ABSTRACT

LAUREN F. KELLY
Fibrin deposition in the malaria-infected placenta: a disruption in the balance between coagulation and fibrinolysis
(Under the direction of DR. JULIE M. MOORE)

Placental malaria is characterized by the accumulation of *Plasmodium falciparum*-infected red blood cells in the human placenta. This leads to maternal anemia and poor fetal outcome including low birth weight and possibly perinatal death. Primigravidae are more susceptible to the devastating consequences of malaria during pregnancy, suggesting the presence of gravidity-dependent immunological resistance among malaria-exposed women. Common features of placental malaria include monocyte infiltration to the maternal blood space and excessive fibrin deposition, an end-product of blood coagulation. The immune factors involved in the recruitment and activation of maternal immune cells to the placenta and their role in local hyper-coagulation are poorly understood. It is hypothesized that syncytiotrophoblasts, fetal cells facing the maternal blood circulation, secrete these cell mediators as well as pro-coagulants or anti-fibrinolytics, resulting in the influx of maternal immune cells and clotting/fibrin accumulation. To address this hypothesis, placental plasma samples were collected in malaria-endemic western Kenya and stratified according to placental malaria status and gravidity. The levels of plasminogen activator inhibitor-1 (PAI-1), which inhibits fibrin degradation, and Tissue Factor Pathway Inhibitor (TFPI), which suppresses clotting, were evaluated by ELISA. Additionally, protein was isolated from placental tissue of these women and semi-quantitative estimation of fibrin and PAI-1 protein by western blot was initiated. Ultimately, elucidation of the coagulation factors expressed during placental malaria, and how they are regulated, will contribute to understanding the immunopathogenic mechanisms occurring at the materno-fetal interface of the malaria-infected placenta.

INDEX WORDS: Malaria, Pregnancy, Coagulation, Fibrinolysis

FIBRIN DEPOSITION IN THE MALARIA-INFECTED PLACENTA: A DISRUPTION IN THE BALANCE BETWEEN COAGULATION AND FIBRINOLYSIS

by

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And for loving this great adventure:

> You do not have to be good.
> You do not have to walk on your knees
> for a hundred miles through the desert repenting.
> You only have to let the soft animal of your body
> love what it loves.
>
> —Mary Oliver

from *Dream Work* published by Atlantic Monthly Press © Mary Oliver

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CHAPTER 1
INTRODUCTION

Malaria continues to be a global health problem, widespread in Sub-Saharan Africa and in the tropical and subtropical regions of Latin America, the Indian subcontinent, and Southeast Asia. The warm climates of these regions provide ideal breeding conditions for the malarial vector and transmitter, the female *Anopheles* mosquito [1]. Accounting for 300-500 million clinical cases and 1-3 million deaths annually, the protozoan *Plasmodium falciparum* is responsible for the bulk of severe disease and death. Perpetuating a complex lifecycle comprised of asexual and sexual stages, this parasite is transmitted by the bites of mosquitoes to humans where it multiplies in liver cells, and then infects red blood cells [2].

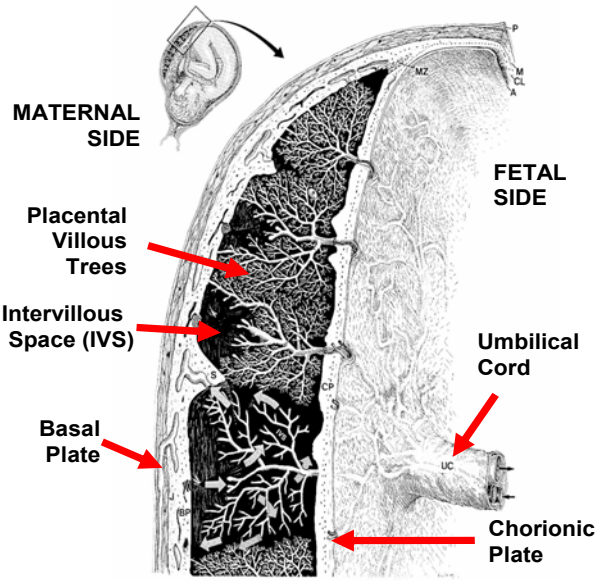
In highly malaria-endemic areas, children under the age of five years are at the greatest risk for this parasitic infection because they lack the acquired immunity protecting most adults. Furthermore, women in endemic areas are highly susceptible to malaria during first and second pregnancies, despite immunity acquired after years of exposure [3]. More than 50 million women are exposed to the risk of malaria in pregnancy every year and these infections result in substantial maternal and especially fetal morbidity, causing 75,000-200,000 infant deaths every year. The disease causes maternal anemia and leads to poor fetal outcome, primarily low birth weight (LBW) due to premature delivery or intrauterine growth restriction (IUGR) [4].

Understanding of the biological basis for susceptibility to malaria in pregnancy was recently advanced by the finding that red blood cells infected with *P. falciparum* (iRBCs) sequester in the placenta. These iRBCs adhere to specific molecules in the placenta, namely

chondroitin sulphate A (CSA), permitting evasion of immune responses acquired before the first pregnancy [2]. Additionally, the parasite interacts with host cells by expressing a surface protein when infecting erythrocytes called *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). Encoded by members of the highly polymorphic *var* gene family, many variants of PfEMP1 exist. To our knowledge, VAR2CSA is the only gene product mediating cytoadherence of iRBCs to host cells by CSA in the placenta and thus, contributes to parasite sequestration in this organ crucial for fetal development [5].

