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Normothermic Machine-perfused Human Donor Livers Produce Functional Hemostatic Proteins

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Background. Normothermic machine perfusion (NMP) is used for the viability assessment of high-risk donor livers before transplantation. The production of hemostatic proteins is one of the major synthetic functions of the liver. The objective of this study was to measure the concentration and functionality of hemostatic proteins concentration in the NMP perfusate of human donor livers. **Methods.** Thirty-six livers that underwent NMP for viability assessment were included in this study. Perfusate samples taken during NMP (start, 150 min, and 300 min) were used for the measurement of antigen and activity levels of hemostatic proteins (factors II, VII, and X; fibrinogen; plasminogen; antithrombin; tissue plasminogen activator; von Willebrand factor; and proteins induced by vitamin K absence). The antigen levels were correlated with hepatocellular function according to previously proposed individual hepatocellular viability criteria: lactate clearance and perfusate pH. **Results.** Antigen levels of hemostatic proteins reached subphysiological levels in the NMP perfusate. Hemostatic proteins that were produced during NMP were at least partially active. All livers produced all hemostatic proteins tested within 150 min of NMP. Hemostatic protein concentrations did not significantly correlate with perfusate lactate and perfusate pH after 150 min of NMP. **Conclusions.** All livers produce functional hemostatic proteins during NMP. The generation of a functional hemostatic system in NMP perfusate confirms the need for adequate anticoagulation of the perfusate to avoid generation of (micro)thrombi that may harm the graft.

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INTRODUCTION

Liver transplantation is the only curative treatment option for end-stage liver disease. The general shortage of donor livers, together with increasing demands for liver transplantation, has generated a shortage in available donor livers and a consequent increase in waiting list mortality approaching 20% worldwide.[1] This donor liver shortage was the reason to start considering more high-risk donor livers for transplantation. These extended criteria donors (ECDs) include organs from donors aged >60 y with a higher body mass index or other relevant comorbidities. ECD livers are more prone to ischemia–reperfusion injury and primary nonfunction and have a higher prevalence of posttransplant nonanastomotic strictures.[2-7] The standard preservation method for donor livers is static cold preservation. However, ex situ machine perfusion for several hours is increasingly used as a preservation method to assess the function and improve organ quality of ECD livers. During normothermic machine perfusion (NMP), at 37 °C, the liver is metabolically active, which allows for assessment of hepatocellular and cholangiocellular function.[7-9] Hepatocellular viability markers are used to assess whether the liver is suitable for transplantation during NMP. These criteria are lactate clearance, the stabilization of perfusate pH, and the production of bile.[7,8] In addition, we use cholangiocellular viability criteria to decide whether a liver is suitable for transplantation.

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All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

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S.B.B. and T.L. contributed to the design of the study and wrote the article. S.B.B. and J.A. performed the experiments and analyzed the data. S.B.B. and B.L. contributed to the machine perfusion experiments under supervision of V.E.d.M. and R.J.P. Experiments and analyses in this study were supervised by T.L. All authors read and edited the final version of the article.

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The liver is the site of synthesis for many hemostatic proteins, including coagulation factors, inhibitors of coagulations, and proteins involved in clot breakdown.[9] Some hemostatic proteins, such as the platelet-adhesive protein von Willebrand factor (VWF) and coagulation factor VIII, are synthesized in endothelial cells rather than hepatocytes. A selected number of coagulation factors (VII, IX, X, II, protein C, and protein S) require vitamin K to establish a posttranslational modification that is required for adequate functioning of these proteins. In the absence or antagonism of vitamin K, these proteins remain in an inactive form, referred to as "proteins induced by vitamin K absence" (PIVKA). When livers produce hemostatic proteins during NMP, appropriate anticoagulant medication should be administrated to avoid clot formation.[10] In most centers, heparin is administered as anticoagulant medication during NMP. Importantly, antithrombin production by the liver is necessary for heparin to be functional as an anticoagulant. Although the liver is fully metabolically active during NMP, the production of hemostatic proteins and the coagulation status during NMP have not yet been studied extensively. Coagulation parameters are also not included in the viability assessment of the liver during NMP, although the production of (hemostatic) proteins is regarded as an important physiological hepatocellular function.[11] In addition, when the liver produces hemostatic proteins during perfusion, the perfusion fluid can start to resemble normal plasma, and undesired activation of the coagulation cascade may lead to the formation of fibrin (micro)thrombi.[12,13]

In this study, we measured the concentrations and activity of hemostatic proteins during NMP to better characterize the hemostatic composition of perfusion fluid. In addition to proteins synthesized by hepatocytes, we measured perfusate levels of VWF and tissue plasminogen activator (tPA) that are produced in endothelial cells rather than hepatocytes. Excessive endothelial release of VWF and tPA is regarded as an indicator of endothelial activation. We hypothesized that hemostatic protein concentration increases during NMP and that the antigen concentration correlates with established hepatocellular criteria.

