UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO
FACULTAD DE BIOLOGÍA

PROGRAMA INSTITUCIONAL DE DOCTORADO EN CIENCIAS BIOLÓGICAS

OPCIÓN EN BIOTECNOLOGÍA MOLECULAR

"ESTUDIO DE LA RESPUESTA INMUNOTROMBÓTICA A LA PROTEÍNA SPIKE DEL VIRUS SARS-CoV-2"

T E S I S
PARA OBTENER EL GRADO DE:
DOCTOR EN CIENCIAS BIOLÓGICAS

P R E S E N T A

ALAN FABRICIO CANO MÉNDEZ

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NOVIEMBRE, 2024

El presente trabajo se realizó en el Laboratorio de Hemostasia y Biología Vascular dela división de estudios de posgrado de la Facultad de Ciencias Médicas y Biológicas “Dr. Ignacio Chávez” de la Universidad Michoacana de San Nicolás de Hidalgo en colaboración con el Centro Multidisciplinario de Estudios en Biotecnología de la Facultad de Medicina Veterinaria y Zootecnia de la Universidad Michoacana de San Nicolás de Hidalgo, bajo la dirección de la Dra. Martha Eva Viveros Sandoval y de la Dra. Alejandra Ochoa Zarzosa.

Para su realización se contó con el apoyo de la Coordinación de Investigación Científica de la UMSNH y de CONAHCyT a través de la convocatoria Ciencia Básica y de Frontera 320085.

Durante la realización del proyecto se contó con el apoyo de CONAHCyT a través de la beca nacional para estudiantes responsables del proyecto, con número de registro de becario 8912049.

“La recompensa se encuentra en el esfuerzo y no en el resultado. Un esfuerzo total es una victoria completa”

Mahatma Gandhi

“Ex favilla nos resurgemus”

A G R A D E C I M I E N T O S

Primeramente, agradezco y dedico este trabajo a mi familia. A mi mamá Liliana Méndez, por su eterno apoyo, su amor incondicional, por nunca soltar mi mano y por ser la mejor amiga que la vida me ha podido dar. A mi papá Fabricio Cano por ser un motor y un pilar en mi vida, por su eterno carisma y por enseñarme que la bondad y la humildad son cualidades indispensables para el desarrollo de nuestra persona. A mis hermanas: Michelle Cano Méndez y Shariat Cano Méndez, por estar siempre presentes, por permitirme amarlas y acompañarlas, por ser grandes y fieles amigas y por formar este gran equipo. A Niko, el cachorro que todos deberían tener en sus vidas. A mis tías y tíos, primas y primos y sobrinas y sobrinos, quienes siempre me han tendido la mano, estado constantes y leales.

A mis abuelos Rosa Gómez y Miguel Méndez, gracias por mostrarme el valor del trabajo constante, la importancia del esfuerzo y la tenacidad, la relevancia de la humildad y el coraje para seguir siempre adelante hasta lograr tus objetivos.

A mi abuelita Estelita Hernández, quién comenzó este camino tomando mi mano hace 12 años, y que hoy su espíritu me acompaña dándome la fuerza y el valor que solo ella tuvo para sortear los desafíos de la vida.

A mi gran mentora, la Dra. Martha Eva Viveros Sandoval. Estoy y estaré profundamente agradecido por todo el apoyo emocional, familiar, intelectual y moral que solo un gran ser humano, una gran madre, una gran maestra y una gran científica puede ofrecer.

A la Dra. Alejandra Ochoa Zarzosa, por su infalible apoyo durante el desarrollo de este trabajo. Por sus consejos y su disponibilidad para siempre orientarme cuando me sentía perdido.

A mis amigas, estupendas científicas, excepcionales compañeras, grandes maestras y doctoras: Nallely García Larragoiti, Sandra López Castañeda y Yesenia Ambriz Murillo. Gracias infinitas por todo lo que han aportado a mi persona. Son, sin temor a equivocarme, uno de los tesoros más grandes que me llevó de este viaje.

A todo el gran equipo de trabajo del Laboratorio de Hemostasia y Biología Vascular: Paty, Lupita, Lau, Tory, More, Sofi, Dr. Areán, gracias por su incondicional

apoyo, gracias por su colaboración y por hacer del Laboratorio un lugar especial.

A mi banda: Michelle, Alexis, Rafa, Cecy, Andy, Val y Paola; así como a Rosana, amigos que son familia y que me han acompañado a lo largo de este trayecto, sin soltar jamás mi mano y sin de alentarme en ningún solo momento.

C O N T E N I D O

RESUMEN

La enfermedad causada por el virus SARS-CoV-2 conocida como COVID-19, es además de ser una enfermedad respiratoria, es una patología inmunotrombótica. El proceso de infección viral está mediado por la glicoproteína Spike (S) localizada en la superficie membranal de este virus. La proteína S está conformada por las subunidades (S1 y S2), donde S1 facilita la interacción entre el virus y la célula diana a través del dominio de unión a receptor (RBD). Debido a lo anterior, resulta imperante estudiar la respuesta de las plaquetas y de células endoteliales a la proteína S completa (S1, S2) y al dominio RBD. Así como evaluar el perfil inmunotrombótico en muestras de pacientes afectados por COVID-19 y en sujetos vacunados con las plataformas vacunales circulantes en el estado de Michoacán durante el periodo 2021-2022.

Los resultados mostraron que la proteína S completa y el dominio RBD inducen la activación plaquetaria, pero no la agregación de estas células; sin embargo, una previa estimulación plaquetaria con proteínas virales, seguida de una dosis de colágeno a mínima concentración (2 µg/ml) favorece el restablecimiento de la agregación de estas células. Se determinó que las plaquetas tratadas con las proteínas virales liberan diversos marcadores inmunotrombóticos. Se confirmó que las proteínas Spike y el dominio RBD inducen la activación de las líneas celulares endoteliales (CE) EA.hy 296 y HUVEC, favoreciendo la liberación de factores procoagulantes y proinflamatorios al medio. Se determinó que existen receptores alternativos a la ECA-2 en plaquetas y en células endoteliales tales como la glicoproteína (GP) 141, GP IIbIIIa, los receptores tipo toll 2, 4 y 7 y las integrinas α5β1, neuropilina 1 y CD 147.

Para el estudio clínico se incluyeron un total de 84 muestras de pacientes con diagnóstico confirmado de COVID-19, los cuales se clasificaron de acuerdo a la gravedad de la enfermedad. Se determinó que las concentraciones elevadas de IL-6, IL-8, P-selectina, PSGL-1, Dimero-D, tPA, PAI-1 y FvW correlacionan con la severidad de la enfermedad. Finalmente, se evaluó el perfil inmunotrombótico de sujetos que recibieron diferentes dosis de inmunización con las diferentes plataformas de vacunación circulantes en el estado de Michoacán. Se encontró un aumento de las concentraciones plasmáticas de IL-6, P-selectina, PSGL-1, tPA y PAI-1 en el grupo inmunizado con la vacuna ChAdOx1-AztraZeneca.

La proteína S y el dominio RBD inducen una respuesta inmunotrombótica en plaquetas y células endoteliales. Clínicamente se observó un estado inmunotrombótico activo en los pacientes afectados por COVID-19 grave y en sujetos vacunados con la vacuna ChAdOx1-AztraZeneca

Palabras clave; SARS-CoV-2, Proteina spike, dominio RBD, Células endoteliales, Vacunación

ABSTRACT

The disease caused by the SARS-CoV-2 virus, known as COVID-19, is not only a respiratory disease, but also an immunothrombotic pathology. The viral infection process is mediated by the Spike (S) glycoprotein located on the membrane surface of this virus. The S protein is comprise by subunits (S1 and S2), where S1 facilitates the interaction between the virus and the target cell through the receptor binding domain (RBD). Due to the above, it is imperative to study the response of platelets and endothelial cells to the complete S protein (S1, S2) and the RBD domain, as well as to assess the immunothrombotic profile in samples of patients affected by COVID-19 and in subjects vaccinated with the circulating vaccine platforms in the state of Michoacán during the period 2021-2022.

Results showed that the complete S protein and the RBD domain induce platelet activation, but not aggregation of these cells; however, a previous platelet stimulation with viral proteins, followed by a dose of collagen at minimum concentration (2 µg/ml) favors the reestablishment of aggregation of these cells. It was determined that platelets treated with viral proteins release several immunothrombotic markers. It was confirmed that Spike proteins and the RBD domain induce the activation of endothelial cell lines (EC) EA.hy 296 and HUVEC, favoring the release of pro-coagulant and pro-inflammatory factors into the medium. It was determined that alternative receptors to ACE-2 exist in platelets and endothelial cells such as glycoprotein (GP) 141, GP IIbIIIa, toll-like receptors 2, 4 and 7 and integrins α5β1, neuropilin 1 and CD 147.

A total of 84 samples from patients with a confirmed diagnosis of COVID-19 were included for the clinical study and classified according to disease severity. Elevated concentrations of IL-6, IL-8, P-selectin, PSGL-1, D-dimer, tPA, PAI-1 and vWF were found to correlate with disease severity. Finally, the immunothrombotic profile of subjects who received different doses of immunization with the different vaccination platforms circulating in the state of Michoacán was evaluated. An increase in plasma concentrations of IL-6, P-selectin, PSGL-1, tPA and PAI-1 was found in the group immunized with the ChAdOx1-AztraZeneca vaccine.

Protein S and the RBD domain induced an immunothrombotic response in platelets and endothelial cells. Clinically, an active immunothrombotic state was observed in

patients affected by severe COVID-19 and in subjects immunized with the ChAdOx1-AztraZeneca vaccine.

I. INTRODUCCIÓN

I.I. El virus SARS-CoV-2: el enemigo común

El SARS- CoV-2 tiene un origen zoonótico y apareció a finales del 2019 en una provincia de Wuhan extendiéndose alrededor del mundo causando predominantemente cuadros que afectan a las vías respiratorias (1–3). El impacto sanitario y económico causado por la pandemia de COVID-19 ha marcado un hito en la historia moderna de la humanidad. Se estima que, a más de 4 años de iniciada la pandemia, se han infectado a alrededor de 780,000,000 personas en el mundo y se han reportado aproximadamente 7,000,000 de defunciones (4).

El SARS-CoV-2 es un virus envuelto perteneciente a la familia de los *Coronaviridae,* género *Betacoronavirus,* subgénero *Sarbecovirus* (5). Este virus se disemina principalmente por vía aérea a partir de aerosoles expulsados por una persona infectada. El ciclo de replicación de este agente viral comienza con su internalización a la célula diana, la cual está mediada por la interacción entre la proteína Spike y la enzima convertidora de angiotensina 2 (ECA-2) como receptor de entrada; una vez internalizado, el agente viral continúa con la replicación del material genético viral, la traducción proteica y el ensamblaje de nuevos viriones (Figura 1) (6,7).

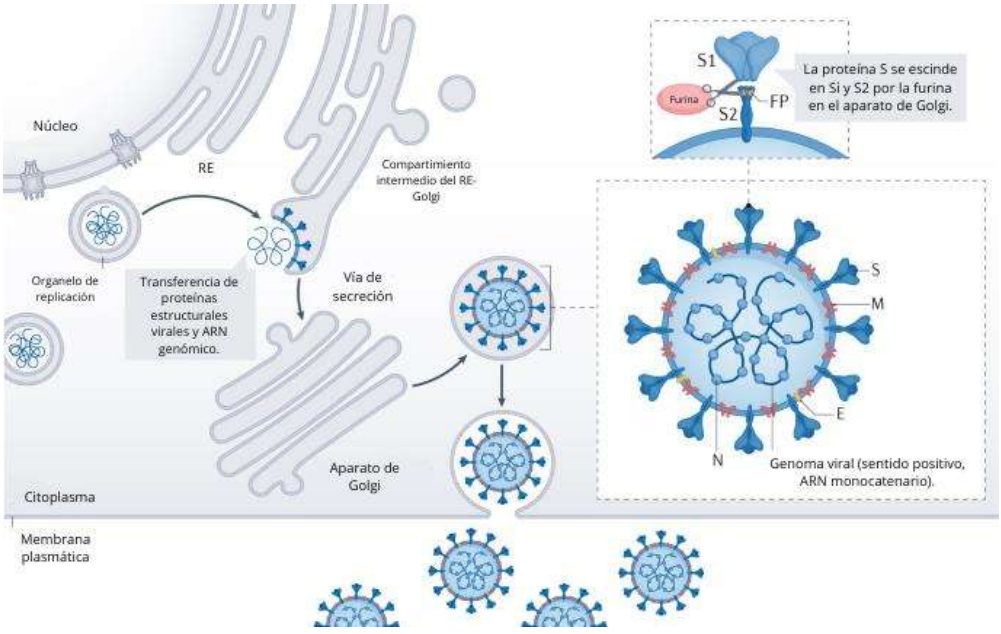

Figura 1. Participación de la proteína Spike en la infección del SARS-CoV-2.