The strong inverse relationship between gravidity and susceptibility to malaria in pregnancy suggests that acquired protection from this disease is mediated by an immune response directed against a target that is pregnancy-specific and highly immunogenic. In malaria-endemic areas, it is thought that women of multiple pregnancies, or multigravidae, develop robust antibody responses, capable of inhibiting parasite adhesion to CSA and thus controlling local parasitemia, as well as T cell memory [6]. To better understand the gravidity-dependent immunological resistance among malaria-exposed women, we are interested in the orchestration of molecular and cellular events mediating pathology at the materno-fetal interface of the placenta.

Parasite sequestration in the intervillous space (IVS) of the placenta is the hallmark of malaria during pregnancy, commonly referred to as placental malaria (PM) [2, 4-6]. A fetal organ attached to the uterine wall during pregnancy, the placenta allows for the delivery of oxygen and nutrients to, and the expulsion of wastes from, the developing fetus (Figure 1). The placenta is comprised of villous trees lined with fetal-derived syncytiotrophoblast (ST)—polynucleated epithelial cells in direct contact with maternal blood of the IVS [7]. Importantly, the role of fetal ST in modulating the maternal immune response of the placental



Benirschke and Kaufmann. *Pathology of the human placenta* (2000).

microenvironment during malaria infection is not yet well understood. Whether immunopathogenic and immunoprotective mechanisms are brought about by maternal or fetal cells is currently under investigation.

In comparison to uninfected placentae, histological sections of placentae from malarious women clearly reveal the accumulation of iRBCs, increased maternal immune cell infiltrate (especially monocytes), excessive fibrin deposition, tl **Figure 1.** Cross-sectional illustration of the materno-fetal interface of the placenta. e, and the buildup of hemozoin (Figure 2). Also called malaria pigment, hemozoin is the byproduct of hemoglobin digestion by the parasite and is minimally present within fibrin during active-acute infection and substantially present in fibrin or in maternal leukocytes during active-chronic infection [4]. It is believed that collectively, these factors contribute to impaired uteroplacental blood flow and placental tissue necrosis and ultimately, the clinical consequences of PM for mother and fetus. However, a direct and comprehensive investigation of the role of excessive coagulation and fibrin persistence in the pathogenesis of PM has not been attempted.

Cytokine and chemokine changes in placental plasma during malaria infection have been associated with poor pregnancy outcomes in humans. Specifically, pro-inflammatory cytokines including tumor necrosis factor (TNF) and interferon-γ (IFNγ) were significantly elevated when a LBW, rather than normal weight, infant was delivered in the context of PM [8]. Enhancing the

inflammatory placental environment, chemokine expression has been localized to the placenta and positively correlated with placental monocyte infiltration [9]. While proinflammatory responses are crucial to the clearance of iRBCs and protection against PM, they also play a major role in pathophysiology and contribute to morbidity.

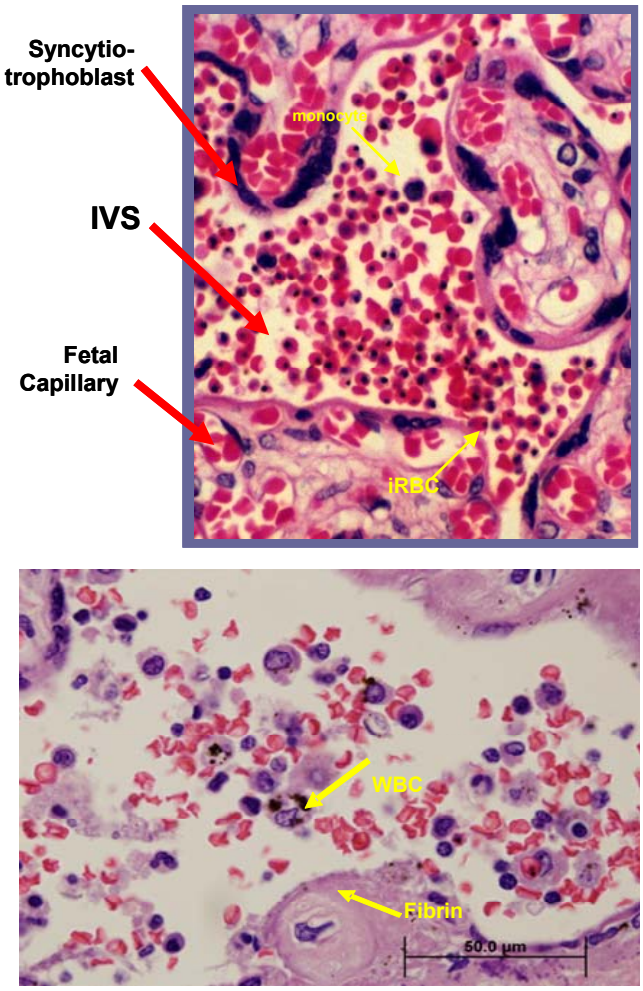


Figure 2. Histological sections of *P. falciparum*-infected placentae of two different women from malaria-endemic Western Kenya illustrating distinct features of placental malaria. (IVS=intervillous space; iRBC=infected red blood cell; Hz-laden WBC=hemozoin-laden white blood cell.)