MATERIALS AND METHODS

Study Design

A retrospective analysis was performed on human donor livers that underwent sequential hypothermic oxygenated machine perfusion (HMP) with University of Wisconsin machine perfusion solution and NMP with red blood cells. Livers were included from January 1, 2019, to December 1, 2021. All included livers were derived from circulatory death donors and were offered to our transplantation center for viability assessment during NMP. The donor characteristics were collected from Eurotransplant.

Organ Procurement and Machine Perfusion

The livers were procured by the local procurement teams and transported to the University Medical Center Groningen using static cold storage. On arrival at the University Medical Center Groningen, the portal vein and hepatic artery were cannulated, and the livers underwent sequential HMP and NMP, as described previously.[7,8]

For the machine perfusion, the Liver Assist (XVIVO, Gothenburg, Sweden) was used. After 1-h HMP, the perfusion solution was switched, and the NMP perfusate (**Table S1, SDC,** http://links.lww.com/TP/C783) was added to the circuit. The livers were rewarmed for 1 h to 37 °C and were perfused for 150 min at 37 °C. If the liver met the predefined viability criteria within 150 min of NMP, it was accepted for transplantation, and the liver was kept on NMP until the recipient hepatectomy was finished. If the liver did not meet the criteria, it was secondarily discarded after 150 min.[7,8]

Viability Assessment

Livers were assessed for hepatocellular and cholangiocellular function within 150 min of NMP.[7,8] The hepatocellular viability markers: arterial lactate <1.7 mmol/L, perfusate pH 7.35 to 7.45, and total bile production >10 mL.[11] The cholangiocellular markers are bile pH, bile bicarbonate, and bile glucose. The liver had to meet both the cholangiocellular and hepatocellular criteria to be accepted for transplantation.

Perfusate Composition and Sampling

All livers were perfused with red blood cells without the addition of plasma for 150 min in normothermic conditions (37 °C). The perfusate was heparinized before the start of machine perfusion with a bolus of 10 000 U unfractionated heparin (LEO pharma BV, Amsterdam, the Netherlands), followed by a 1000 U bolus every hour from the start of NMP until the end of the perfusion. Vitamin K was not added to the perfusion solution. The complete

TABLE 1.

Donor characteristics and preservation times

Variable	All (N = 36)
Gender	
Female	16 (56)
Male	20 (44)
Age (y)	66 (55–69)
Body mass index (kg/m^2)	26.0 (24.3–28.8)
Cause of death	
Anoxia	9 (25)
Cerebral vascular accident	17 (47)
Trauma	7 (19)
Other	3 (8)
Functional donor warm ischemia time (min)	30 (26–34)
Warm ischemia time (min)	16 (15–18)
Hepatectomy time (min)	39 (33–45)
Cold ischemia time (min)	271 (230–296)
ET-DRI[14]	2.84 (2.54–3.16)
DRI[15]	2.78 (2.50–3.19)

Continuous data are presented as median (IQR) and categorical data as number (percentage). The cold ischemic time was defined as the time between the start of the in situ cold flush of the liver in the donor and the start of HMP. The warm ischemic time was defined as the time from circulatory death in the donor until the start of the in situ cold flush of the liver in the donor. The functional warm ischemia time was defined as the time from the donor saturation of <80% or mean arterial pressure of <60 mm Hg to the start of in situ cold flush in the donor.[12] DRI, Donor Risk Index; ET-DRI, Eurotransplant Donor Risk Index; HMP, hypothermic machine perfusion; IQR, interquartile range.

perfusate composition is presented in **Table S1** (**SDC**, http://links.lww.com/TP/C783).

Perfusate samples were taken from the arterial port of the Liver Assist every 30 min during NMP. The perfusate was centrifuged at 1500*g* at 18 °C for 10 min. The perfusate was pipetted in 2 mL Eppendorf tubes and stored at –80 °C. Perfusate samples taken at the start of NMP and at the end of viability assessment (150 min) were used in this study. From those livers that were accepted for transplantation, a sample was taken and analyzed when the liver was still perfused, awaiting transplantation at 300 min of NMP (n = 15).