1. El virus SARS- CoV-2 es un agente que posee como material genético ARN de polaridad positiva, este virus infecta a las células humanas a través de la proteína Spike,

localizada en la superficie membranal del virus. **2.** La proteína S está conformada por dos subunidades. S1 posee el dominio de unión a receptor (RBD) el cual interacciona con receptores de las células diana. La S2 está anclada a la membrana viral y facilita la fusión de la membrana. **3-4.** La proteína se ancla al receptor en la célula hospedera y proteasas presentes en la célula hospedera (furina y TMPRSS2) escinden la S2 de la proteína S y facilita la fusión de las membranas. **5-6.** La fusión de membranas permite la inserción del material genético viral. **7-9.** Se lleva a cabo la replicación viral. **10.** El ARN viral permite la síntesis de ARN complementario y utilizando la maquinaria ribosomal de la célula hospedero traduce proteínas virales que por un lado arman una maquinaria que permite sintetizar más copias de material genético. Por otro lado, ensambla nuevas estructuras virales que salen de la célula e inician el ciclo viral. Adaptado de: Jackson et al., 2022. (19).

I.II. La proteína Spike, la llave de entrada a nuestras células

El SARS-CoV-2 posee un genoma de ARN monocatenario de polaridad positiva de aproximadamente 26 a 33 kb que codifica un total de 16 proteínas no estructurales (nsp 1-16), involucradas típicamente en la replicación viral, y cuatro proteínas estructurales: envoltura (E), membrana (M) y nucleocápside (N) y la proteína de espiga o Spike (S) (5,8,9). La proteína S está localizada en la superficie membranal del SARS-CoV-2, cuenta con un peso molecular de alrededor de 180 kDa y pertenece a una familia de proteínas transmembrana de tipo I organizada en dos subunidades: una N- terminal S1, y una C-terminal S2 (7). La proteína S se puede encontrar en dos conformaciones estructuralmente distintas, prefusión y postfusión (7,10).

El dominio de unión al receptor (RBD), situado en la subunidad S1 de la proteína, se compone de una secuencia de 200 aminoácidos y se caracteriza por ser un sitio con un gran número de mutaciones relacionadas con todas las nuevas variantes de SARS-CoV-2 (11,12). El RBD está conformado por dos dominios: el núcleo, rico en residuos no polares, y el motivo de unión al receptor (RBM), correspondiente a los residuos 443-503 de la proteína S, que es mayoritariamente polar (11,13,14) (Figura 2).

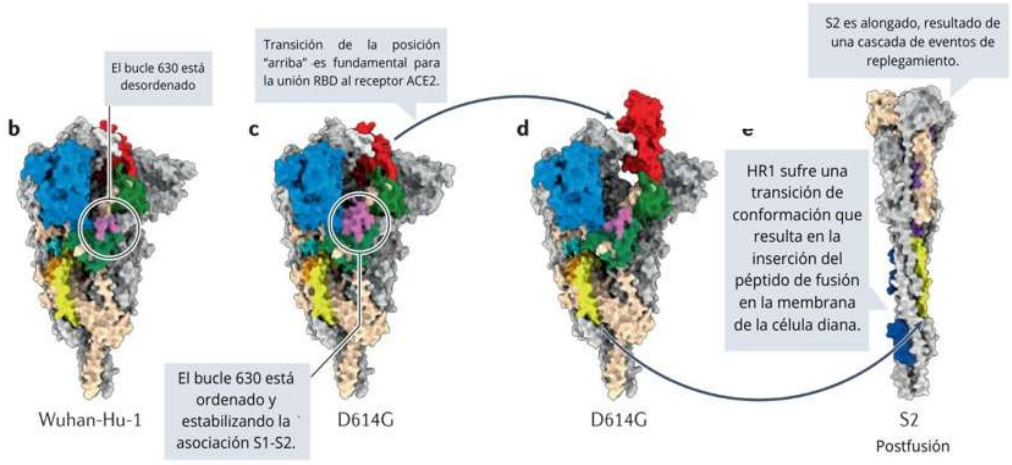

Figura 2. Estructura de la proteína Spike. La proteína Spike consta de aproximadamente 1,273 aminoácidos. La proteína cuenta con diversos dominios: el dominio N terminal, que es el más expuesto (azul), seguido de este dominio se encuentra el RBD, sitio que permite la interacción con el receptor en las células diana. El dominio CTD1 y CTD2 son los sitios que permiten el ensamblaje del homotrímero. Estos 3 sitios representativos están aglomerados en la subunidad 1. Adaptado de: Jackson et al., 2022. (19).

Se ha propuesto a la ECA-2 como el principal receptor utilizado por el SARS- CoV-2 (16). La función principal de la ECA-2 es convertir a la angiotensina I y la angiotensina II, generadas por la renina y la ECA, en angiotensina-(1-9) y angiotensina-(1-7), respectivamente (17). La ECA-2 se encuentra ampliamente expresada en diversos tejidos del cuerpo humano, desde el parénquima pulmonar, gastroenterológico, el sistema nervioso central, sistema urogenital, el endotelio vascular y las plaquetas (16).

Se han reportado receptores alternativos a la ECA-2, expresados en diversas células. Entre ellos, los receptores tipo Toll (TLR) 2, 7 y 9, o el cluster de diferenciación 147 (CD147), también llamado basigina o inductor de la metaloproteinasa de la matriz extracelular (EMMPRIN) (18–22). Lo anterior ha originado una amplia variedad de manifestaciones clínicas.

I.III. COVID-19: manifestaciones clínicas y mecanismos fisiopatológicos

La infección viral inicia con la migración del virus desde el epitelio nasal al tracto

respiratorio superior, dónde la enfermedad se manifiesta con síntomas como: fiebre, malestar general y tos seca (23). Durante esta fase se produce una respuesta inmunitaria que implica la liberación del ligando de quimiocina 10 (CXCL-10) y de interferones (IFN-β e IFN-λ) por parte de las células infectadas por el virus (24). En la mayoría de los pacientes el reto inmunológico se resuelve en esta fase, ya que la respuesta inmunitaria montada es suficiente para contener la propagación del virus. Sin embargo, una parte importante de los pacientes pueden desarrollar síntomas graves de la enfermedad. Esto es consecuencia de la invasión y penetración de células epiteliales alveolares de tipo 2 (neumocitos tipo 2) a través del receptor del hospedero ECA-2 (25). El virus comienza a replicarse para producir más viriones; como consecuencia, los neumocitos tipo 2 liberan citocinas y marcadores inflamatorios como interleucinas (IL-1, IL-6, IL-8, IL-120 e IL-12), factor de necrosis tumoral-α (TNF-α), interferones (IFN-λ e IFN-β), CXCL10, proteína quimioatrayente de monocitos-1 (MCP- 1) y proteína inflamatoria de macrófagos-1α (MIP-1α) (25,26,27). Este proceso infeccioso e inflamatorio compromete la pérdida de neumocitos de tipo 1 y 2, generando un daño alveolar que culmina en un síndrome de dificultad respiratoria aguda (SDRA) (24).

Como consecuencia de la dispersión viral a través del torrente sanguíneo y del proceso inflamatorio exacerbado, una serie de síntomas sistémicos pueden presentarse en el paciente como son: la anosmia, disgeusia, síntomas gastrointestinales, disfunción cardíaca, hepatobiliar, renal, así como alteraciones del sistema vascular y de la coagulación (23,24).

Existen reportes que confirman que los pacientes más graves presentan coagulopatía, formación masiva de coágulos intravasculares tanto en vasos pequeños (microangiopatía trombótica), como en grandes arterias (tromboembolia pulmonar). La presentación clínica de la coagulopatía asociada a COVID-19 es principalmente disfunción orgánica, mientras que los eventos hemorrágicos son poco frecuentes (28). La coagulopatía asociada a COVID-19 implica una serie de interacciones complejas entre la respuesta inmunitaria innata, las vías de coagulación y fibrinolítica, la actividad de las plaquetas y del endotelio vascular, lo que da lugar a un estado procoagulante (24,29,30).

Por otro lado, se ha reportado un estado de disfunción endotelial que provoca un aumento de la liberación del factor von Willebrand (FvW) multimérico y la posibilidad de

una mayor adhesión de las plaquetas al endotelio, así como la disminución de proteínas anticoagulantes en la superficie de las células endoteliales (31). La hiperreactividad plaquetaria da lugar a un aumento de los agregados plaqueta-neutrófilo y plaqueta-monocito, estimulando a los neutrófilos a liberar trampas extracelulares (NETs), que atrapan plaquetas y proteínas protrombóticas contribuyendo a las complicaciones trombóticas pulmonares (32). A este proceso en el que convergen el sistema inmunológico y el sistema de la coagulación se le denomina inmunotrombosis, el cual en un estado exacerbado puede culminar en una tromboinflamación, lo que compromete el estado de salud del individuo afectado tanto en el plazo inmediato como en el largo plazo (33,34) (Figura 3).

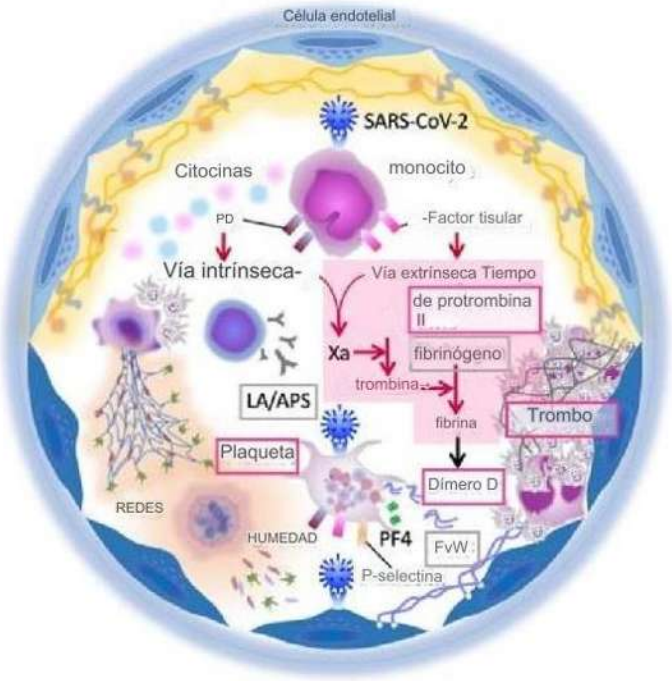

Figura 3. Esquematización del proceso inmunotrombótico en el nicho alveolar. La activación endotelial ocasionada por el SARS-CoV-2 favorece la liberación de moléculas procoagulantes como el FvW y la P-selectina generando una superficie protrombótica. El estado inflamatorio induce la liberación exagerada de citocinas que activa monocitos y neutrófilos los cuales favorecen la activación de la coagulación. Finalmente, la activación plaquetaria subsecuente al proceso inflamatorio, netosis y lesión endotelial propician la formación de coágulos intravasculares. Tomado de: Iba, T et al., 2024. (35).

I.IV. Inmunotrombosis y tromboinflamación: mismos componentes, distintos desenlaces

La inflamación y la coagulación son respuestas esenciales del hospedero en estados homeostáticos y en enfermedades críticas como COVID-19 (35). El término inmunotrombosis se acuñó por primera vez en 2013 por Engelmann y Massberg (33). En la inmunotrombosis intervienen células inmunitarias innatas, plaquetas y factores de coagulación, que contribuyen al control y la eliminación de las infecciones. En su forma fisiológica, representa un proceso de micro coagulación que no produce síntomas clínicos adversos y simplemente ayuda a inmovilizar patógenos invasores o estructuras extrañas dañinas para su posterior eliminación por las células inmunitarias (36,37).