In general, the complications of malaria due to inflammation are further compounded by *P. falciparum* infection activating the blood coagulation system [10]. Tissue factor (TF) serves as the initiator of the extrinsic pathway for the clotting cascade and it is broadly accepted that TNF is involved in the upregulation of TF expression. It has been demonstrated that *P. falciparum*-iRBCs induce TF expression in microvascular endothelial cells *in vitro*, and support the assembly of multimolecular coagulation complexes [11]. Recently, the link between inflammation and coagulation has been explored in the context of malaria pathogenesis. It is proposed that a *coagulation-inflammation cycle* is mounted and sustained at sites of parasite sequestration [12], such as the placenta. The mechanisms triggering and propagating coagulation activation in malaria infection remain vague.

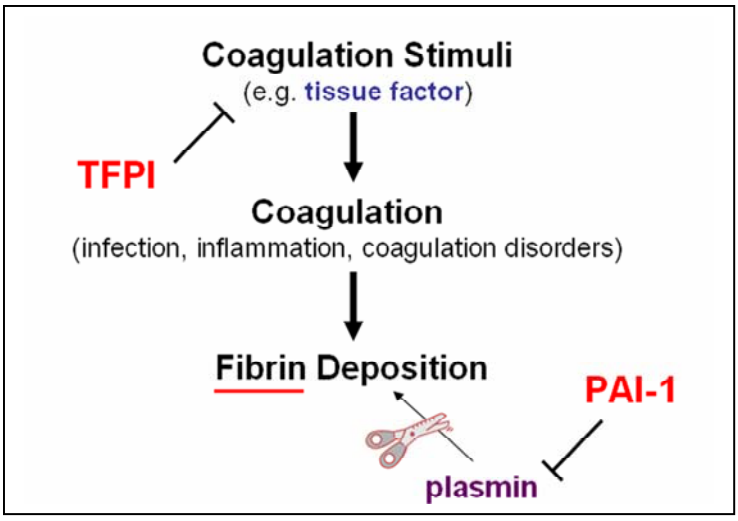


Figure 3. Simple model highlighting players of interest regulating the coagulation cascade and degradation of fibrin.

In this study, we explore cell mediators involved in coagulation and fibrinolysis that may contribute to the influx of maternal immune cells and clotting/fibrin accumulation in the malaria-infected placenta. Plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor found in plasma that inhibits fibrinolysis by inactivating urokinase-type and tissue-type plasminogen activator (uPA and tPA, respectively). Usually, uPA and tPA act in concert to convert plasminogen to plasmin which in turn proteolyses fibrin; therefore, without sufficient plasmin, appropriate fibrin degradation does not occur (Figure 3). In this scenario, fibrin clots persist. In preeclamptic women, an increase in PAI-1 levels during maternal and fetal perfusion of the human placenta was observed [13]. Additionally, placentae from pregnant women with preeclampsia and/or fetal IUGR demonstrated higher PAI-1 expression, compared to normally pregnant women, and this expression was localized by immunohistochemical analysis to the ST [14]. Similar to preeclamptic women, those infected with malaria during pregnancy experience hemodynamic stress and hypoxia, therefore, these data suggest PAI-1 dysregulation could be a common upstream event giving rise to comparable pathologic outcomes in different conditions.

Furthermore, a recent study assessing cytokine imbalance in the placentae of women with preeclamptic and normal pregnancies elucidated relationships among blood coagulation related factors in placental tissue and peripheral blood. Notably, these findings demonstrated that

trophoblast cell cultures with high levels of TNF exhibited increased expression and secretion of TF and PAI-1 and decreased expression and secretion of TF pathway inhibitor (TFPI) [15]. As proinflammatory cytokines and players in the blood coagulation cascade, PAI-1 and TFPI are particularly interesting in the study of the immunopathogenesis of malaria during pregnancy.

Fibrin deposition in the IVS of malaria-infected placentae is both excessive and common, possibly contributing to disease pathogenesis. Curious about molecular players involved in the dysregulation of coagulation and/or fibrinolysis, we asked if PAI-1 is found in placental plasma collected from women in malaria-endemic western Kenya and attempted to quantify PAI-1 and fibrin in placental tissue isolates from these women by Western blotting. To further evaluate the disruption in coagulation and fibrinolysis, levels of TFPI were assessed in placental plasma. Lastly, whether ST could produce PAI-1 in response to malaria infection by stimulation with hemozoin was investigated.

CHAPTER 2
MATERIALS AND METHODS

Collection of human placental plasma in Kenya

Placentae were obtained from mothers who delivered at the Nyanza Provincial General Hospital in Kisumu, and Siaya District Hospital, areas in western Kenya where malaria is holoendemic and human immunodeficiency virus (HIV) is epidemic. Written, informed consent was obtained. Mothers were screened for malaria parasites on a Giemsa-stained thick blood smear and for HIV-1 and HIV-2 antibodies. This study was approved by the Kenya Medical Research Institute Ethical Review Committee and the Institutional Review Board (IRB) of the University of Georgia, Athens, Georgia.