Enzyme-linked Immunosorbent Assay and Activity Measurements

We used commercially available enzyme-linked immunosorbent assays to determine the antigen concentration of hemostatic proteins and endothelial activation markers in the NMP perfusate. The following proteins were investigated: factors II, VII, and X; fibrinogen; plasminogen; antithrombin; tPA; VWF; and PIVKA-II (**Table S2**, **SDC**, http://links.lww.com/TP/C783).

Activity assays were performed to quantify factors VII, X, and II; antithrombin; and fibrinogen by using an automated coagulation analyzer (STA Compact Max 3, Stago, Breda, the Netherlands). Activity levels were indicated as percentage relative to normal pooled plasma (NPP). Specific activities were calculated by dividing activity by antigen values.

Definitions

The cold ischemic time was defined as the time between the start of the in situ cold flush of the liver in the donor and the start of HMP. The warm ischemic time was defined as the time from circulatory death in the donor until the start of the in situ cold flush of the liver in the donor. The functional warm ischemia time was defined as the time from the donor saturation <80% or mean arterial pressure <60 mm Hg to the start of in situ cold flush in the donor.[12]

Statistical Analysis

Continuous data in the study were presented as the median and interquartile range. Categorical data were presented as frequencies and percentages. A linear mixed-effects model was used to model the fixed effects of time on the concentration of the hemostatic proteins. The correlation between 2 variables was analyzed using the Pearson correlation test. The level of significance was set at a *P* value of <0.05. All