La tromboinflamación por su parte, describe diversos aspectos de la participación de las plaquetas en los procesos inflamatorios, como las interacciones plaqueta-leucocito, o la activación plaquetaria a través de receptores TLR los cuales se asocian clásicamente a la detección de Patrones Moleculares Asociados a Patógenos (PAMP) (38). La tromboinflamación es un estado que ha permitido describir la fisiopatogenia de diversos escenarios como son el ictus o los problemas cardiovasculares con desenlace trombótico, la trombosis observada en cáncer o fases graves de COVID-19 (39). En la actualidad, la relación entre inmunotrombosis y tromboinflamación se entiende comúnmente como fenómeno dinámico de causa y efecto. La inmunotrombosis se refiere a la influencia del sistema inmune en la formación de un trombo, mientras que la tromboinflamación se refiere al impacto del trombo en el sistema inmunitario (34) (Figura 4).

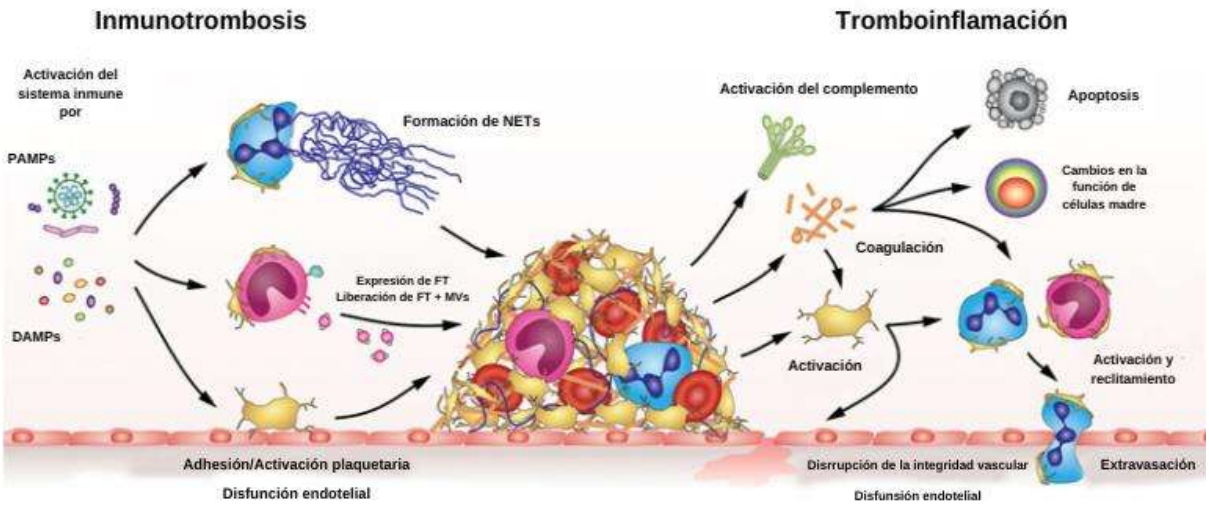

Figura 4. Inmunotrombosis vs Tromboinflamación. La inmunotrombosis se define como la activación de la coagulación en consecuencia a un estímulo inflamatorio como los Patrones Moleculares Asociados a Daño (DAMPs) o a Patógenos (PAMPs). Por otro lado, la deficiencia en el control de este proceso procoagulante conlleva a la activación del sistema inmune por parte de productos del proceso inmunotrombótico tales como plaquetas activadas, productos de degradación de fibrina y proteínas del complemento. Tomado de: Schrottmaier et al., 2024. (34).

I.V. Las plaquetas, células multifacéticas

Las plaquetas son células anucleadas derivadas del megacariocito que circulan en el torrente sanguíneo en una concentración que va desde las 150,000 hasta las 450,000 células por microlitro (40). Morfológicamente están constituidas por 3 tipos de gránulos: gránulos alfa (α), densos y lisosomales, los cuales son preformados durante su génesis en el megacariocito (41). Los gránulos α contienen proteínas asociadas a la adhesión celular, la coagulación, la inflamación, el crecimiento celular y la defensa del hospedero (42). El contenido de estos gránulos es liberado posterior a la activación plaquetaria, y pueden ser expresados en la membrana o liberados al espacio extracelular. Por otro lado, Los gránulos densos contienen altas concentraciones de nucleótidos de adenina como el Adenosín Di Fosfato (ADP) y el Adenosín Tri Fosfato (ATP), uracilo, guanina, calcio y potasio. Además, son ricos en polifosfatos y aminas bioactivas como la serotonina e histamina (41). Finalmente, los gránulos lisosomales plaquetarios contienen enzimas

degradadoras de proteínas como las catepsinas, la elastasa y la colagenasa; enzimas degradadoras de carbohidratos como la glucosidasa y la galactosidasa; y fosfatasa ácida como enzima hidrolítica de ésteres de fosfato (43).

Las plaquetas clásicamente se han estudiado desde su función trascendental en los procesos de hemostasia y coagulación. Estas células son pivotes en la contención de la ruptura de la integridad vascular, generando la formación de un conglomerado de células que impiden la pérdida de fluido sanguíneo (40). Una vez formado el tapón plaquetario, se activa el sistema de coagulación para permitir la formación de fibrina, que ancla el tapón plaquetario a la rotura del vaso. Por último, una vez resuelto el daño, la fibrina se elimina mediante fibrinólisis (44,45). Sin embargo, en años recientes se ha reportado la participación de las plaquetas como células centinela de la respuesta inmune (46–49).

El ejemplo más reciente, es la participación de las plaquetas como células inmunes frente a la infección por SARS-CoV-2. En los pacientes con COVID-19 a menudo se observa un aumento de la activación y agregación plaquetaria, que puede contribuir al estado de hipercoagulabilidad observado en los casos graves (31). Además, se ha implicado a las plaquetas en la cascada inflamatoria de COVID-19 mediante interacciones con las células inmunitarias y la liberación de citocinas y quimiocinas proinflamatorias (46). Estudios recientes han descrito las posibles implicaciones terapéuticas dirigidas a la función plaquetaria en el tratamiento de COVID-19 (25).

La naturaleza biológica de las plaquetas les confiere una variedad de herramientas que utilizan para participar activamente en la respuesta inmunitaria. Estas células poseen una amplia variedad de receptores que les permiten detectar patógenos, entre ellos los TLRs, las glicoproteínas de membrana como la glicoproteína IV, IIb-IIIa y los receptores de quimiocinas (46,47). Asimismo, estas células tienen la peculiaridad de poseer concentraciones abundantes de ARNm que codifica para proteínas proinflamatorias, lo que las posiciona como células centinela del sistema inmunitario innato (43).

Por otro lado, las plaquetas pueden activar a los neutrófilos a través de la P-selectina, induciendo su activación y la generación de trampas extracelulares de neutrófilos (NETs) en estados de sepsis y algunas infecciones víricas como COVID-19 (49). Las plaquetas pueden activar las células linfoides a través de la generación de MVs

plaquetarias ricas en CD40L (47).

Existe también una estrecha interacción entre las plaquetas y el endotelio vascular. Cuando el endotelio está sometido a estados de inflamación, aumenta la expresión membranal de la E-selectina, la proteína de Adhesión de Células Vasculares 1 (VCAM1) y Molécula de Adhesión Intercelular 1 (ICAM1), lo que aumenta la adhesión plaquetaria al endotelio, induciendo un estado procoagulante y proinflamatorio (50,51)

I.VI. Células endoteliales y su relevancia en los procesos infecciosos

Las células endoteliales (CE) forman el revestimiento interno de los vasos sanguíneos y son cruciales para mantener la homeostasis vascular (52). Estructuralmente las CE se caracterizan por ser una sola capa de células unidas por uniones estrechas, uniones adherentes y uniones tipo “gap” (53). Las CEs poseen complejos granulares llamados “corpúsculos de Weibel Palade” (CWB) los cuales conservan proteínas procoagulantes como el FvW, los factores VII y IX de la coagulación, así como proteínas vinculadas con los procesos inflamatorios, tales como la P-selectina (52).

En un estado de reposo, las células endoteliales poseen características protectoras antiinflamatorias y anticoagulantes, lo anterior mediado por la producción de óxido nítrico, prostaglandinas, antitrombina y el complejo proteíco S/C. Sin embargo, el endotelio activado o disfuncional muestra un fenotipo proinflamatorio, mediado por la producción de citocinas infamatorias a través de la vía NF-Kβ, y la baja o nula producción de reactantes de oxígeno y eicosanoides. Paralelamente, se convierte en una superficie procoagulante, expresando moléculas de adhesión leucocitaria y plaquetaria y disminuyendo las concentraciones de moléculas fibrinolíticas (54).

En el contexto de COVID-19, la infección por SARS-CoV-2 tiene un profundo impacto en las células endoteliales denominado endotelitis, contribuyendo a la disfunción vascular sistémica observada en los casos graves (55) (Figura 5).

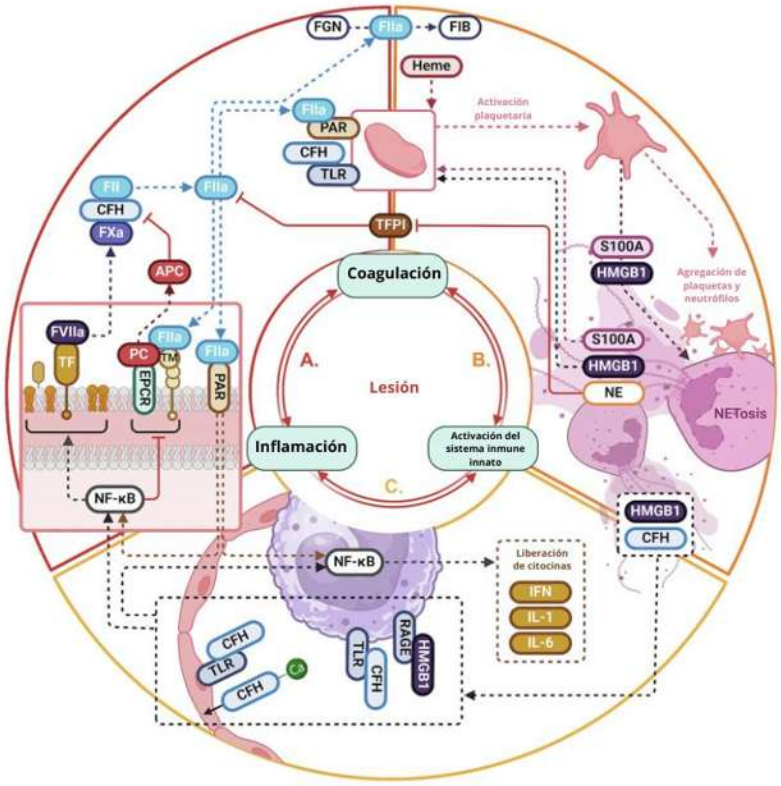

Figura 5. Modelo convergente de la coagulación. Se muestra las correlaciones que existen entre un proceso inflamatorio agudo caracterizado por la liberación de DAMPs y PAMPs a la luz vascular, y la subsecuente activación de la cascada de la coagulación y activación de plaquetas, las cuales potencian la respuesta del sistema inmune innato, favoreciendo una retroalimentación positiva y aumentando el estado inflamatorio. Tomado de: Yong et al., 2024. (54).

I.VII. Vacunas: herramienta de salud pública

La propagación acelerada del SARS-CoV-2 provocó la rápida infección de millones de personas en todo el mundo y la afectación no sólo de la sanidad pública, sino también de las infraestructuras sociales, educativas y económicas. Cómo respuesta, el sistema de salud pública mundial, en conjunto con farmacéuticas trasnacionales, centros de investigación multidisciplinarios y universidades, lograron el rápido desarrollo de las vacunas contra COVID-19. Las primeras vacunas que recibieron autorización reglamentaria en función de su eficacia fueron: la vacuna BNT162b2 (Pfizer-BioNTech), la mRNA-1273 (Moderna), la ChAdOx1 nCoV-19 (AstraZeneca) y la Janssen (Johnson &

Johnson) (56).