Placentae were collected immediately upon expulsion into sterile containers with anticoagulant (heparin), carefully examined, and immediately prepared for sample processing if intact and undamaged. Placental plasma was collected by the prick method as described previously [16]. Briefly, this method of collection accesses placental intervillous spaces (IVS) through the chorionic plate. Within approximately 2 min of placenta expulsion, the placenta was carefully placed with the chorionic plate (fetal side) down on a raised sterile wire mesh stand. This orientation promotes blood accumulation and intervillous blood (IVB) space accessibility.

A large-bore, 14-gauge needle attached to a syringe was directed approximately 0.5 cm deep through the wire mesh into the IVS, denoted as dark-purple regions, while puncture of the surrounding fetal vessels—on the surface of the chorionic plate—was carefully avoided. The syringe was gently pulled to create a vacuum initiating blood flow, followed by withdrawal to

allow for dripping blood, about 1 ml, to be collected into microcentrifuge tubes containing 25 µl of a 1:4 heparin (stock concentration, 1,000 units/ml) dilution. Tubes were centrifuged, plasma was extracted and transferred to cryostorage tubes, and stored at -85°C until used for ELISA.

Samples were stratified according to the mother's infection status (placental malaria-positive only, HIV-positive only, doubly positive, or uninfected) and gravidity (primigravidae, secundigravidae, and multigravidae).

Isolation of human primary trophoblasts from placentae

Term placentae were obtained from women delivering by elective cesarean section at Athens Regional Medical Center, Athens, Georgia. Written, informed consent was obtained. The study design and involvement of human subjects were reviewed and approved by the University of Georgia and Athens Regional Medical Center institutional review boards. These placentae were both malaria and HIV negative as determined from information obtained from the study subjects' questionnaires.

Primary placental cytotrophoblast cells were isolated from the obtained fresh human placentae (used within 30 min to 2 h post removal). The chorionic villous tissue was removed from the placenta using a sharp sterile scalpel. The tissue was then minced and subjected to four rounds of 30 min enzymatic digestion using trypsin and DNase I (Sigma, St. Louis, MO). The released cells were then washed and layered on a 5—75% freshly prepared percoll (Sigma, St. Louis, MO) gradient to separate the cells and obtain trophoblast preparations.

In order to obtain a pure trophoblast preparation, these cells were incubated with mouse-anti-human CD9 antibody (Pharmingen, San Diego, CA) (which binds all cells expect the trophoblasts) and anti-major histocompatibility complex (MHC) class 1 antibodies (W6/32,

ATCC; antibodies purified from murine ascites). The unbound cells were then immunopurified by negative selection over a column containing glass beads coated with goat anti-mouse polyclonal antibodies. The immunopurified cells were cryopreserved in liquid nitrogen until use or were placed in culture immediately.

Cell culture of human primary trophoblast and hemozoin stimulation

Primary cytotrophoblasts were cultured, induced to form a syncytium, and stimulated with hemozoin as described previously [17]. Briefly, cells were thawed quickly in a 37°C water bath and then washed in Iscove's Modified Dulbecco's medium (IMDM; Mediatech, Inc., makers of cellgro®, Manassas, VA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 10 ng/ml recombinant human epidermal growth factor (rhEGF; Sigma, St. Louis, MO; used to induce syncytium formation), 100 units/ml of penicillin and 100 μg/ml streptomycin (Gibco, Billings, MT) (complete IMDM). Cells were then plated in 60 mm and 100 mm tissue culture plates at 1 x 10^6 cells/ml and placed in a 37°C incubator with an atmosphere of 5% CO_2. After 4 h in culture the non-adherent cells were washed off and the plates filled with complete IMDM medium. The medium was changed every day for 5 days after which the cells were grown in EGF-free medium.

The ST began to form on day 6 after plating and experiments were performed on culture day 10. The purity of the ST was determined by staining the ST with anti-vimentin monoclonal antibodies (clone # V9; Sigma, St. Louis, MO) to ascertain that there was no contamination with fibroblasts or other vimentin-positive cells. Preparations that had more than 1% fibroblast contamination were discarded.

Briefly, crude hemozoin (Hz) was isolated from *in vitro* culture of *Plasmodium falciparum*-infected red blood cells (strain FCR3 selected for ST binding). A 10% parasitemia culture was lysed with 1% saponin for 10 min. The parasites were washed seven times in phosphate-buffered saline (PBS) by spinning at 500g for 15 min. The pellet was then resuspended in 5 ml PBS and extensively sonicated to disperse the Hz and residual lipids. This process was repeated as many times as was necessary to wash away the lipids. The final pellet was dried, weighed, and reconstituted in endotoxin-free water at a concentration of 1.0 mg/ml. The Hz was ascertained to be endotoxin-free by the use of the Limulus Amoebocyte Lysate gel-clot test (Cambrex Corp., East Rutherford, NJ).

To assess if ST cells could produce PAI-1 in response to Hz stimulation, 10 μg/ml Hz was added to cells, or cells were left unstimulated, over a given time course (2 to 24 h). Supernatants were collected from Hz-stimulated and unstimulated ST cells from six individual placentae and stored at -85°C until used for ELISA.