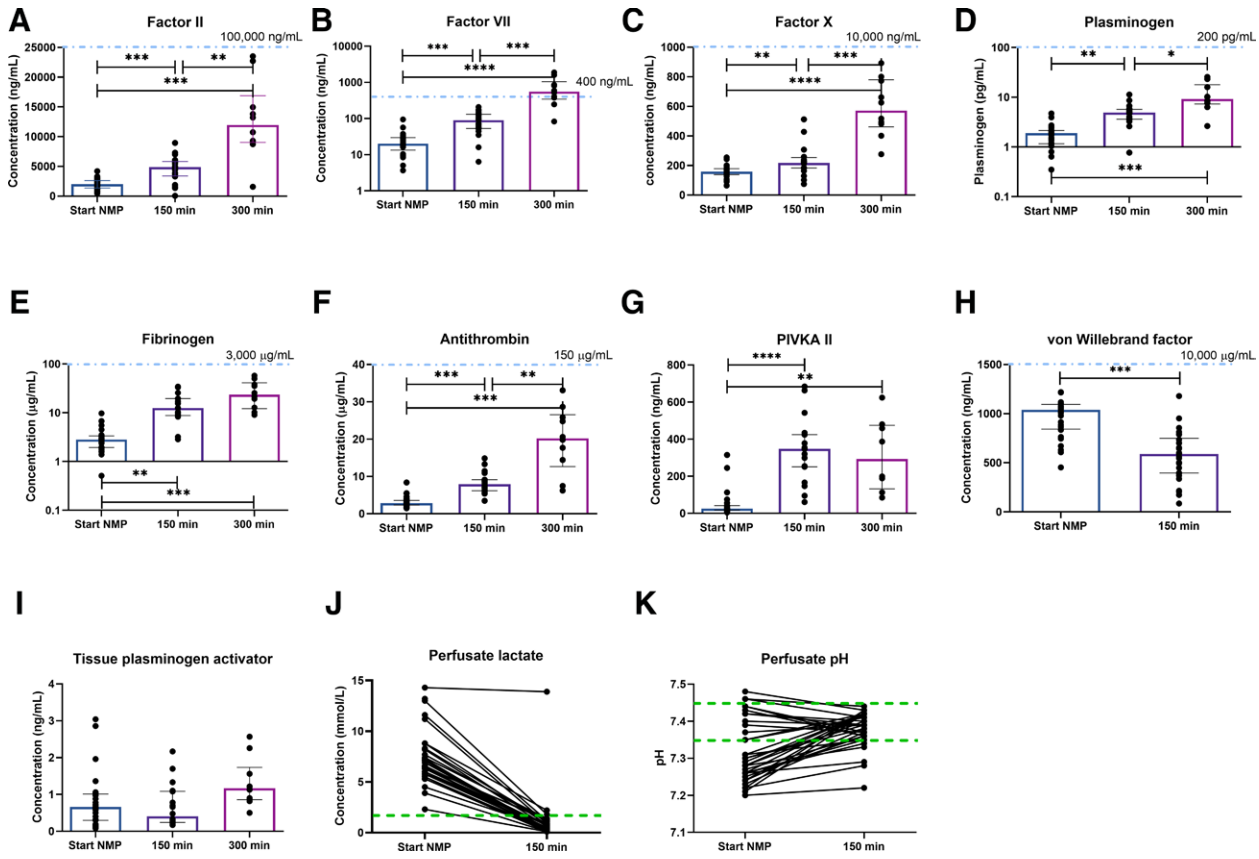

FIGURE 1. Concentration of proteins during NMP. A–E, Hemostatic proteins factor II, factor VII, and factor X. D, Plasminogen and antithrombin concentration. E, Fibrinogen concentration. F, Antithrombin concentration. G, Protein-induced vitamin K absence or antagonism-II levels. H and I, Endothelial activation markers VWF and tPA. J–L, The hepatocellular viability markers perfusate lactate levels and perfusate pH during the first 150 min of NMP. Normal plasma concentrations of the proteins are indicated by the blue dotted lines in (A–F) and (H). The normal plasma concentration of tPA is <10 ng/mL (I). Viability criteria of perfusate lactate and pH are visualized as green striped lines in (J) and (K). Some samples are missing because of hemolysis and incorrect processing of the samples. *A statistically significant difference $P < 0.05$. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. NMP, normothermic machine perfusion; tPA, tissue plasminogen activator; VWF, von Willebrand factor.

analyses were conducted using SPSS software version 23.0 for Windows (SPSS Inc, Chicago, IL) and Graphpad Prism version 9.0 (Graphpad Software, San Diego, CA).

TABLE 2.

Hepatocellular function and hemostatic protein concentration after 150 min of NMP

Variable	All (N = 36)
Perfusate pH	7.38 (7.36–7.41)
Perfusate lactate (mmol/L)	0.55 (0.27–1.10)
Factor II (ng/mL)	4893 (3377–5816)
Factor VII (ng/mL)	90 (53–130)
Factor X (ng/mL)	217 (183–253)
Fibrinogen (µg/mL)	12 (8–19)
von Willebrand factor (ng/mL)	559 (395–700)
Tissue plasminogen activator (ng/mL)	0.40 (0.24–1.08)
Plasminogen (pg/mL)	4.94 (3.63–4.94)
Antithrombin (µg/mL)	7.91 (6.18–9.11)

Data are presented as median (IQR).
IQR, interquartile range; NMP, normothermic machine perfusion.

RESULTS

Donor and Preservation Characteristics

We included 36 livers, of which 23 livers were successfully transplanted and 13 livers were secondarily discarded after viability assessment using NMP. Of the 23 transplanted livers, graft survival at 1 y was 100%. The median age of the donors was 66 y (55–69 y), and the donors had a median body mass index of 26.0 kg/m^2 (24.3–28.8). The livers had a median warm ischemia time of 16 min (15–18 min). From the start of cold flush to the start of HMP, the cold ischemia time was 271 min (246–296 min). Further donor and preservation characteristics are presented in Table 1.

Hemostatic Protein Concentration During NMP

Antigen levels of vitamin K–dependent coagulation factors II, VII, and X increased during NMP (Figure 1A–C). Although levels of factor X and II reached perfusate levels that were substantially lower than those found in plasma, factor VII levels reached physiological levels after 300 min of NMP (Figure 1B). Antigen levels of fibrinogen, antithrombin, and plasminogen concentrations