Durante las fases de inmunización, se notificaron diversos efectos adversos que iban desde: dolor en el sitio de aplicación, hiperemia y edema, hasta: fatiga, cefaleas, mialgias y artralgias. De manera relevante, un grupo pequeño de pacientes refirió el desarrollo de eventos trombóticos (57). A este proceso se le ha denominado trombocitopenia trombótica inmune inducida por la vacunación (VITT), y se ha observado principalmente en personas que recibieron la vacuna ChAdOx1 nCoV-19 (58).

La fisiopatología de la VITT se ha vinculado a la formación de un complejo inmune entre el factor 4 plaquetario (PF4) y la inmunoglobulina tipo G (IgG) anti PF4, los cuales inducen la agregación plaquetaria, favoreciendo un estado procoagulante (Figura 6) (59). Aunque la incidencia de VITT en todo el mundo es muy baja, oscilando entre 0,5 y 6,8 casos por cada 100,000 vacunas aplicadas, en México la información epidemiológica es escasa.

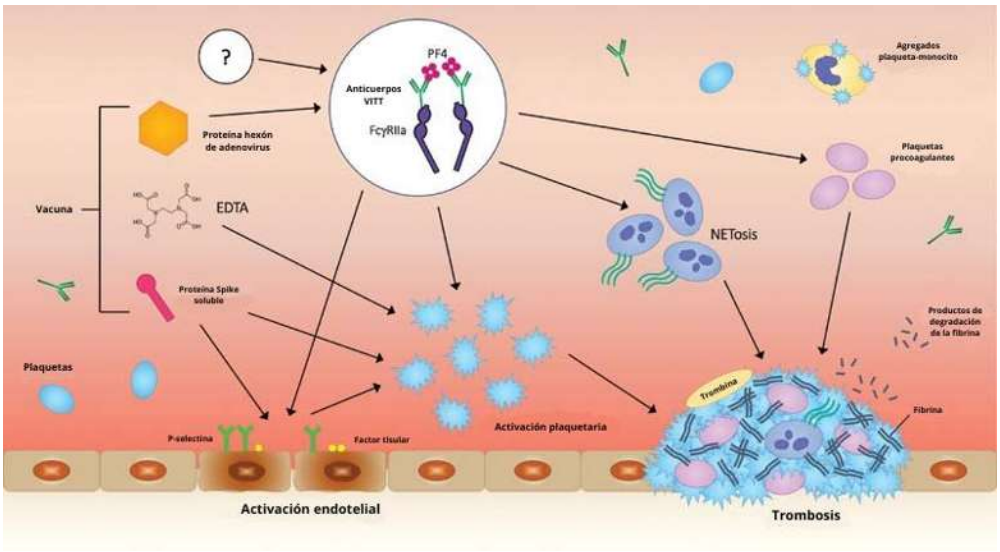

Figura 6. Posibles mecanismos de trombosis en la VITT. La trombosis en la VITT se produce debido a la formación de anticuerpos IgG contra el PF4, que activan las plaquetas a través de FcγRIIa. Estos anticuerpos pueden unirse a complejos de PF4 con componentes de la vacuna como las proteínas del adenovirus. Tomado de: Selvadurai et al., (2023). (59).

II. ANTECEDENTES

El virus SARS-CoV-2 utiliza a la proteína spike como mecanismo de entrada a las células del hospedero a través del dominio RBD, utilizando diversos receptores, siendo ECA-2 su principal receptor (6-7, 13). La proteína spike se ha posicionado como un blanco de diagnóstico y terapéutico en el curso de la enfermedad; así como en el diseño de vacunas contra COVID-19 (56).

COVID-19 ha demostrado ser una patología inmunotrombótica, en donde las alteraciones en el proceso inflamatorio y de la coagulación correlacionan con el pronóstico de los pacientes afectados por esta enfermedad (26,31). El termino inmunotrombosis describe un mecanismo fisiopatológico que involucra a la activación del sistema de la coagulación, incluyendo plaquetas y células endoteliales, como consecuencia de una respuesta inflamatoria exacerbada subsecuente a un proceso infeccioso (33,36).

El papel de las plaquetas y las células endoteliales como como células inmunológicas fue descrito recientemente y ha permitido entender la fisiopatología de diversas patologías que afectan al sistema circulatorio, como COVID-19 (47-48, 50, 52-53).

Se ha demostrado que la proteína spike tiene un rol fundamental en la activación de células involucradas en la respuesta inmunotrombótica (36). Sin embargo, la participación de estas células en el establecimiento del estado procoagulante y proinflamatorio observado en COVID-19 presenta áreas de estudio sin resolver.

III. JUSTIFICACIÓN

El virus SARS-CoV-2 ha representado un evento catastrófico de salud pública a nivel mundial. La enfermedad COVID-19 ha costado la vida de millones de personas alrededor del mundo y actualmente se mantiene con circulaciones ondulantes entre la población. El SARS-CoV-2 utiliza la glicoproteína Spike, localizada en su superficie, para infectar a la célula diana. Se ha identificado a la ECA-2 como su principal receptor; sin embargo, se han propuesto receptores alternativos en diversas células. Estudiar las interacciones y respuestas que guardan las células involucradas en los procesos de inmunotrombosis a estructuras virales, permitirá entender de una mejor manera la fisiopatología de diversas enfermedades que tienen afectaciones al nicho vascular.

El cuadro clínico observado durante las primeras oleadas de la pandemia se caracterizó por un estado inmunotrombótico activo, el cual correlacionaba con un peor pronóstico para el paciente. Dentro del proceso inmunotrombótico se involucra la activación paralela de 3 sistemas: el sistema inmunológico, el sistema de la coagulación y el sistema vascular.

Evaluar la respuesta plaquetaria y endotelial a la proteína estructural Spike del virus SARS-CoV-2 permitirá entender mejor la fisiopatología de COVID-19, identificar blancos de diagnóstico, pronóstico y terapéuticos que beneficien a la población en general.

Conocer los mecanismos inmunotrombóticos desencadenados por una inmunización contra SARS-CoV-2, permitirá identificar biomarcadores de riesgo trombótico y evaluar la seguridad de las plataformas biológicas de las vacunas.

IV. PREGUNTA DE INVESTIGACIÓN

¿Cuál es la respuesta de las plaquetas y de las células endoteliales a la proteína Spike completa (S1, S2) y al dominio RBD del virus SARS-CoV-2?

V. HIPÓTESIS

La proteína Spike completa (S1, S2) y su dominio RBD inducen la activación de las plaquetas y de células endoteliales, favoreciendo el desarrollo de un estado inmunotrombótico.

Existe un estado inmunotrombótico activo en pacientes afectados por COVID-19 y en sujetos vacunados con plataformas de vector viral no replicante, lo cual condiciona el desarrollo de un evento trombótico.

VI. OBJETIVOS

VI.I Objetivo general

Estudiar la respuesta de las plaquetas y de células endoteliales a la proteína S completa (S1, S2) y al dominio RBD. Además, evaluar el perfil inmunotrombótico en plasma de pacientes afectados por COVID-19 y en sujetos vacunados con las plataformas circulantes en el estado de Michoacán.

VI.II Objetivos particulares

1. Evaluar la activación plaquetaria y de células endoteliales inducida por la proteína Spike completa y el dominio RBD.

2. Identificar biomarcadores del microambiente tromboinflamatorio en el sobrenadante de plasma rico en plaquetas (PRP) y cultivo de células endoteliales tratadas con la proteína Spike completa y con el dominio RBD.

3. Determinar biomarcadores inmunotrobóticos en muestras sanguíneas de pacientes infectados por SARS-CoV-2 y correlacionar estos con el estado de salud del paciente.

4. Evaluar biomarcadores inmunotrombóticos en muestras de sujetos vacunados con los diferentes biológicos utilizados en los esquemas de vacunación en México.

VII. ESTRATEGIA EXPERIMENTAL

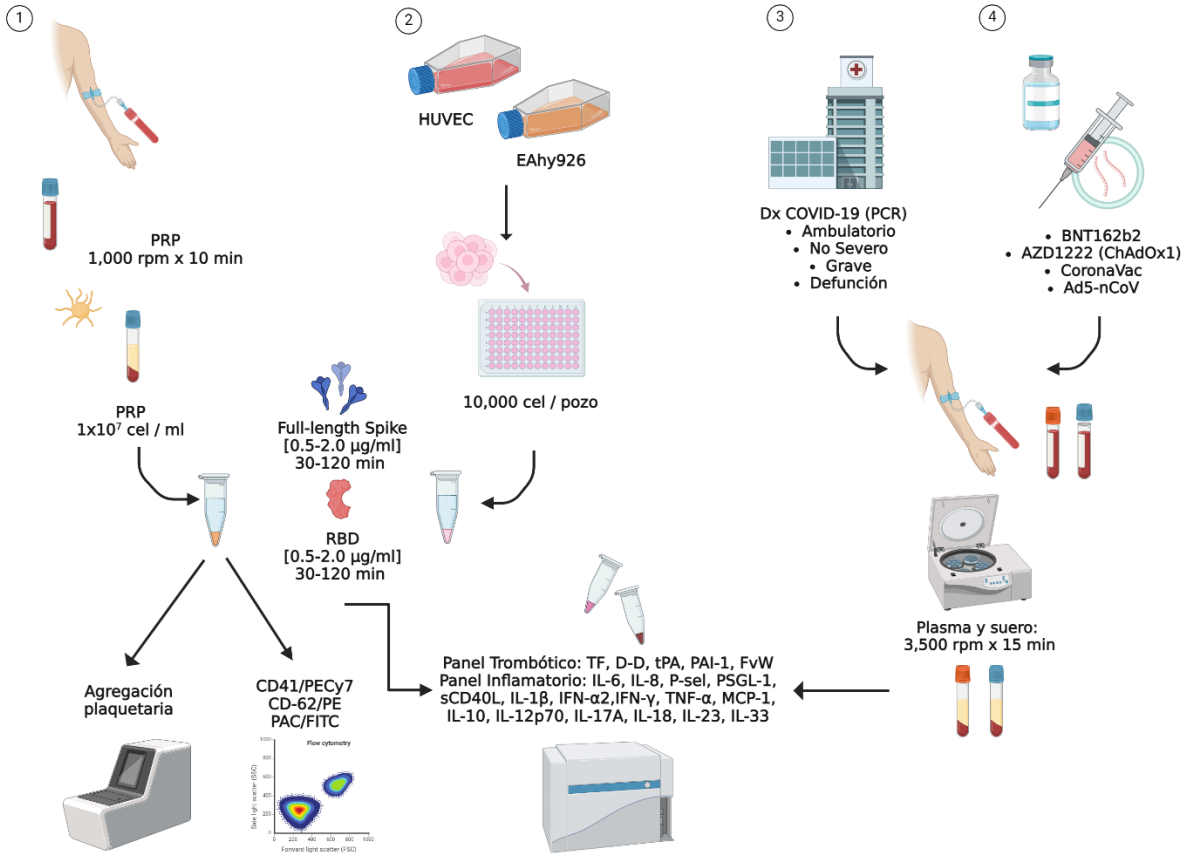

Figura 7. Estrategia experimental. Estrategia seguida para la realización del proyecto "Estudio de la respuesta inmunotrombótica a la proteína Spike del virus SARS-CoV-2". **1)** Determinación de la reactividad plaquetaria a la proteína Spike y al dominio RBD del SARS-CoV-2. **2)** Evaluación de la actividad y respuesta endotelial a la proteína Spike y al dominio RBD del SARS-CoV-2. **3)** Estudio de perfil inmunotrombótico en pacientes afectados por COVID-19 y correlación con la gravedad de la enfermedad. **4)** Análisis del estado inmunotrombótico en sujetos vacunados con las diferentes plataformas de inmunización circulantes en el estado de Michoacán.