ELISAs

Levels of PAI-1 in human placental plasma and in supernatant from primary human trophoblast stimulated with Hz were measured by an in-house developed ELISA. 96-well plates were coated with monoclonal mouse anti-human PAI-1 primary antibody (Innovation Research, Inc., Southfield, MI) at 5 μg/ml in Tris-buffered saline (TBS) and incubated overnight. SuperBlock (Pierce, Rockford, IL) in TBS was used as a blocking buffer for one hour. Human PAI-1 (stable mutant form; Innovative Research, Inc., Southfield, MI) was added as the protein standard in 3% BSA/TBS by serial dilution starting at 50 ng/ml and then samples were added. Placental plasma samples were diluted 1:300 and supernatant samples were diluted 1:50, both in

3% bovine serum albumin (BSA)/TBS. The standard and samples were incubated for 2 h, preceding 1 h incubation of the polyclonal biotinylated sheep anti-human PAI-1 IgG secondary antibody (Innovative Research, Inc., Southfield, MI) at 10 ng/ml in 3% BSA/TBS. For detection, streptavidin-horseradish peroxidase (strep-HRP; R&D systems, Minneapolis, MN) in 3% BSA/TBS at 1:200 was added to the plate in the dark, followed by substrate A:B (R&D Systems, Minneapolis, MN), or 1:1 solution of hydrogen peroxide and tetramethyl benzidine (TMB). To terminate the enzymatic reaction of the peroxidase catalyzed by the substrate, phosphoric acid (H_3PO_4) was used. Absorbance was read at 450 nm and 570 nm on a SpectraMax PLUS spectrophotometer and data were recorded in SOFTmax PRO. Protein concentrations were determined using a standard curve obtained from the known concentration of protein standards included in each assay plate. Plates were washed three times between steps with Tris-buffered saline with 0.05% Tween-20 (TBS-T).

Levels of TFPI in placental plasma were measured by ELISA as described above. A monoclonal mouse anti-human TFPI primary antibody (R&D Systems, Minneapolis, MN) was used at 1.0 μg/ml in PBS. A biotinylated goat anti-human TFPI secondary antibody (R&D Systems, Minneapolis, MN) was used at 0.1 μg/ml in 0.1% BSA-Tris + 2% goat serum. Recombinant human TFPI (R&D Systems, Minneapolis, MN) was used as the protein standard at a high concentration of 250 μg/ml followed by serial dilutions.

Collection of placental tissue in Kenya

Following blood collection, full thickness sections of fresh placental tissue were isolated and inserted into cryovials. Tissues were snap-frozen in liquid nitrogen and kept frozen at <-80°C until use for isolation of protein.

Isolation of protein from placental tissue

Snap frozen placental tissue samples, stored in liquid nitrogen or at -85°C, were put on dry ice to prevent thawing. Mortar and pestle apparatuses were cleaned with 70% ethanol and flamed. Each tissue sample was added to a cold mortar filled with liquid nitrogen and ground by the pestle into smaller blocks. Liquid nitrogen was added to the mortar as needed. About 40 mg of tissue was solubilized with 1 ml of ice cold radioimmunoprecipitation (RIPA) buffer containing freshly added sodium orthovanadate (Na_3VO_4), phenylmethylsulphonyl fluoride (PMSF) and a protease inhibitor cocktail comprised of aproptinin, leupeptin, pepstatin. Ground tissue in RIPA buffer was homogenized by sonication and tubes were centrifuged at 10,000 revolutions per minute for 5 min. For each sample, the liquid part of the suspension (containing protein) was extracted and stored in a fresh Eppendorf tube at -85°C until used for the Bradford reaction.

Semi-quantification of isolated protein

To semi-quantify concentrations of PAI-1 and fibrin in placental tissue, the concentration of total isolated placental protein was determined by spectrophotometry quantification using the Bradford reaction with Bio-Rad Protein Assay as a dye reagent concentrate and bovine serum albumin (BSA) as standard (Bio-Rad Laboratories, Hercules, CA). Forty μg/lane of isolated protein was separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane.

Membranes were incubated at room temperature for 1 h in blocking buffer, 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween-20 (TBS-T) and then probed with primary antibodies against fibrin and PAI-1 overnight at 4°C. Antibodies were diluted 1:500 for the rabbit anti-human PAI-1 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)

and 1:200 for the murine monoclonal antibody against fibrin neotope β-chain IgG (American Diagnostica, Inc., Stamford, CT), both in blocking buffer. Final detection was performed with appropriate horseradish peroxidase-tagged secondary antibodies (Sigma, St. Louis, MO) in blocking buffer. Immunoreactive bands were visualized using enhanced chemiluminescence (SuperSignal; Pierce, Rockford, IL).

Each membrane was stripped with freshly prepared stripping buffer (2% SDS; 62.5 mM Tris—HCl, pH 6.8; 100 mM 2-mercaptoethanol) and reprobed with antibodies against the housekeeping protein β-actin. This was detected and visualized as described above. β-actin was used as a loading control for densitometry analysis performed using ImageJ, a Java-based image processing program developed by the NIH and available online.

Statistical analysis

The SAS statistical software package (version 8.02; SAS institute, Inc., Cary, N.C.) was used for data analysis. In cases of non-normally distributed data, the nonparametric Wilcoxon rank sum test was used. To compare the averages of the ranked data in more than two groups, the nonparametric Kruskal-Wallis (KW) test was performed; the permutation method from the MULTITEST procedure (SAS proc multtest) was used to obtain the adjusted *P* value for each pair of groups in multiple comparisons.

A student's t test in Microsoft Excel was used to analyze ELISA data in Table 1. No significant differences were found.