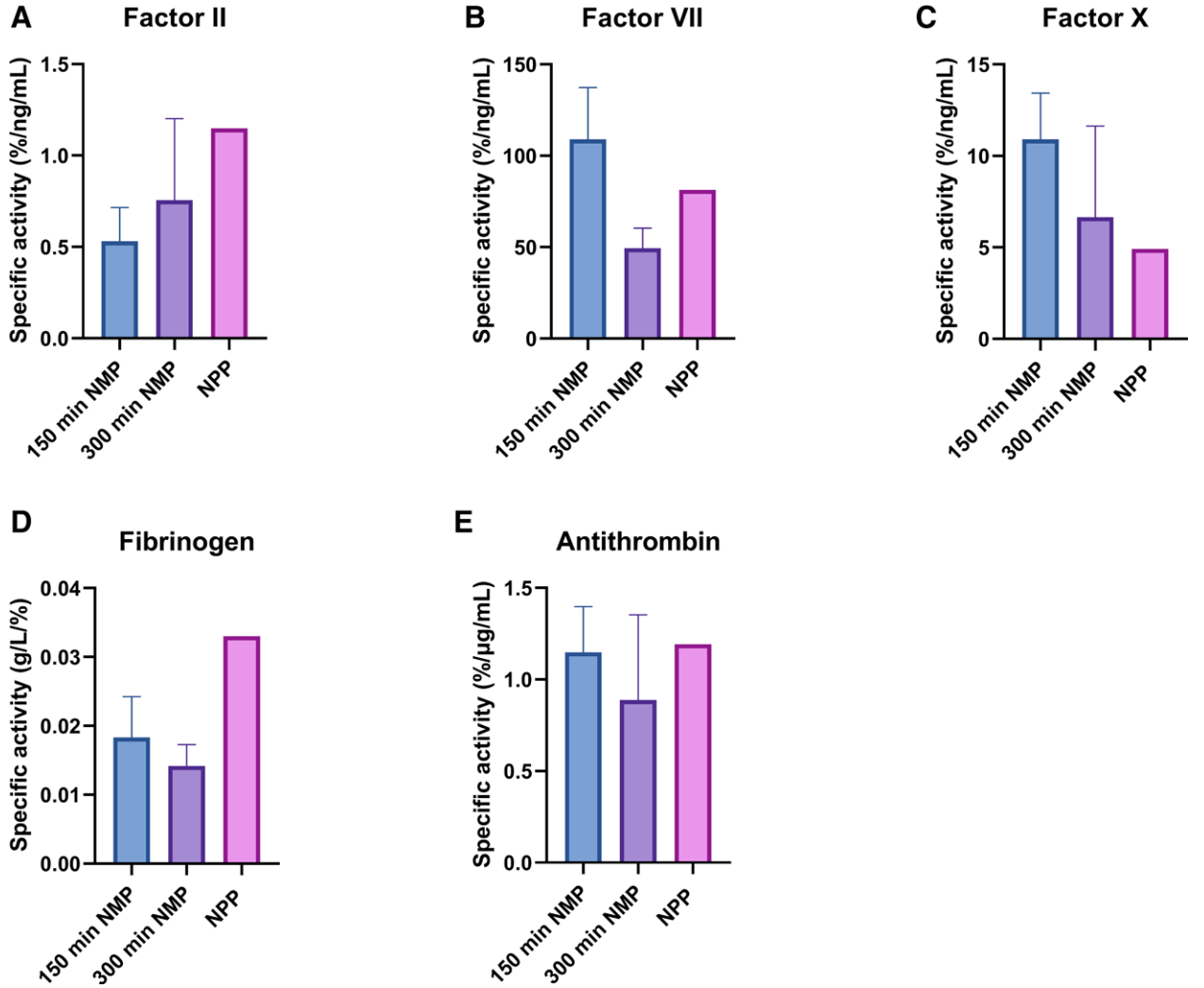

FIGURE 2. Specific activity of individual coagulation factor. This figure presents the specific activities of various coagulation factors, calculated by dividing activity by antigen values: (A) factor II (vitamin K dependent), (B) factor VII (vitamin K dependent), (C) factor X (vitamin K dependent), (D) fibrinogen, and (E) antithrombin. NMP, normothermic machine perfusion; NPP, normal pooled plasma.

significantly increased over time to subphysiological levels (Figure 1D–F). The median hemostatic protein concentrations after 150 mins of NMP are presented in Table 2.

During the first 150 min of NMP, PIVKA-II concentration increased, indicating an increased production of functionally defective prothrombin (Figure 1G). PIVKA-II concentration decreased during more prolonged NMP perfusion compared with the end of the viability assessment.

Specific Activity of Individual Coagulation Factors

We measured activity levels of factors VII, X, and II and fibrinogen in a subset of 15 perfusates, including perfusate from livers that were transplanted. We first validated that activity assays can be reliably performed in the presence of high concentrations of heparin (**Figure S1**, **SDC**, http://links.lww.com/TP/C783). The specific activity of factors VII, and X and antithrombin was comparable with or even somewhat higher than the specific activities measured in NPP (Figure 2A–C, E). The specific activity of factor II and fibrinogen was notably lower in perfusates than in normal plasma, although the specific activity of factor II approached normal levels after 300 min of perfusion (Figure 2D).

Endothelial Activation Markers

A decrease in VWF and tPA antigen levels was observed during the first 150 min of NMP (Figure 1H and I). However, tPA concentration significantly increased between 150 and 300 min of NMP. Both endothelial activation markers remained below physiological limits (VWF: 8–12 µg/L and tPA: <10 ng/mL).

Individual Hepatocellular Viability Criteria and Hemostatic Protein Concentration

Of the 36 livers, 28 met the hepatocellular criteria, but 5 of these did not meet the lactate clearance (<1.7 mmol/L) and perfusate pH (7.35–7.45) criteria. All livers produced >10 mL of bile and therefore fulfilled this hepatocellular criterion. The correlations between perfusate lactate concentration and pH and concentration of hemostatic protein after 150 min of NMP were analyzed. None of the hemostatic protein concentrations correlated significantly with the lactate concentration at 150 min (Figure 3), with the exception of VWF ($r^2 = 0.33$, $P = 0.008$; Figure 3G). We did not observe any significant correlations between perfusate pH and hemostatic protein concentration (Figure 4).