VIII. RESULTADOS

VIII.I. Capítulo 1: Los resultados de este apartado se encuentran publicados enel artículo científico titulado: *Platelet Reactivity and Inflammatory Phenotype Induced by Full-Length Spike SARS-CoV-2 Protein and Its RBD Domain*

***Cano-Mendez, A**., García-Larragoiti, N., Damian-Vazquez, M., Guzman-Cancino, P., Lopez-Castaneda, S., Ochoa-Zarzosa, A., & Viveros-Sandoval, M. E. (2022). Platelet Reactivity and Inflammatory Phenotype Induced by Full-Length Spike SARS-CoV-2 Protein and Its RBD Domain. International journal of molecular sciences, 23(23), 15191. https://doi.org/10.3390/ijms232315191.*

International Journal of
Molecular Sciences

MDPI

Article

Platelet Reactivity and Inflammatory Phenotype Induced by Full-Length Spike SARS-CoV-2 Protein and Its RBD Domain

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Abstract: A state of immunothrombosis has been reported in COVID-19. Platelets actively participate in this process. However, little is known about the ability of SARS-CoV-2 virus proteins to induce platelet activity. Platelet-rich plasma (PRP) was incubated with spike full-length protein and the RBD domain in independent assays. We evaluated platelet activation through the expression of P-selectin and activation of glicoprotein IIbIIIa (GP IIbIIIa), determined by flow cytometry and the ability of the proteins to induce platelet aggregation. We determined concentrations of immunothrombotic biomarkers in PRP supernatant treated with the proteins. We determined that the spike full-length proteins and the RBD domain induced an increase in P-selectin expression and GP IIbIIIa activation ($p < 0.0001$). We observed that the proteins did not induce platelet aggregation, but favored a pro-aggregating state that, in response to minimal doses of collagen, could re-establish the process ($p < 0.0001$). On the other hand, the viral proteins stimulated the release of interleukin 6, interleukin 8, P-selectin and the soluble fraction of CD40 ligand (sCD40L), molecules that favor an inflammatory state $p < 0.05$. These results indicate that the spike full-length protein and its RBD domain can induce platelet activation favoring an inflammatory phenotype that might contribute to the development of an immunothrombotic state.

Keywords: platelet activation; platelet aggregation; SARS-CoV-2; spike protein; RBD domain

Citation: Cano-Mendez, A.; García-Larragoiti, N.; Damian-Vazquez, M.; Guzman-Cancino, P.; Lopez-Castaneda, S.; Ochoa-Zarzosa, A.; Viveros-Sandoval, M.E. Platelet Reactivity and Inflammatory Phenotype Induced by Full-Length Spike SARS-CoV-2 Protein and Its RBD Domain. *Int. J. Mol. Sci.* **2022**, *23*, 15191. https://doi.org/10.3390/ijms232315191

Academic Editor: Isabella Russo

Received: 30 September 2022
Accepted: 28 November 2022
Published: 2 December 2022

1. Introduction

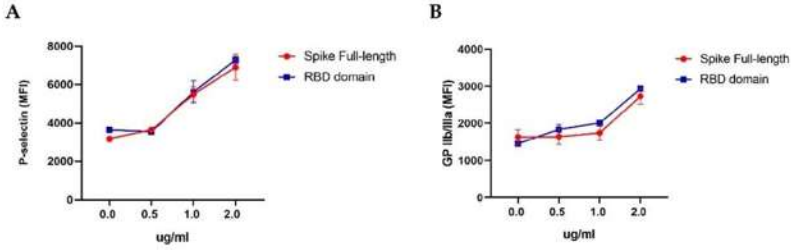
The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is an enveloped positive-sense single-stranded RNA virus that causes Coronavirus Disease 2019 (COVID-19) and has spread worldwide, causing predominantly respiratory illness [1,2]. The SARS-CoV-2 genome encodes a total of 16 nonstructural proteins (nsp 1-16) and four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N) [3]. The S protein is located on the surface of SARS-CoV-2, with a molecular weight of around 180 kDa, and belongs to a type I transmembrane protein family [4] organized in two subunits: an N-terminal S1, and a C-terminal S2. Viral entry depends on an engagement between the Receptor Binding Domain (RBD), located in the S1 subunit of the protein, and the angiotensin-converting enzyme 2 (ACE2) as the entry receptor [5,6]. The RBD is composed of a sequence of 200 amino acids and is now characterized as a site with a vast number of mutations related to all the novel SARS-CoV-2 variants [7,8]. Critically ill COVID-19 patients show acute respiratory distress syndrome (ARDS), accompanied by a hemostasis imbalance characterized by an active coagulation process and platelet activation associated with systemic inflammation (cytokine storm) in a process defined as immunothrombosis [9]. Platelets are circulating anucleate cells of the bloodstream, traditionally associated with hemostatic processes through their

rapid response to vascular damage [10]. Recently, platelets have been positioned as sentinel cells of the immune system due to their unique structural, functional, and generational characteristics, and have been shown to be key mediators in thrombosis and inflammation [11–14]. In addition, platelets can directly interact with viruses and participate in the immune response, since platelets express a wide range of receptors that they use to interact with pathogens [15–17]. Different platelet receptors involved in the recognition of SARS-CoV-2 have been proposed to lead to a state of platelet hyperactivity and an increase in aggregation and adhesion [9,18–21]. The role of SARS-CoV-2 spike protein and its domain in the platelet–virus interaction, as well as its ability to promote the development of a proinflammatory and prothrombotic state, requires further clarification. The aim of this study was to evaluate the platelet response to SARS-CoV-2 spike protein and domains.

2. Results

2.1. SARS-CoV-2 Spike Full-Length Protein and RBD Domain Induce Platelet Activation

Firstly, we evaluated different concentrations (0.5, 1, and 2 μg/mL) of the full-length (complete) SARS-CoV-2 spike protein and of its RBD domain, and their ability to induce platelet activation. We observed the highest cell activation at a protein concentration of 2 μg/mL. This result coincided for both proteins; therefore, subsequent assays were performed using this same concentration. Mean fluorescence expression of platelet P-selectin and GP IIbIIIa activity are shown in (Figure 1A,B). Then, we explored the full-length spike protein's and RBD domain's capacity to activate platelets in a time-dependent manner. The results of fluorescence expression of platelet activation markers P-selectin and GP IIbIIIa are shown in (Figure 1C,D). Compared with the control, the full-length spike protein was able to induce the maximum expression of platelet activation markers at 30 min after stimulation ($p < 0.0001$). Interestingly, the RBD domain induced the higher expression of these proteins at 120 min, and a statistical difference was observed when compared with the unstimulated platelets ($p < 0.0001$). The negative control was treated under the same conditions as the experimental groups but without the stimulation of viral proteins. Finally, we compared the highest expression results of these activation markers for each protein and the percentage of cells positive for the expression of cell activation markers, with the expression of these biomarkers induced by known activation agonists, unstimulated cells, and with the stimuli induced by Tyrode's buffer used as a vehicle. Our results show that the RBD domain induced a higher expression of both P-selectin and GP IIbIIIa than epinephrine and ADP, but lower than collagen ($p < 0.0001$). Additionally, the expression of activation markers generated by full-length spike protein was similar to that induced by epinephrine but statistically lower than ADP and collagen ($p < 0.0001$). These results are shown in (Figure 1E–H) and confirm that both the spike protein and the RBD domain are able to activate platelets.



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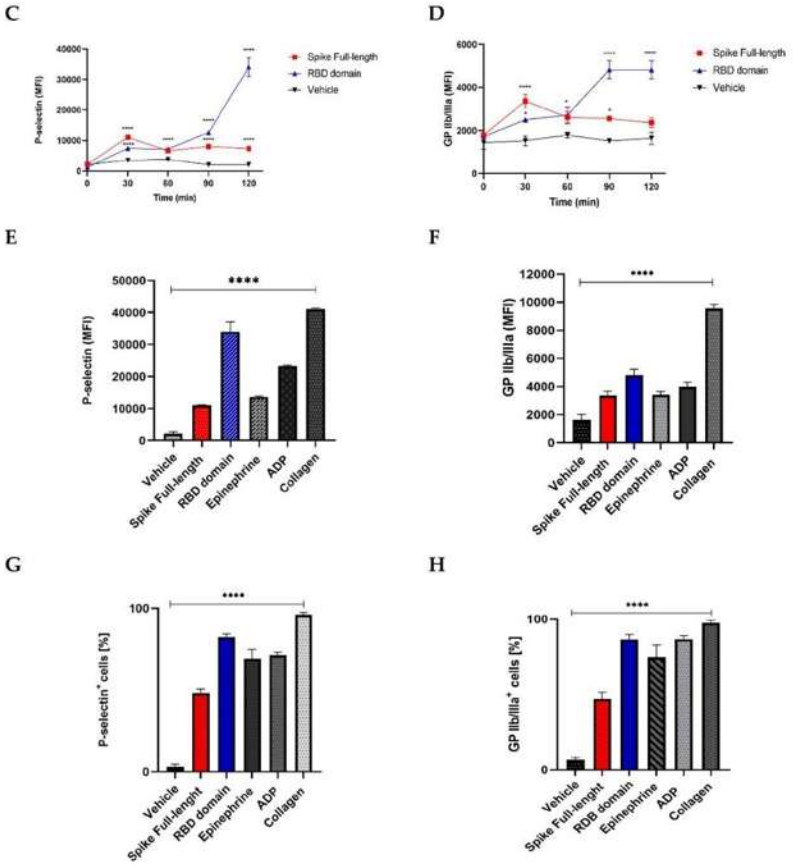

Figure 1. Platelet stimulation with full-length spike protein and RBD domain. Platelet-rich plasma was incubated for 60 min at 37 °C with spike full-length and RBD domain at different concentrations. Mean fluorescence expression of platelet activation markers when incubated with the spike full-length and RBD domain (**A**,**B**). Platelet-rich plasma was incubated for different times at 37 °C with spike full-length and RBD domain at a concentration of 2.0 µg/mL. Tyrode's buffer was used as control (**C**,**D**). Mean Fluorescence Intensity expression of P-selectin and GP IIbIIIa, respectively, on platelets' surface when incubated with spike full-length protein, RBD domain, and Tyrode's buffer at different times (**E**,**F**). Comparison between maximal expression of activation markers P-selectin (CD62) and GP IIbIIIa (PAC1). Spike full-length protein for 30 min (2.0 µg/mL), RBD domain 120 min 2.0 µg/mL, Tyrode's buffer as vehicle and ADP 20 µM (20 min), epinephrine 100 µM (40 min) and collagen 20 µM (30 min) as known activation agonists (**G**,**H**). Comparison of maximum expression of activation markers indicating the percentages of P-selectin (**G**)- and GP IIbIIIa (H)-positive cells after stimulation with viral proteins spike full-length and RBD domain, known activation agonists and vehicle. *p* values were calculated using one-way ANOVA and Tukey's post-hoc tests ($n = 3$). Data presented as Mean ± SD. * $p < 0.05$, ****: Statistical difference $p < 0.0001$.

2.2. Full-Length Spike Promotes Platelet Aggregation in the Presence of Low Doses of Collagen

After we determined that both proteins were able to induce platelet activation, we decided to investigate whether the spike protein or its RBD domain were capable of inducing platelet aggregation, another important function of platelets.

Our results demonstrate that neither the complete spike protein nor the RBD domain induce platelet aggregation on their own when compared to known aggregation agonists, which achieve aggregation percentages of more than 90% ($p < 0.0001$) (Figure 2A–H). Once we observed that proteins induced activation but not aggregation, based on previous results reported in our lab by García-Larragoiti et al. [22] and in a previous work performed by Chiao-Hsuan et al. [23], we decided to assess if platelet hyperreactivity induced by viral protein stimulation, in addition to subthreshold doses of known aggregation agonists, could restore platelets' aggregation capacity.

