner in pregnant pigs. As this method is used to predict possible pregnancy-related complications, like the risk of postpartum haemorrhage in women with VWD, it might also be regarded as an application possibility for pigs. It is important to note that the utilisation of the C/EPI cartridge, along with the interpretation of measurements for porcine samples, requires careful handling. We encountered challenges related to non-laboratory conditions in the barn, such as vibrations during sampling and transport, as well as fluctuating temperatures, which may interfere with the sensitive PFA measurement system. Furthermore, the hematocrit and the platelet count should be measured in future studies to ensure the significance of these PFA measures and to point out influencing factors. Additionally, larger numbers of samples would help to increase the power of the analysis. However, haemorrhage around parturition seems to be rare in sows, even in those affected by VWD. This might be due to the different types of placenta. While humans show a haemochorial placentation [36] with expulsion of decidual tissue after birth, leaving a "large wound" prone to bleeding, pigs show an epitheliochoral placentation [37] without loss of uterine tissue during birth. Porcine PFA reference values can be easily adjusted in pigs for the different cartridges. A rough porcine reference is provided in this study. However, to determine the precise reference ranges, larger studies with more animals would have to be performed.

We observed significant variations in the coagulation factors chromogen factor VIII (FVIII:C) and von Willebrand factor activity (VWF:GPIbM) throughout pregnancy. FVIII:C levels were upregulated during pregnancy, with significantly higher levels in LP for WT compared with VWD T1 animals, mirroring similar patterns of an increase during pregnancy seen in human VWD patients [38,39]. Regarding VWF:GPIbM, VWD T1 animals exhibited a similar, significant trend. Consistently lower mean values were observed during the cycle corresponding to the pre-implantation period. Mattoso et al. (2013) reported

identical observations in pregnant dogs with VWD, which also aligns with findings in humans [38–40]. The higher mean value of 134% for VWF:GPIbM in the WT pigs BI appears to be an outlier without relevance. BI represents the same time points as the oestrus-to-metoestrus cycle stages. In these cycle phases, the average is lower at only 105–122%.

The upregulation of FVIII:C may be attributed to elevated VWF:GPIbM levels. VWF serves to protect FVIII from degradation, and the half-life of VWF might increase during pregnancy. In addition, as VWF is expressed in endothelial cells and a placenta contains numerous newly developing vessels, VWF production can also be expected to be increased by this pathway. Consequently, if VWF is more stable and present in larger quantities, a larger amount of FVIII may be bound and thus protected. It is important to note that our methods only access plasmatic VWF, while the role of platelet VWF remains unclear. In the future, it might be very meaningful to also find a way to analyse the VWF Antigen (VWF:Ag) and the VWF collagen binding activity (VWF:CBA) in porcine blood and to combine the results with VWF:GPIbM to evaluate the ratio during pregnancy and the oestrus cycle. With the assays and detection systems used for this study, it was not possible to measure valid VWF:Ag and VWF:CBA values. In both VWD T1 and WT animals, repeatedly, no values could be measured.

Cyclical variation was not observed in VWF:GPIbM and FVIII:C levels. This is consistent with the results reviewed by Knol et al. (2012), where no difference for VWF and FVIII plasma levels was found in most studies [41].

Regarding VWF activity, we did not observe any fluctuations as described by Kadir et al. (1998), who found a peak in the luteal phase for WT and VWD T1 animals [38]. The plasma levels of VWF remained steady, which is consistent with findings in female dogs [7] and women with VWD [42]. The influence of steroid hormones on VWF:Act levels is widely discussed [27].

4.2. Multimers

Multimer analysis is commonly employed to determine the subtype of VWD and complement VWF antigen and activity measurements. In VWD T1 pigs, all multimers were present, but in lower amounts than in healthy individuals, showing a general loss instead of a specific loss of certain multimer groups. The total quantity of multimers always varies proportionally to the VWF level, and a reduced quantity combined with non-specific loss is typical for VWD T1 [19]. In addition, a loss of HMWM may be observed during pregnancy. In a study conducted by Drury et al. (2014), 17 out of 21 pregnant, healthy women lost HMWM and gained intermediate molecular weight multimers instead [6]. This trend aligns with the findings in VWD T1 animals of the present study. The haemostaseological demands of pregnancy lead to an increase in VWF production, and structural changes acquired during pregnancy may contribute to the prolonged survival time of VWF. The loss of HMWM and the corresponding gain of IMWM can be seen as an adaptive mechanism employed by the pregnant body to enhance VWF levels. As greater multimerisation usually results in enhanced collagen binding and platelet aggregation [3], our findings seem to contradict the shortening of CT observed in PFA measurements. This may suggest that IMWM indeed have sufficient ability to aggregate platelets. Additionally, the slight increase in VWF:GPIbM during pregnancy might cause the shortening of CT. It is important to note that clear results for WT animals were not yielded in this study. Overall, interpreting multimers using Hydragel-11 (SEBIA GmbH, Mainz, Germany) and Hydrasis 2 Scan Focusing (SEBIA GmbH, Mainz, Germany) for porcine samples was challenging. The antibody used for VWF multimer detection seems to be more sensitive to human VWF than to porcine VWF. Moreover, pigs might show lower VWF:Ag than humans in standardised human testing, and as a consequence, the intensity of VWF multimer bands would also be lower. Although human and porcine VWF exhibit similarities [43], the structural differences seem to be clear enough to impede complete specific binding to porcine VWF multimers, as evidenced by the blurred bands on the gel and less distinguishable peaks

in the densitometric diagrams. The second method of SDS agarose gel electrophoresis produced clearer bands, suggesting a better cross-species response with the human antibody used. Combining SDS agarose gel electrophoresis with the densitometric capabilities of the Hydrasis 2 Scan Focusing (SEBIA GmbH, Mainz, Germany) enables us to gain more reliable information for porcine VWF multimer analysis.