One of the livers that were secondarily discarded did not clear any lactate. The perfusate lactate level after 150 min of NMP was 13.90 mmol/L compared with a median of 0.55 mmol/L (0.28–1.10). This liver showed low concentrations of factors II and VII and very low concentrations of plasminogen compared with the other livers.

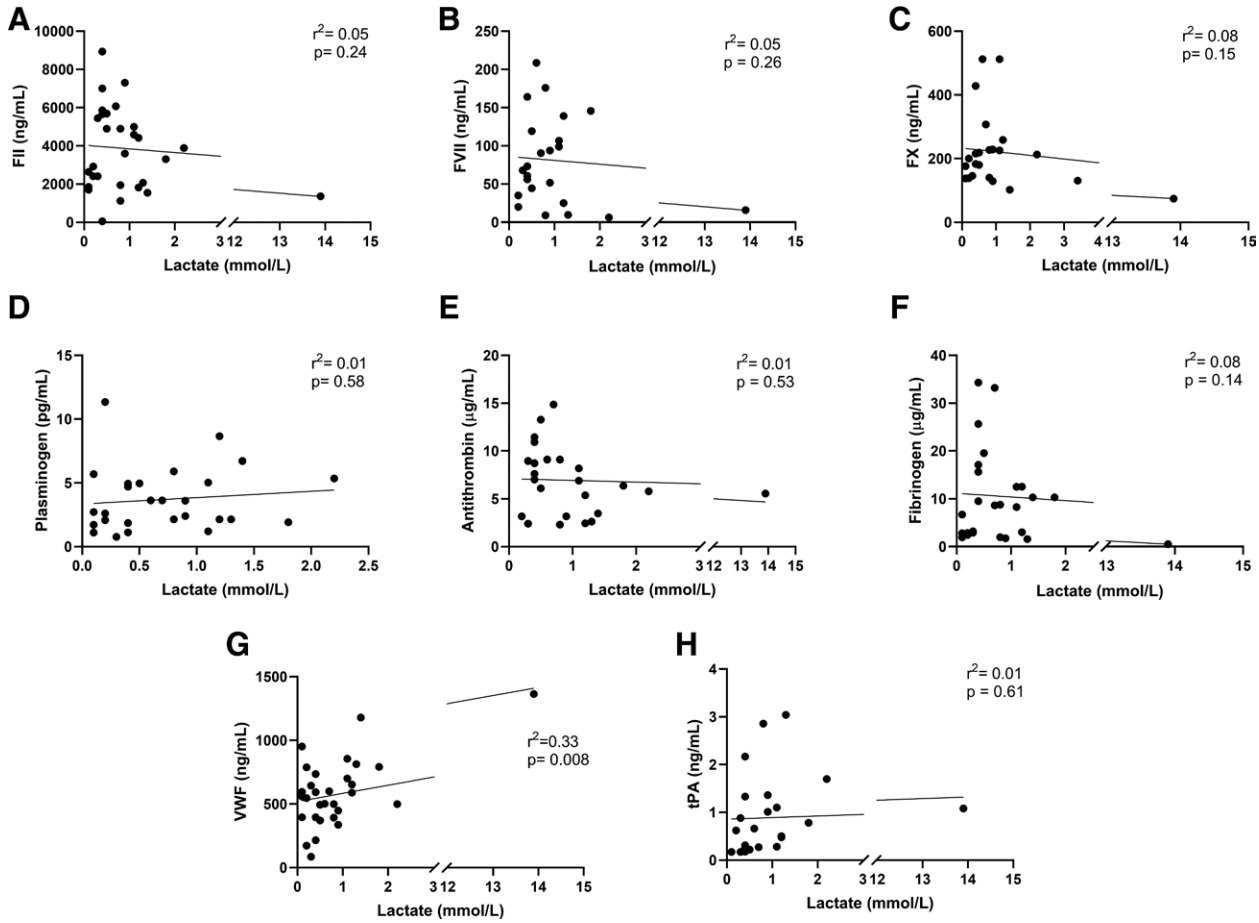

FIGURE 3. Correlations between perfusate lactate and hemostatic protein concentration after 150 min of NMP. A–C, Hemostatic proteins factor II, factor VII, and factor X. D, Plasminogen. E, Antithrombin. E, Fibrinogen. G, VWF. H, tPA. NMP, normothermic machine perfusion; tPA, tissue plasminogen activator; VWF, von Willebrand factor.