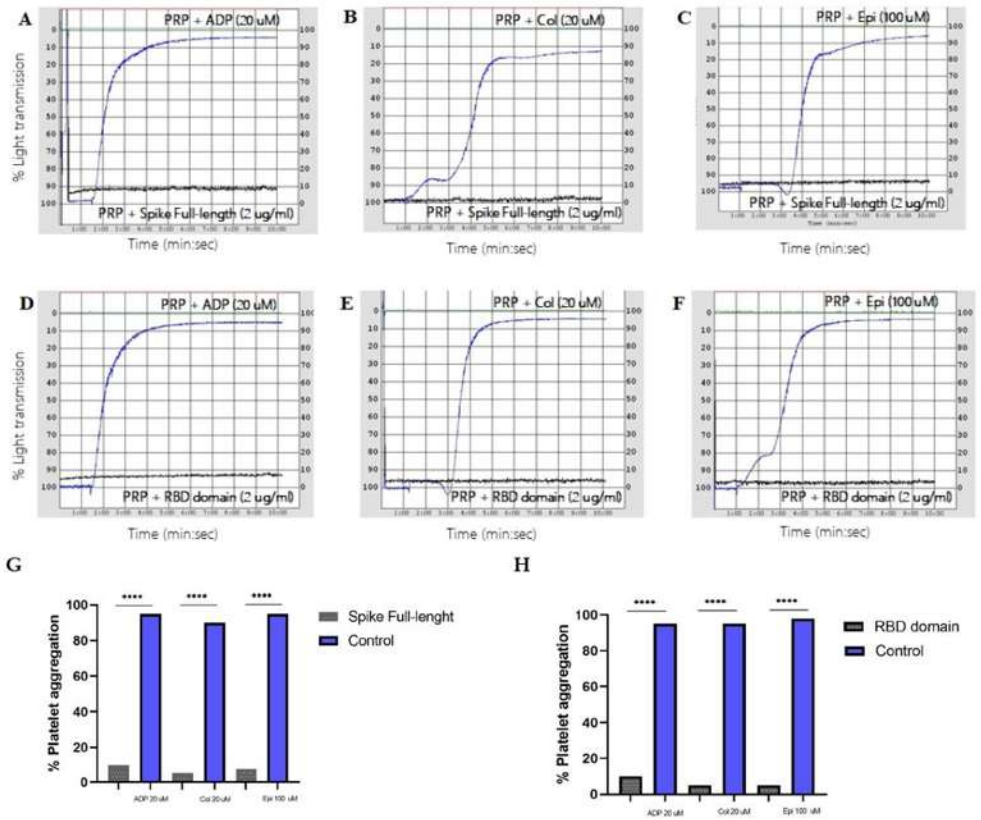

Figure 2. Determination of the aggregation capacity of platelets in response to spike full-length and RBD domain (**A**–**C**). Platelet-rich plasma (PRP) was incubated with spike full-length protein (2.0 µg/mL) for 30 min and compared to positive controls stimulated with a dose of 20 µM ADP, 20 µM collagen, and 100 µM epinephrine (**D**–**F**). PRP was incubated with RBD domain (2.0 µg/mL) for 120 min and compared to positive controls stimulated with a dose of 20 µM ADP, 20 µM collagen, and 100 µM epinephrine (**G**,**H**). Comparison of platelet aggregation induced by known agonists when compared to PRP treated with virus proteins. Platelet agonists, but not proteins, were able to induce greater than 80% of aggregation. *p* values were calculated using one-way ANOVA and Tukey's post-hoc tests ($n = 3$). Data presented as Mean ± SD; ****: Statistical difference $p < 0.000$.

Interestingly, our results shown in Figure 3 prove that minimal doses of collagen were able to re-establish platelet aggregation up to 90% in cells previously treated with the full-length spike protein ($p < 0.0001$) (Figure 3B); however, this was not observed in

assays to which ADP or EPI were added (Figure 3A–C). On the other hand, as is shown in (Figure 3D–F), none of the minimal doses of known agonists were able to induce platelet aggregates in those cells previously stimulated with the RBD domain. Our results suggest that SARS-CoV-2 full-length proteins induce a hyperreactive state in platelets forming aggregates when exposed to aggregating factors.

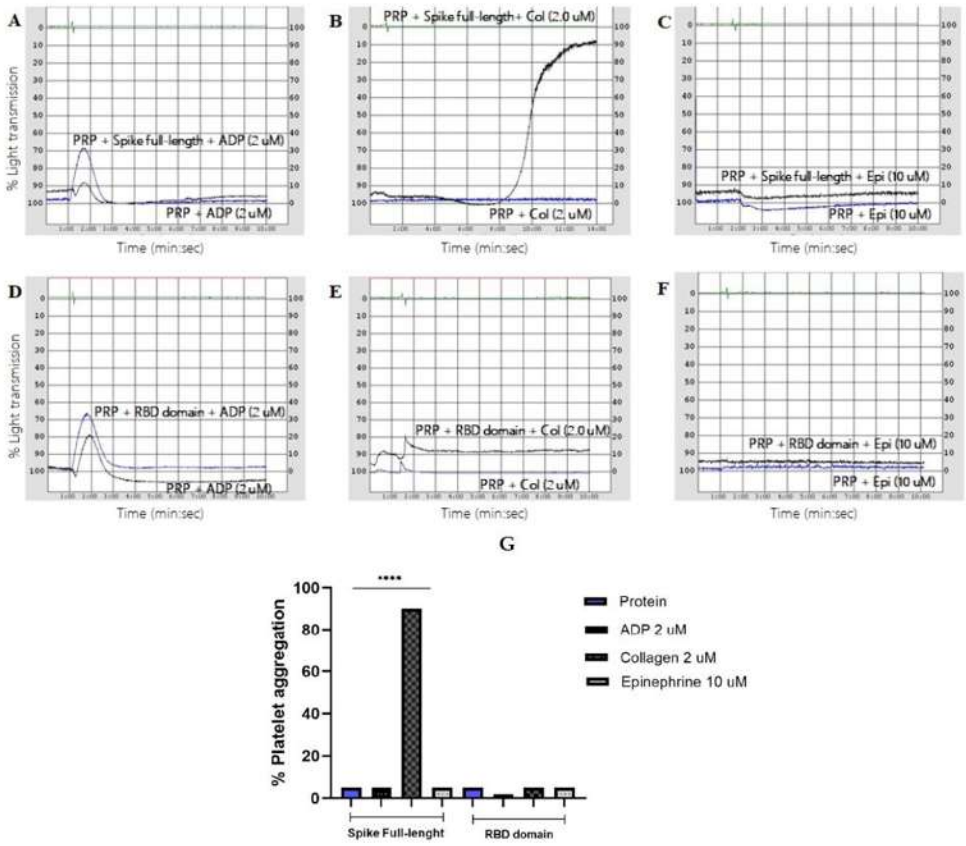

Figure 3. Determination of the aggregation capacity of platelets incubated with spike full-length or RBD domain in response to subthreshold doses of known aggregation agonists (**A**–**C**). PRP was incubated with spike full-length protein (2.0 µg/mL) for 30 min and a dose of 2 µM ADP, 2 µM collagen, and 10 µM epinephrine was added. Only the minimal dose of collagen was able to induce aggregation of PRP when compared to the negative control (PRP + 2 µM ADP, 2 µM collagen, and 10 µM epinephrine, respectively) (**D**–**F**). PRP was incubated with RBD domain (2.0 µg/mL) for 120 min and a dose of 2 µM ADP, 2 µM collagen, and 10 µM epinephrine was added (**G**). Comparison of platelet aggregation induced by minimal doses of known agonists in PRP incubated with full-length spike or RBD proteins when compared to PRP added with minimal doses of known aggregation agonists. None of the known agonists were able to induce aggregation of PRP when compared to the negative control (PRP + 2 µM ADP, 2 µM collagen, and 10 µM epinephrine, respectively). *p* values were calculated using one-way ANOVA and Tukey's post-hoc tests ($n = 3$). Data presented as Mean ± SD. ****: Statistical difference $p < 0.0001$.

2.3. The Spike Protein and Its RBD Domain Stimulates Platelets' Release of Pro-Inflammatory Factors

In order to approach the role of platelets in response to viral proteins, we decided to assess the concentration of different biomarkers related to inflammation in the supernatant of PRP stimulated with each SARS-CoV-2 protein at different times. We found that IL-6 concentration was higher in PRP after stimulation for 30 and 60 min (min) with the RBD domain when compared with unstimulated PRP ($p < 0.01$). Interestingly, full-length spike protein shows a raise in IL-6 concentration at 60 min ($p < 0.05$), presenting the main pick at 90 min ($p < 0.0001$) when compared with controls (Figure 4A). Moreover, only RBD was able to induce platelet release of IL-8 after a stimulus of 30 min ($p < 0.001$). sCD40L at 60 min of stimulation with the RBD domain showed differences in concentration when compared to control ($p < 0.0001$) (Figure 4B,C). Finally, we observed that both proteins stimulated the significant release of P-selectin, but not the expression of PSGL-1 (Figure 4D,E). Significant RBD-induced P-selectin release was observed at 60 min ($p < 0.0001$), while the full-length spike protein maintained significant concentrations during 30, 60, 90, and 120 min, when compared with unstimulated PRP ($p < 0.0001$). Curiously, none of the known activation agonists such as ADP, EPI or collagen showed an increase in the concentration of inflammatory-related biomarkers. These results suggest that platelets may contribute to the cytokine storm reported in SARS-CoV-2 infection by showing a pro-inflammatory phenotype.

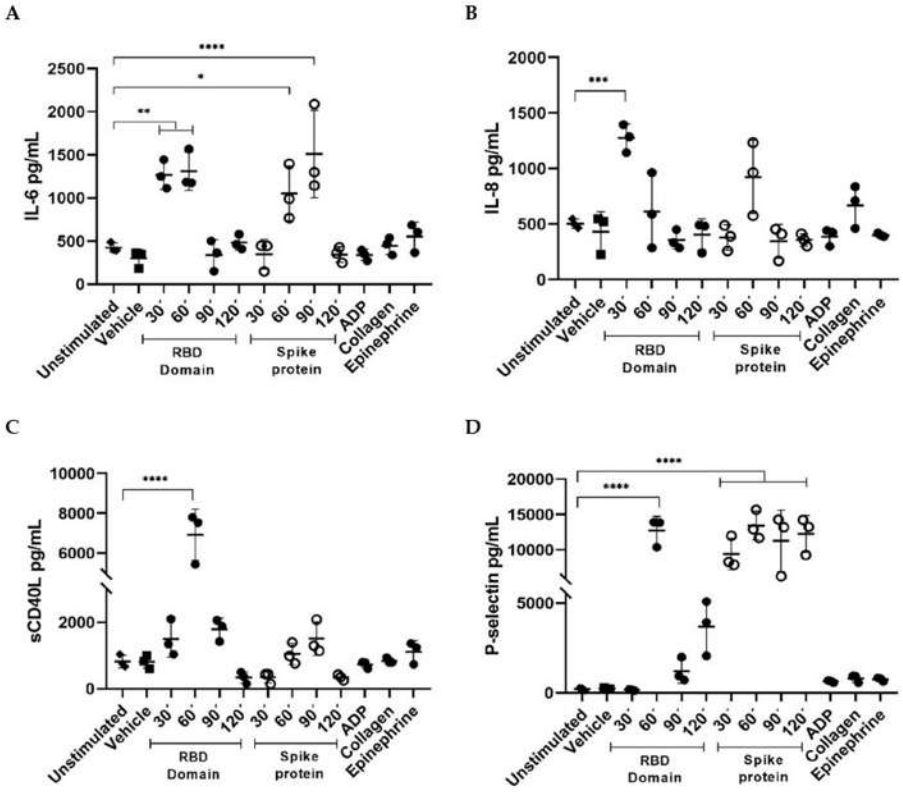

Figure 4. *Cont.*

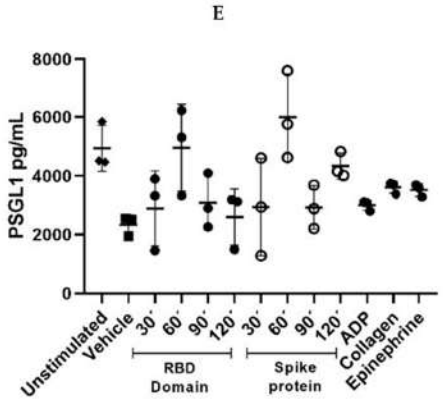

Figure 4. The spike protein and its RBD domain stimulate platelets' release of pro-inflammatory factors. Comparison of supernatant concentration of (**A**) IL-6, (**B**) IL-8, (**C**) sCD40L, (**D**) P-selectin and (**E**) PSGL-1 in PRP incubated with spike full-length protein (2.0 µg/mL) and RBD domain (2.0 µg/mL) at different times. Known platelet aggregation agonists were used as activation controls (20 µM ADP, 20 µM collagen, and 100 µM epinephrine), Tyrode's buffer was used as vehicle and PRP treated under the same conditions but without external stimuli as a negative control. *p* values were calculated using one-way ANOVA and Tukey's post-hoc tests ($n = 3$). Data presented as Mean ± SD. Statistical difference $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

2.4. Platelets Contribute to a Procoagulant Microenvironment when Stimulated with Full Length Spike Protein and RBD Domain

Hemostasis and fibrinolysis processes are altered in severe COVID-19, and platelets are an important source of coagulation and fibrinolytic factors. In order to measure whether protein-stimulated platelets contribute to this misbalance, we determined five different biomarkers related to a prothrombotic state. D-dimer concentrations were found to be significantly higher in platelets stimulated with the RBD domain for 60 min ($p < 0.01$) and with the full-length protein at 60 min ($p < 0.0001$) (Figure 5A). Moreover, only the full spike protein induced a significant increase in Factor IX concentrations after 60 min of stimuli ($p < 0.0001$). Only EPI showed increments of FIX ($p < 0.05$) (Figure 5B). Interestingly, both full-length spike protein and RBD domain induced a time-dependent release of PAI-1, 60 min being the time where the highest concentrations were found ($p < 0.0001$). (Figure 5C). We did not find differences in TF and tPA at any time (Figure 5D,E). These results indicate that platelets release coagulation factors that contribute to a prothrombotic state when exposed to an immune insult, such as that reported in COVID-19.