CHAPTER 3
RESULTS

Excessive fibrin deposition seen in the malaria-infected placenta may result from a disruption in the balance between coagulation and fibrinolysis. Data concerning the cell mediators involved in the accumulation of fibrin and whether these factors are gravidity-dependent are incomplete. To determine the influences of malaria and HIV infection, as well as gravidity, on placental concentrations of PAI-1, an inhibitor of fibrinolysis, placental plasma samples from women living in malaria-endemic western Kenya were compared (Table 1). While the level of PAI-1 in placental plasma was highest in malaria-positive/HIV-negative women, it did not significantly differ from the levels of PAI-1 in the placentae of women of other infection statuses. Furthermore, it does not appear that the concentration of PAI-1 is gravidity-dependent per lack of detectable pattern in levels within or between infection group(s), with one exception: the concentration of PAI-1 decreases with increasing gravidity in placental plasma of malaria-negative/HIV-positive women.

Table 1. Level of PAI-1 in placental plasma stratified by gravidity and infection status

	Mal+, HIV+	**Mal-, HIV-**	**Mal+, HIV-**	**Mal-, HIV+**	**Combined**
G1	2575 ± 1640 (6)	2717 ± 397 (45)	3472 ± 622 (23)	3985 ± 1024 (15)	3116 ± 326 (89)
G2	2177 ± 768 (12)	4354 ± 1059 (26)	2486 ± 934 (16)	3120 ± 897 (8)	3291 ± 543 (62)
G>2	3611 ± 860 (11)	2394 ± 347 (48)	4566 ± 1550 (6)	2061 ± 478 (20)	2624 ± 278 (85)
Combined	2803 ± 557 (29)	2940 ± 312 (119)	3267 ± 502 (45)	3055 ± 556 (43)	

Values are reported as mean ± standard error in ng/ml. Sample sizes are reported as (n).
Mal=malaria, HIV=human immunodeficiency virus. G=gravidity.

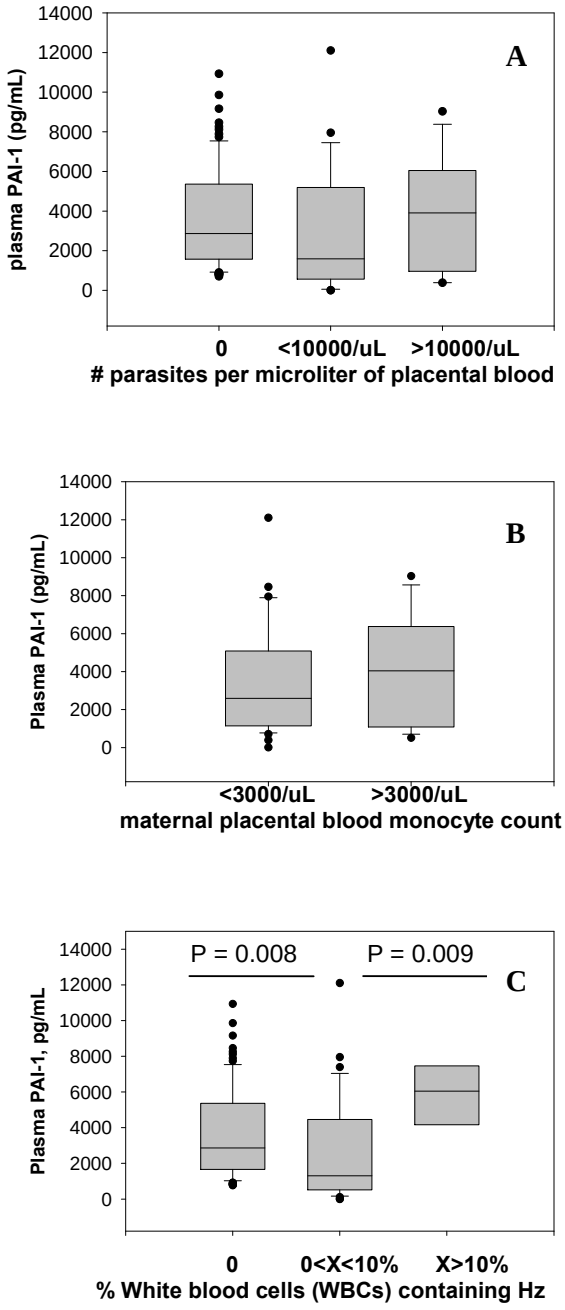


Figure 4. Concentration of PAI-1 in placental plasma does not change as a function of parasite density (A) nor does it change as a function of maternal blood monocyte count (B); however, placental PAI-1 increases with high levels of hemozoin-laden white blood cells. Levels of PAI-1 in placental plasma from Kenyan women were detected by ELISA. Parasite density, monocyte count, and presence of hemozoin were assessed by thick blood smear (A-C).

Considering the deposition of fibrin in the IVS characteristic of PM [6], significantly higher levels of PAI-1 in placentae infected with *P. falciparum* compared to uninfected were anticipated. Contrary results stimulated further exploration of the role of PAI-1 in these placentae. The concentration of PAI-1 was compared to various parameters corresponding to different aspects of the biological events of malaria during pregnancy. The level of PAI-1 in placental plasma did not change as a function of parasite density (Figure 4A), nor as a function of maternal monocyte infiltration to the IVS (Figure 4B). However, placental PAI-1 increased with the amount of white blood cells (WBCs) laden with hemozoin (Hz) (figure 4C).