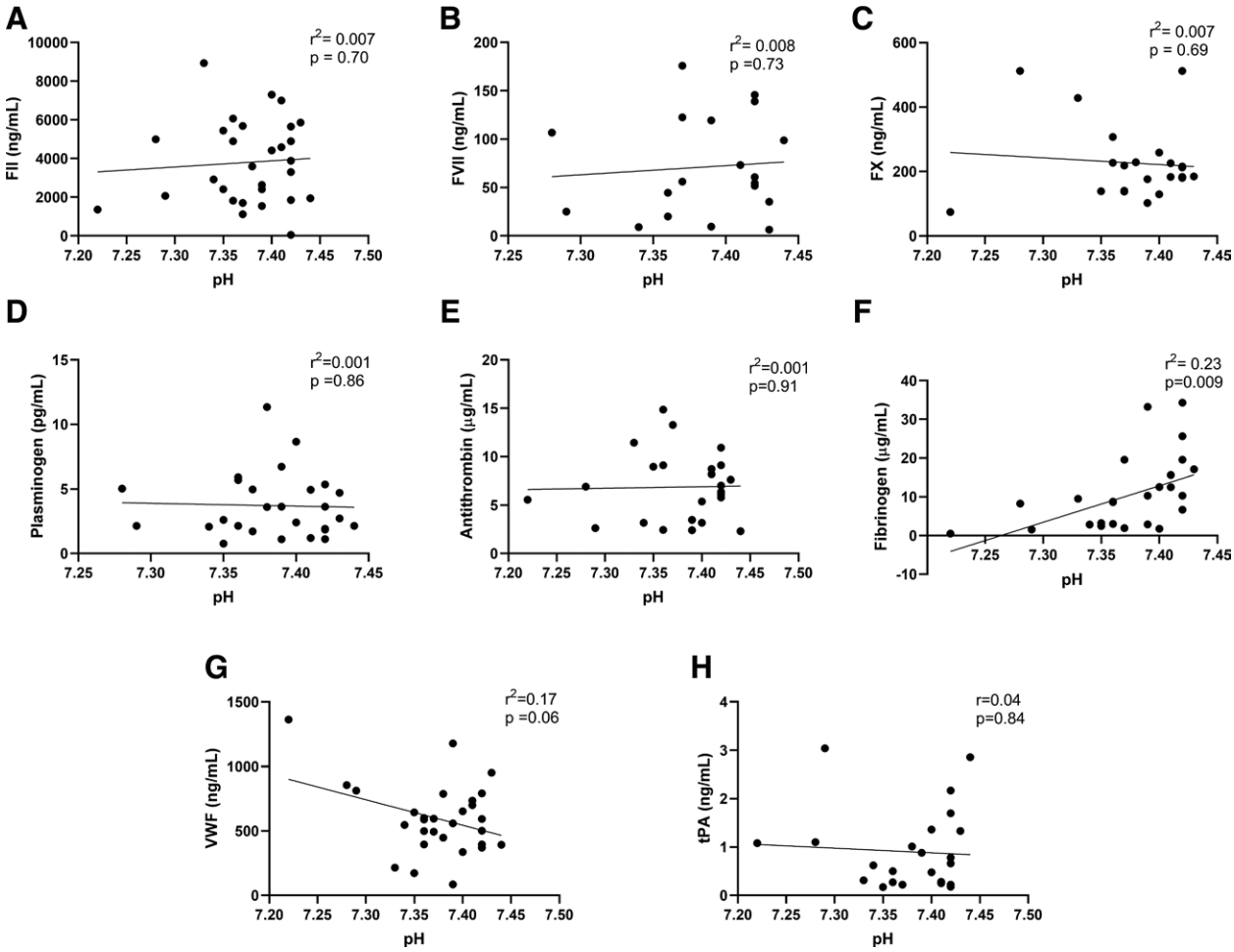

FIGURE 4. Correlations between perfusate pH and hemostatic protein concentration after 150 min of NMP. A–C, Hemostatic proteins factor II, factor VII, and factor X. D, Plasminogen. E, Antithrombin. F, Fibrinogen. G, VWF. H, tPA. NMP, normothermic machine perfusion; tPA, tissue plasminogen activator; VWF, von Willebrand factor.