4.3. Oestrus Cycle and Haemostaseological Changes

The oestrus cycle and its haemostaseological changes are significant factors in pregnancy. In humans, the oestrus cycle comprises four distinct phases with various sub-phases: ovulation, early luteal phase, mid luteal phase, late luteal phase, menstruation/early follicular phase, and late follicular phase. Similarly, the oestrus cycle in pigs is commonly categorized into four phases: oestrus, metoestrus, dioestrus, and prooestrus. A comparison of hormone levels in the different phases reveals a correspondence between the pig and human cycles: ovulation in humans aligns with oestrus in pigs, the early luteal phase in humans corresponds to metoestrus in pigs, the mid/late luteal phase in humans corresponds to dioestrus in pigs, and the follicular phases in humans correspond with prooestrus in pigs [44–46].

In the immunohistochemistry results of WT pigs, we observed cyclic variation in VWF staining intensities in the ovary, with a peak in the metoestrus and the lowest level in the oestrus. This might be attributed to significant changes in the ovary of pigs, where multiple follicles (approximately 15 to 30) mature for ovulation [46], compared to only one ovulating follicle in women [47]. The development of more corpora haemorrhagica from the follicular cavities leads to larger injuries and the recruitment of VWF for proper coagulation. During prooestrus, the organism prepares for the following oestrus and might produce more VWF. In oestrus, the VWF produced in advance may be used up. In the following metoestrus, with the peak of VWF staining in IHC, it might be produced in retrospect to the bleeding of the corpora haemorrhagica to compensate for the loss. In pigs, these three phases are not followed by menstruation, as seen in humans. However, the associated blood vessels in the corpora haemorrhagica are also rebuilt in pigs. Hypercoagulability serves as a suitable mechanism across species to prepare for the next oestrus cycle. In VWF deficiencies, the absence of hypercoagulability during human menstruation poses a significant problem and increases the likelihood of impaired angiogenesis [42,48]. Our findings are in line with the hypothesis of James et al. (2007) concerning ovarian cysts reported in women affected by VWD [23]. They consider that bleeding in the follicular cavity around ovulation enhances the risk of ovarian cyst formation.

Plasma FVIII levels are indeed influenced by von Willebrand factor (VWF) to a certain extent. As with VWF:GPIbM, we did not notice significant changes during the cycle in WT or VWD T1. However, some trends were observed. The highest levels were measured in WT and VWD T1 animals during oestrus, followed by a decrease until the next dioestrus. In healthy women, similar results regarding plasma VWF levels were found, with higher values during ovulation and the luteal phase and lower values during menstruation and the early follicular phase [49]. Interestingly, Govorov et al. (2018) reported opposite results in women with VWD, with higher levels in the follicular phase and lower levels in the luteal phase [42]. Altogether, VWF:GPIbM and FVIII:C seem to be somewhat interdependent, but not to the full extent.

5. Conclusions

In summary, our animal model proves to be suitable for haemostaseological studies on VWD. To our knowledge, we report for the first time the course of parameters around VWF and FVIII in the pregnant VWD T1 pig compared with WT pigs. The changes detected are widely consistent with findings in healthy pregnant women and pregnant women with VWD. Pigs showing the same procoagulant changes will allow future research on bleeding complications at this vulnerable time. These changes can be interpreted as necessary adaptations to prepare the pregnant body for the challenge of childbirth and to better

overcome postpartum haemorrhage. The observed changes in VWF expression in ovarian tissue during the oestrus cycle indicate the influence of endothelial cells on newly formed blood vessels. Up-regulation of VWF during haemostaseologically demanding periods of pregnancy and the oestrus cycle appears to be present in pigs, similarly as in humans. The results of the highest procoagulant changes in late pregnancy and around ovulation give the opportunity to shed light on possible points of best interference or therapy in human VWD patients.

Nevertheless, to corroborate our findings, a larger number of animals should be sampled during pregnancy in the future. It would be interesting to also analyse samples after delivery to investigate the return of VWF- and FVIII-parameters to baseline levels.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/hemato5010006/s1, Table S1: Mean values for blood and immunohistochemistry; Table S2: Fractions of multimers during pregnancy.

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Conflicts of Interest: Stefanie Lehner, Carsten Detering, Mahnaz Ekhlasi-Hundrieser, Rabea Möller, Katharina Kaiser, and Mario von Depka Prondzinski are employees of the Werlhof-Institute MVZ GmbH. Otherwise, there are no financial or non-financial conflicts of interest to declare.

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