While villous ST from preeclamptic women clearly produced PAI-1 [14], whether ST can synthesize and secrete PAI-1 in the context of malaria had yet to be demonstrated. Therefore, primary human trophoblast cells were isolated from placentae donated by women who

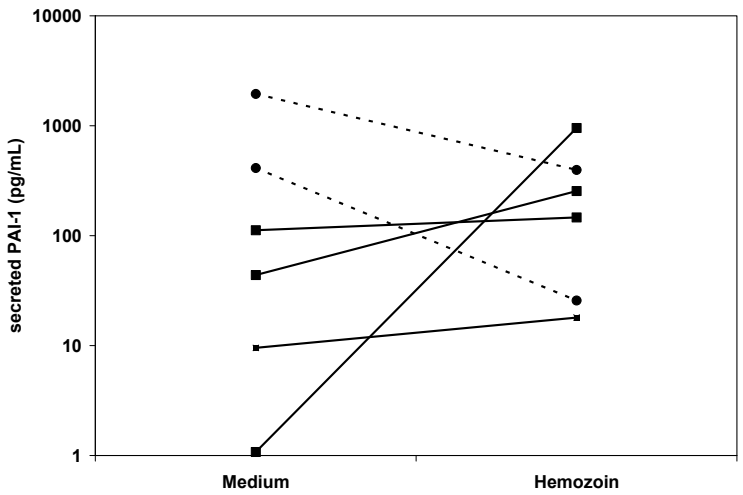


Figure 5. Cultured syncytiotrophoblast produce PAI-1 in response to hemozoin. Trophoblast cells were isolated from placental tissue, donated from women who delivered at Athens Regional Hospital, and induced to form a syncytium. Supernatant from unstimulated (medium) and stimulated (hemozoin) syncytiotrophoblast was collected at 24 hours and concentration of PAI-1 was assessed by ELISA.

delivered at Athens Regional Medical Center in Athens, Georgia. These cells were induced to form a synctium and then stimulated with Hz, the byproduct of parasite hemoglobin digestion which is often found in intervillous fibrin or WBCs of placentae of active-chronic and past infections with *P. falciparum*. PAI-1 was detected in supernatant collected at 24 h from all six placentae and in four out of the six, the concentration of PAI-1 increased when ST were stimulated with Hz compared to ST in the presence of medium only (Figure 5). Therefore, ST are capable of producing PAI-1 and this production may increase in the context of PM.

In addition to studying anti-fibrinolytics, another approach to evaluating local hyper-coagulation in the placenta is to assess failures of inhibitors of the clotting cascade. TF initiates the extrinsic clotting pathway and if inhibited, it is supposed that less fibrin (the product of coagulation) will result. Therefore, it was hypothesized that PM-positive women, those with substantial fibrin deposition in their placental IVS, would have lower levels of TFPI in placental plasma. The concentration of TFPI in placental plasma from primigravidae living in malaria-endemic western Kenya was assessed by ELISA (Figure 6). Consistent with the hypothesis that malaria during pregnancy leads to increased fibrin deposition, PM-positive women had significantly lower levels of TFPI (P=0.005).

To our knowledge, fibrin deposition in the malaria-infected placenta has been elucidated by histological readings and immunohistochemical evaluation of fixed placental tissue only. Therefore, we attempted to semi-quantify fibrin as well as PAI-1 by using isolated protein from snap-frozen placental tissue of women from malaria-endemic western Kenya. To assess if the amount of fibrin changes with the concentration of PAI-1 in placental plasma, samples were chosen based on the level of PAI-1 measured previously by ELISA. Protein from placental tissue was used from the 10 women with the greatest amount of placental PAI-1 and the 10 women with the least amount of placental PAI-1, for both PM-positive and PM-negative infection groups. Samples from primigravidae, those lacking protective immunity during *P. falciparum* infection and with more fibrin by histological analysis [6], were used. Following the protein isolation, protein was separated by SDS-PAGE (using the housekeeper β-actin as a loading control) and then transferred to nitrocellulose membranes and probed with appropriate antibodies (Figure 7). Western blots are ongoing for PAI-1 and fibrin and currently, data are insufficient to draw definitive conclusions.

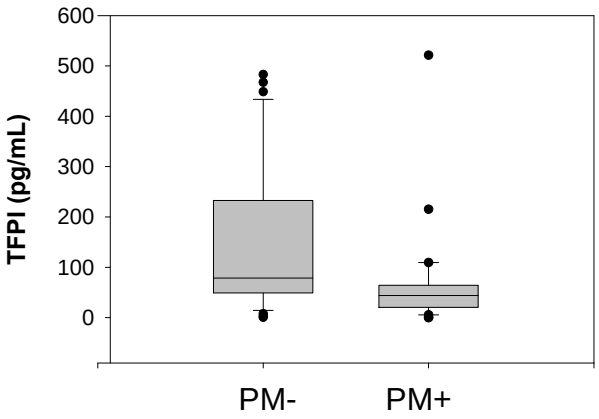


Figure 6. For primigravid women, the concentration of TFPI in placental plasma is significantly higher in uninfected women (PM-) compared to malaria-infected women (PM+) (P=0.005). Placental plasma was collected from women in western Kenya by the prick method and TFPI was measured by ELISA.