DISCUSSION

Here we demonstrated that human livers preserved by NMP produce relevant quantities of functional hemostatic proteins within 150 min of NMP. These data confirm and extend previous findings by us[10,16] and others.[17] Whereas production of hemostatic proteins during human NMP was previously assessed by antigen assays, we now demonstrate that the hemostatic proteins produced are functional, although the specific activity of fibrinogen was notably lower in perfusates than in normal plasma. The generation of a functional hemostatic system during NMP confirms the need for adequate anticoagulation of the perfusate to avoid generation of (micro)thrombi that may harm the graft.[10]

The production of hemostatic proteins was generally not correlated to individual viability criteria. These results are in line with data by Gilbo et al[17] and somewhat in contrast to our earlier pilot study in which we showed correlations of hemostatic proteins with bile production and lactate concentrations.[16] According to our findings, there was a statistically significant positive correlation between VWF and absolute lactate concentration after 150 min of NMP in the context of transplantation. This observation may be attributed to the impaired ability of damaged livers to clear lactate, leading to the release of more VWF into the perfusion solution.

We demonstrate for the first time that hemostatic proteins produced during human NMP are functionally active, which is in line with previous data in pig NMP.[16] Activity assays are performed by mixing a sample with plasma deficient in the analyte of interest and are based on clot formation, which is potentially affected by the presence of large quantities of heparin in the sample. However, the sample is diluted substantially in factor-deficient plasma, and we validated that the composition of the perfusate did not interfere with the assays (**Figure S1, SDC**, http://links.lww.com/TP/C783). The specific activity of the vitamin K–dependent proteins factor VII and factor X was comparable with that observed in NPP, but the specific activity of FII was decreased, particularly after 150 min of perfusion. The levels of PIVKA-II increased over time, suggesting a relative vitamin K deficiency in the circuit, which may result in a decline in specific activity during longer perfusion times. Notably, the specific activity of fibrinogen was clearly decreased in perfusates compared with normal plasma at both time points. The apparent synthesis of functionally defective fibrinogen may relate to alterations in posttranslational modifications. In particular, hypersialyation is known to impair fibrinogen polymerization, as has, for example, been demonstrated in neonates and patients with cirrhosis.[18,19] Additional studies will be required to clarify the nature of this functional fibrinogen defect.

We demonstrated a lack of endothelial activation during NMP by low levels of the endothelial-derived proteins VWF and tPA in the perfusate. The absence of relevant levels of VWF in the perfusate is beneficial because VWF is a protein that may interact with circulating cells, including platelets and white blood cells.[20] However, the absence of large amounts of tPA may not be desired. We have previously demonstrated lysis of preexisting fibrin clots during NMP by generation of plasmin during NMP.[16,21] The generation of plasmin is likely accomplished by production of plasminogen and a plasminogen activator (such as tPA) by the graft. Removal of preexisting microthrombi might be beneficial for the graft because this would improve perfusion of the microvasculature. It may therefore be that in livers releasing low amounts of tPA, adding exogenous tPA may benefit.

The activity of coagulation factors in the perfusate reaches detectable levels (>1% of normal) within 150 min of perfusion. Therefore, it is conceivable that during prolonged perfusion, (supra)physiological levels of coagulation proteins will be produced, at which time the perfusion fluid can start to resemble normal plasma. Indeed, Eshmuminov et al[22] demonstrated substantial production of selected hemostatic proteins during a 4- to 7-d NMP procedure. Therefore, it will be of the utmost importance to ensure adequate anticoagulation during these prolonged perfusions to avoid micro- or macrovascular clot formation, which may compromise function when these livers are transplanted after prolonged perfusion.[23]

Different types of devices are used for NMP, for example, the OrganOx Metra device, the Liver Assist device, or custom-made devices.[8,11,22] Also, many different NMP protocols and differences in composition and amount of perfusion solution are currently being used. These differences make it difficult to directly compare the absolute concentrations of hemostatic proteins produced between studies. Also, because the liver not only produces but also clears hemostatic proteins, the levels measured in the perfusate are per definition a result of production and clearance. It is thus yet unclear whether livers that yield low levels of hemostatic proteins in their perfusates are low producers or perhaps fast clearers. The half-life of the measured coagulation factors varies widely, ranging from minutes to days, and the timing of the perfusate samples collected may not have captured the full spectrum of changes in these factors over time. In addition, the power of our study may have been limited because of the small sample size, which limits the validity of subgroup analyses. As a result, we may not have been able to detect smaller differences between groups or to fully explore the potential impact of other variables, including variations in vitamin K status between different livers on coagulation status.

In conclusion, here we demonstrate that human livers produce substantial amounts of functional hemostatic proteins during 150 to 300 min of perfusion, although the functionality of some of these proteins is partly compromised. Our results stress the need for adequate anticoagulation during short- and long-term NMP.

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