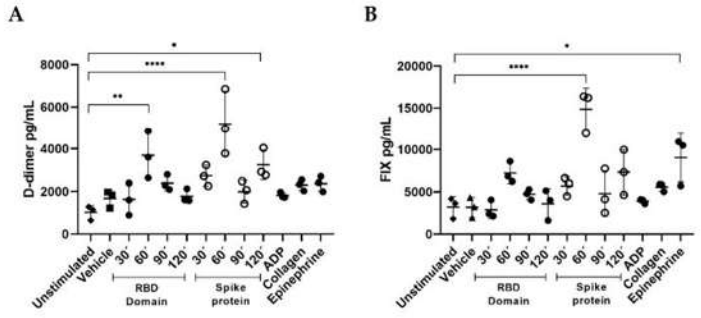

Figure 5. *Cont.*

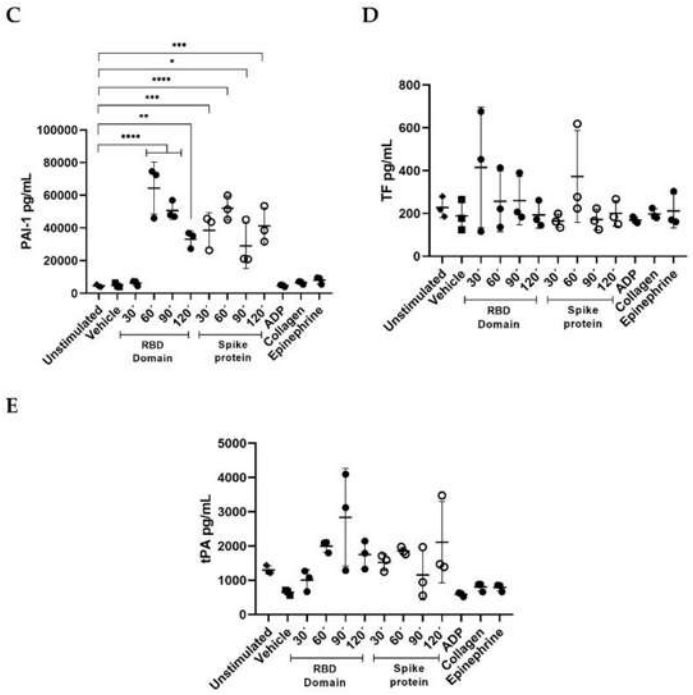

Figure 5. The spike protein and its RBD domain stimulate platelets' release of pro-inflammatory factors. Comparison of supernatant concentration of (**A**) D-dimer, (**B**) FIX, (**C**) PAI-1, (**D**) TF and (**E**) tPA in PRP incubated with spike full-length protein (2.0 μg/mL) and RBD domain (2.0 μg/mL) at different times. Known platelet aggregation agonists were used as activation controls (20 μM ADP, 20 μM collagen, and 100 μM epinephrine), Tyrode's buffer was used as vehicle and PRP treated under the same conditions but without external stimuli as a negative control. p values were calculated using one-way ANOVA and Tukey's post-hoc tests ($n = 3$). Data presented as Mean ± SD. Statistical difference * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Discussion

Platelets can be found in large numbers in peripheral blood, and due to their functional characteristics, these cells can play an important role in the immune response. Platelet count in COVID-19 varies according to disease severity. Severe thrombocytopenia is rarely reported in COVID-19 patients and correlates with greater morbidity/mortality [24]. Moreover, mild thrombocytopenia has been detected in most cases. This drop in platelet count may be related to a worsening thrombotic state [25,26]. However, information on the ability of structural proteins and their domains to activate platelets is not entirely clear. Regarding the interaction of these viral proteins with platelets in this study, we demonstrated using flow cytometry that full-length spike protein and RBD can induce platelet activation and degranulation. Initially, we observed that the full-length spike protein and the RBD domain induced the highest platelet activation at a concentration of 2 μg/mL. Our results concur with those reported by Zhang et al., who also used this concentration to perform platelet stimulation assays with spike protein and the S1 subunit of the protein [20]. Both the full protein and its domain were able to induce high expression of P-selectin and the glycoprotein IIbIIIa, known markers of platelet degranulation and activation, respectively [10,27]. Grobbelaar et al. previously reported that the spike subunit 1 (S1) of SARS-CoV-2 can induce fibrin resistance to fibrinolysis and induce platelet activation, contributing to clot

formation [28]. We are one of the first groups to report the ability of the RBD domain to induce platelet activation.

However, there is no consensus on the hypothesis that platelet activation in SARS-CoV-2 is due to the ACE2 receptor [20,21,29–31]. It has been reported that platelets do express ACE2 and that spike protein binding induces platelet activation and degranulation with the increased surface expression of P-selectin. Moreover, the expression of GP IIbIIIa induced by SARS-CoV-2 virions, contributing to enhanced thrombosis in COVID-19, has also been reported [20]. On the other hand, other groups have proposed different mechanisms of platelet activation in COVID-19 based on the expression of platelet EMMPRIM (CD147) [21], ACE2-independent mechanisms [29,30], and the presence of aberrant glycosylation in antibodies against spike [31]. Our results confirm that the spike full-length protein and the RBD actively participate in SARS-CoV-2 immunopathogenesis by inducing platelet activation and degranulation. This process may be mediated by not only one receptor but by the participation of different platelet surface membrane proteins. Further molecular docking studies may be useful to evaluate the variety of possible receptors on the platelet membrane surface to the spike protein and domain.

Platelet physiology indicates that activation is followed by an aggregation process that contributes to microthrombi formation. In order to address whether proteins can induce platelet aggregates, we incubated the platelets with the SARS-CoV-2 proteins. Our aggregation results indicate that these proteins cannot induce platelets' aggregation, but we report that platelets that were incubated with full-length spike protein and stimulated with low doses of collagen recovered aggregation capacity. This concurs with the results of Zhang et al., which showed that cells prestimulated with the spike protein or its subunits induce a hyperreactive state in platelets that can aggregate when low doses of known agonists are added [20]. Furthermore, there is evidence that platelets' responses to known aggregation agonists differ depending on the agonist and the dose used [32,33]. Differences in inducing platelet phenotype by virions, proteins, or domains may be due to alterations in cell proteome, which may explain why we observed recovery of aggregation when a strong agonist like collagen is used. This behaviour has been previously reported with other viral proteins. Garcia-Larragoiti et al. found that dengue virus non-structural protein 1 (NS1) induces platelet reactivity that favors the formation of platelet aggregates when low doses of epinephrine are added [22].

Hyperactivation of platelets in SARS-CoV-2 infection may not only contribute to thrombotic risk, but also to the inflammation process. COVID-19 patients with worse outcomes present high plasmatic levels of inflammatory cytokines and pro-thrombotic factors [33,34]. Platelets represent a rich source of these factors, since they contain granules with large amounts of molecules participating in inflammatory and hemostasis processes. Our results demonstrate that full-length spike protein and its RBD domain promote the release of pro-inflammatory factors in a time-dependent manner. We found differences in platelet response when comparing an inflammatory insult with an aggregating stimulus. This may be regulated by signaling pathways activated by platelets according to the stimulus that induces their activation [35]. It is known that the kinetics and concentration of molecules released by platelets depend on the activation agonist used, as well as the concentration of the same. Differences in the amount of these biomolecules released may be related to a selective degranulation process where these are stored, and our results contribute to the hypothesis of the presence of distinct subpopulations of α-granules in platelets [36,37]. On the other hand, our results are similar to those found by van Holten et al., who observed using immunoassays and proteomic assays that there is no homogeneous release profile of platelet granular content. Rather, it is a heterogeneous process that depends on the characteristics of the activation agonist used [38]. Variations in the concentrations of thrombotic inflammatory factors released by platelets following stimulation with known viral proteins and aggregation agonists may be associated with different aspects. The half-life of some of these factors is only a few minutes. Subsequent denaturation of these factors may be mediated by enzymatic mechanisms. Another important factor is the possible presence of soluble cy-

tokine receptors, generated by proteolytic cleavage of membrane receptors upon activation of the cells that express them. These receptors act as competitive inhibitors of these factors, decreasing their concentration [39]. In this area, we report that SARS-CoV-2 proteins induce the release of anti-fibrinolytic and pro-thrombotic factors by platelets. These results confirm that viral proteins in COVID-19 are able to induce a pro-inflammatory and pro-coagulant phenotype in platelets that may contribute to an immunothrombotic process. Our result concurs with previously reported works where platelets showed a procoagulant phenotype in COVID-19 [20,36,40]. On the other hand, platelets' differential response to other viruses has been reported. Assinger et al. demonstrated that human cytomegalovirus–platelet interaction induced a pro-inflammatory and proangiogenic response [41].

In conclusion, in this work we demonstrate the ability of platelets to actively participate in the immune response against SARS-CoV-2 structural proteins. These results sustain platelet's role in the development of a pro-inflammatory and pro-coagulant state. We found that platelets have a granular content release response that is differential according to the stimuli received. The response by platelets directed against the viral stimulus also favors the activity of other platelets, perpetuating this response. Our results confirm that platelets are important cells in the response against pathogenic agents; however, it is important to carry out more studies on the physiological response of platelets to pathological agents in the face of the immunological challenges that constantly arise today.

4. Materials and Methods

4.1. SARS-CoV-2 Spike Full-Length and RBD Domain Proteins

Recombinant SARS-CoV-2 full-length protein was provided by Bio Vision Human CellExp® (Waltham, MA, USA). Cat # P1525, Size: P1525-50 SARS-CoV-2 Spike protein, and the SARS-CoV-2 Spike protein RBD was provided by GenScript® (Piscataway, NY, USA). Cat # Z03491 SARS-CoV-2 spike protein (RBD, mFc Tag).

4.2. Blood Sample Collection

Whole human blood was obtained from healthy volunteers by clean venipuncture after signing the informed consent form. Healthy donors were individuals within a normal range of body mass index that were not taking any kind of medication and did not exhibit any abnormal findings on routine blood chemistry and hematological tests. All experiments were performed on donors who did not present previous exposure to SARS-CoV-2, previous exposure of at least 1 year, or exposure to SARS-CoV-2 vaccines, to ensure the absence of antibodies to the virus or its viral proteins that could interfere with platelet stimulation assays. Blood was obtained by clean venipuncture and the first tube (2 mL) of blood was discarded to ensure the accuracy of platelet testing. Samples were collected in vacutainer tubes with 3.2% (0.109 mol/L) sodium citrate solution as an anticoagulant (Becton Dickinson) and processed within 4 h after collection.