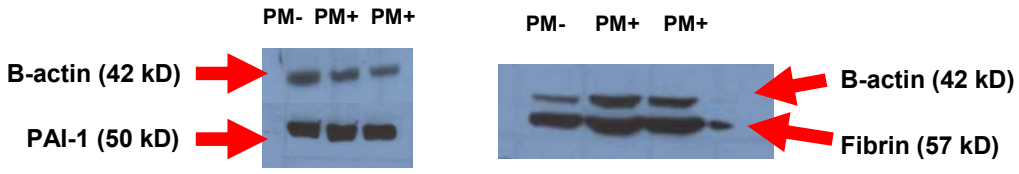


Figure 7. Protein was isolated from snap-frozen placental tissue from women in western Kenya. The total protein concentration for each sample was quantified by Bradford protein assay. Samples were separated on a gel (SDS-PAGE) and then transferred to a nitrocellulose membrane for antibody-staining and developing (Western blot). A housekeeping factor, β-actin was used as a loading control for PAI-1 (left) and fibrin (right).

CHAPTER 4
DISCUSSION

The cell types responsible for the production of coagulation and immune factors found in placental plasma of malaria-positive women have not yet been definitively identified. However, maternal and fetal cells are both suspect. It has been shown that ST become immunologically active upon stimulation with *P. falciparum*-iRBCs [17]. This activity is associated with altered expression and secretion of cytokines and chemokines, however, production of factors regulating coagulation and fibrinolysis had not yet been addressed. Results from this study demonstrate for the first time that the ST is capable of producing PAI-1 in response to stimulation with Hz. An antifibrinolytic, PAI-1 may be a major player involved in the accumulation of fibrin deposits which are found extensively in the IVS of malaria-infected placentae, especially among primigravidae.

A hallmark of PM is the robust infiltration of maternal immune cells, primarily monocytes, to the IVS of infected placentae. It has been shown that the interaction between ST and iRBCs stimulate the chemotactic migration of peripheral blood mononuclear cells (PMBCs) to the IVS [17]. Additionally, TF expression on macrophages in malarious placentae elucidated by immunohistochemistry [18], further highlights the need to consider both fetal and maternal cell types as modulators in the *inflammation-coagulation cycle* ongoing in the malaria-infected placenta. Therefore, maternal monocytes infiltrating the IVS may also produce PAI-1 in the context of malaria during pregnancy and need to be further investigated.

Levels of PAI-1 in placental plasma varied among primigravidae, secundigravidae, and multigravidae of different infection statuses, though placental PAI-1 was greatest in malaria-positive/HIV-negative women. Importantly, placental PAI-1 increased with the number of Hz-laden WBCs, assessed by thick blood smear, occupying the IVS of women with malaria infection. Placental histology is commonly used to diagnose the presence and severity of *P. falciparum* infection. Hz-laden WBCs describe an active-chronic infection and interestingly, chronic infection has been most closely associated with decreased birth weight due to fetal growth restriction [4].

During development, materno-fetal cellular contact is mediated by villous trophoblast cells invading the maternal deciduas, resulting in the remodeling of maternal spiral arteries to ensure sufficient blood flow. Contrarily, failures in this invasion have been associated with gestational diseases such as preeclampsia or severe forms of uterine growth restriction. Recent data suggests PAI-1 plays a critical role in mediating the TNF-inhibitory effect on trophoblast invasion of decidual stromal cells [19]. PAI-1 leads to sustained fibrin clots and while fibrin deposition at the syncytial surface is essential for physiological repair and differentiation of the placental villous, an unusually high level of intervillous fibrin is a histological hallmark of pregnancies with preeclampsia and IUGR [13,19].

The relationship between PAI-1 and fibrin and how their interaction may contribute to the hypercoagulated environment of malaria-infected placentae remains vague. Therefore, levels of PAI-1 and fibrin in protein isolated from placental tissue of women living in malaria-endemic western Kenya are currently being measured. Quantification of these proteins in PM-positive and PM-negative primigravidae and multigravidae compared with levels of protein (PAI-1,

TFPI, and others) in plasma will further our understanding of how fibrin accumulates in the placentae of women infected with *P. falciparum* during pregnancy.

Furthermore, the concentration of TFPI in PM-positive women was significantly lower than that of PM-negative women. TF should be measured and compared between PM-positive and PM-negative women to investigate its role in the hypercoagulated placental environment. It has been demonstrated that *P. falciparum*-infected RBCs induce TF expression in endothelial cells [11] and it would be interesting to investigate if this induced expression could occur by ST.

PM is a unique biological event interfacing a complex parasitic infection with the immunological changes brought about by pregnancy. Women chronically infected with *P. falciparum* during pregnancy are plagued by a crowded placental environment comprised of sequestered iRBCs, excessive fibrin deposition, robust maternal immune cell infiltrate, and a buildup of hemozoin in IVS fibrin or WBCs. It is believed that the presence and dysregulation of these many players, and their products, results in an *inflammation-coagulation cycle* that hinders proper fetal development. Further understanding of the scientific basis of malaria during pregnancy is needed to adequately confront this global health challenge persisting in the developing world. An increased understanding of pathogenic mechanisms of PM will lead to potential therapeutic interventions by which maternal anemia and poor fetal outcomes can be prevented.

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