4.3. Platelet Rich Plasma Isolation

Platelet-rich plasma (PRP) was obtained by centrifuging at 100× *g* for 10 min at room temperature (RT). The supernatant was carefully collected to avoid disrupting the buffy coat and it was left to rest for 30 min in darkness before analysis by flow cytometry.

4.4. Platelet Stimulation with Spike SARS-CoV-2 and RBD Domain

Platelet-rich plasma was adjusted to a concentration of 1×10^7 with Tyrode's buffer to maintain the integrity of the platelets. They were incubated directly with SARS-CoV-2 full-length spike protein and RBD for different concentrations (0.5, 1.0, and 2.0 µg/mL) to determine the best protein concentration to stimulate platelets. Subsequently, PRP was stimulated with viral proteins at a concentration of 2 µg/mL for a minimum of 30 min to a maximum of 120 min at a temperature of 37 °C in individual assays. Platelet activation was confirmed by activation of the heterodimeric complex GP IIbIIIa (PAC1-FITC) and expression of P-selectin (CD62-PE) determined by flow cytometry. The supernatant was

obtained after stimulation and stored at −70 °C until use for immunothrombotic biomarker determination. Assays were designed based on previous published studies using different concentrations of SARS-CoV-2 spike full-length protein, its domains and subunits, as well as different incubation times [20,21,28,42].

4.5. Flow Cytometry Assays

CD41/PECy7 (BioLegend Cat. No. 303718) was used as an identity marker for platelets, PAC-1/FITC (BioLegend Cat. No. 362804) for glycoprotein GP IIbIIIa and CD62/PE (BioLegend Cat. No. 304906) for P-selectin were used as activation markers. IgG1 k (BioLegend Cat. No. 400125), FITC Mouse IgM k Isotype (BioLegend Cat. No. 401605) and Mouse IgG1 k Isotype (BioLegend Cat. No. 400111) were used as isotype control, respectively. The gating strategy of the cell populations was performed according to previously reported by the research group in García-Larragoiti et al. [22]. Dark conditions and minimal handling were used during the assay to avoid external activation of platelets. Adenosine Di Phosphate (ADP) (20 µM) for 20 min, collagen (20 µM) for 30 min, and epinephrine (EPI) (100 µM) for 40 min were used as positive platelet activation controls [27]. Concentrations were used following the instructions suggested by the supplier PAR/PAK II® BIO/DATA CORPORATION (Horsham, PA, USA). The acquisition was performed in a CytoFLEX, BECKMAN COULTER® (Brea, CA, USA). Results were analyzed using FlowJo v 10.8.0.

4.6. Determination of Immunothrombotic Biomarkers in Platelet Stimulated Supernatant

As previously described, PRP stimulated within viral proteins during different times was carefully collected with a pipette. Separate assays of PPR treated with ADP (20 µM) for 20 min, collagen (20 µM) for 30 min, epinephrine (100 µM) for 40 min, Tyrode's buffer, and unstimulated PRP were used as controls. A panel of 10 thrombotic-inflammatory biomarkers was analyzed: Interleukin 6 (IL-6), Interleukin 8 (IL-8), P-selectin, P-selectin ligand 1 (PSGL-1), sCD40L, D-dimer, tissue plasminogen activator (tPA), tissue plasminogen activator inhibitor 1 (PAI-1), tissue factor and coagulation factor IX. Determination of these biomarkers was performed by flow cytometry using the BioLegend® (San Diego, CA, USA) LEGENDplex Kit TM Human Thrombosis Panel Standard following the instructions suggested by the supplier. Samples were read in a CytoFLEX, BECKMAN COULTER®. Briefly, samples were incubated with beads that differed in size for 2 h. Each bead group was conjugated with specific antibodies on its surface that captured a specific analyte. After washing, a cocktail of biotinylated detection antibodies was added for 1 h, forming a detection complex between the capture bead–analyte–detection antibody. Finally, Streptavidin-phycoerythrin (SA-PE) was added and incubated for 30 min. Samples were taken to the CytoFLEX cytometer for analysis.

4.7. Light Transmission Aggregation Assays

Whole blood was obtained from healthy donors who did not present previous exposure to SARS-CoV-2 or previous exposure of at least 1 year in tubes with sodium citrate (3.2%). PRP was obtained under the conditions described above and incubated for 30 min with SARS-CoV-2 full-length spike protein and for 120 min with RBD both at a concentration of 2 µg/mL in separate assays. Platelet-poor plasma (PPP) was separated by centrifugation at 2500× *g* for 15 min and used as a blank. Subsequently, 0.5 mL of the PRP previously incubated with the SARS-CoV-2 proteins and 0.5 mL of unstimulated PRP were placed in the cuvette of the aggregometer, containing a siliconized magnetic bar, at a constant temperature of 37 °C; the results were compared against positive controls (PRP + ADP 20 µM, PRP + Collagen 20 µM, PRP + Epinephrine 100 µM). To investigate if the addition of minimal doses of known aggregation agonists in platelets previously treated with SARS-CoV-2 proteins could improve platelet aggregation, doses of ADP (2.0 µM), collagen (2.0 µM), and epinephrine (10 µM) were added. Assays were performed by triplicate. Light transmission was measured on a Chronolog 560ca aggregometer (Chrono-log). All data were analyzed with AGGRO/LINK®8 software.

4.8. Statement of Ethics

All the studies with healthy donors were approved by the ethics committee of the Faculty of Medical and Biological Sciences "Dr. Ignacio Chávez" of the UMSNH registration number 004/P/5/2021 and the General Hospital "Dr. Miguel Silva"; registration number CEI/2021/III-269. Morelia, México.

4.9. Statistical Analysis

Comparisons between two groups were made with an unpaired *t*-test. Multiple group comparisons were made using a one-way analysis of variance. Tukey's test was used as a post hoc test for pairwise comparisons. The data that were not normally distributed were analyzed using nonparametric statistical analysis. All experiments were independently repeated three times. $p < 0.05$ was considered statistically significant. All data were presented as means ± standard deviation. Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, Inc, San Diego, CA, USA).

Author Contributions: M.E.V.-S. designed the research; M.E.V.-S. and A.O.-Z. conceived and designed the experiments; A.C.-M., N.G.-L. and M.D.-V. performed the flow cytometry experiments and data analysis; S.L.-C. participated in research design and ethical approvals; A.C.-M., M.D.-V. and P.G.-C. designed and performed platelet aggregation experiments; M.E.V.-S. and N.G.-L. performed data analysis; A.C.-M. wrote the original manuscript and M.E.V.-S., N.G.-L., S.L.-C.; and A.O.-Z. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by CIC-UMSNH and CONACyT México in its call for proposals "Ciencia de Frontera: Paradigmas y controversias 2021", grant number 320085. A. Cano-Méndez and P. Guzmán-Cancino are recipients of CONACYT-Mexico fellowships for post-graduate students. The sponsors of this study are public or non-profit organizations that support science in general and had no role in gathering, analyzing, or interpreting the data.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board and Ethics Committee of UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO. Facultad de Ciencias Médicas y Biológicas "Dr. Ignacio Chávez". Protocol code CEI/2021/III-269, 10 March 2021.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

Acknowledgments: R. Villa, D. Hernández-Hernández, and A.G. Vargas-Ruíz Laboratorio de Trombosis, Departamento de Hematología y Oncología, Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán".

Conflicts of Interest: The authors declare no conflict of interest.

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VIII.II. *Capítulo 2*: Los resultados de este capítulo se encuentran en el manuscrito: "*Endothelial cell activation by SARS-CoV-2 Spike protein and its RBD domain: central player in the establishment of the immunothrombotic response in COVID-19".*

Endothelial cell activation by SARS-CoV-2 Spike protein and its RBD domain: central player in the establishment of the immunothrombotic response in COVID-19.

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Abstract

Background: COVID-19, caused by SARS-CoV-2, has been associated with an active immunothrombotic process with an acute inflammatory state, exacerbated coagulation and endothelopathy. The mechanisms by which endothelial activation takes place in COVID-19 have been linked to direct interaction with the SARS-CoV-2 Spike (S) protein with endothelial receptors. ACE-2 has been proposed as the principal endothelial cell receptor.

Aim: To assess the immunothrombotic phenotype induced in endothelial cells by protein S and the RBD domain.

Methods: Human endothelial cells HUVEC and EAhy926 were exposed to SARS-CoV-2 protein S and to the RBD domain. Inflammatory, thrombotic and fibrinolytic mediators were determinate in the supernatant by an immunoenzymatic linked assay using flow cytometry.

Results: Activation of both cell lines was protein concentration and time of stimulation dependent. Increased production of IL-6, IL-8, P-selectin, PSGL-1, MCP-1 and sCD40L; as well as TF, PAI-1, tPA, D-D and vWF were observed when compared to control. The concentrations of immunothrombotic biomarkers were similar to those observed when treating endothelial cells with immune insults such as IL-6 and LPS and with known endothelial activation agonists such as VEGF. Through molecular docking assays we suggest different possible receptors present in ECs that may interact with protein S favoring the activation of these cells.

Conclusion: We confirm that S and RBD induce an immunothrombotic phenotype in ECs, characterized by the release of proinflammatory and prothrombotic mediators. We propose different endothelial receptors that may be the activation pathway in these cells.

Key words: Endothelial cell, SARS-CoV-2, spike protein, RBD, Immunotrombosis,

1. Introduction

COVID-19 disease has been circulating worldwide since 2020 [1,2], affecting millions of people. Vaccines have decreased morbidity and mortality [3–6], but not the ongoing spread of infection, which remains recurrent and intermittent among the population, with cases of severe illness and even associated deaths reported [7,8].

SARS-CoV-2 uses the Spike (S) protein as the main route of entry to host cells [9]. The S protein is an homotrimer located in the viral membrane, and possesses several glycosylation sites [10]. Several mutations have been reported in this protein leading to the different variants of the virus that have circulated around the world [11]. Protein S is divided in subunit 1 (S1) used by the virus to infect host cells and subunit 2 (S2) which is attached to the viral membrane [12,13]. The spike protein possesses a domain located in the S1 subunit known as receptor binding domain (RBD) which interacts with specific receptors on target cells [14]. The main receptor used by this virus is Angiotensin Converting Enzyme II (ACE-2), a protein widely expressed in several cells of the human body [9,10]. The vascular endothelium it's an ACE2-rich tissue, this inevitably compromises the infection and activation, leading to a dysfunction in the vascular physiology [15,16]. Alternative ACE-2 receptors have been proposed in various cells that can be used by the virus to achieve its internalization and infection [17,18].

Among the clinical manifestations described during the course of the infection, it is well known that pneumological and respiratory alterations are the most frequently reported. However, COVID-19 is also a vascular disease [19]. There are several reports that directly link hemostatic and endothelial abnormalities with the severity of the disease in COVID-19 patients [20]. Also, the persistence of symptoms in subjects recovered from COVID-19, a syndrome known as Long COVID, has also been associated with endothelial dysfunction, coagulation disorders and blood circulation of S protein [21–23].

Understanding the endothelial response to SARS-CoV-2 structural proteins could help us to understand the pathophysiological role of ECs during the endothelial dysfunction observed in COVID-19. The aim of this work was to study the ECs response to the SARS-CoV-2 full-length Spike protein and to the RBD domain.

2. Methods

2.1. SARS-CoV-2 Spike Protein and Receptor Binding Domain

Recombinant SARS-CoV-2 Spike Full-Length was provided by Bio Vision Human CellExp® (Waltham, MA, USA). Cat # P1525, Size: P1525-50 and the SARS-CoV-2 Spike protein RBD Domain was provided by GenScript® (Piscataway, NY, USA). Cat # Z03491 SARS-CoV-2 spike protein (RBD, mFc Tag). Both proteins belonged to the ancestral lineage.

2.2. Cell Culture

For this work, two well-characterized endothelial cell lines were used to analyze the effects of both viral proteins and to compare to other stimuli´s. The Human Umbilical Vein Endothelial Cell (HUVEC) was purchased from Lonza and maintained in commercial complete endothelial growth medium-2 (EGM-2) (Lonza, Basel, Switzerland).

The Human Endothelial Cell Line EAhy926 (line established by primary fusion of human umbilical cord cells with clone A549 from lung adenocarcinoma) was generously donated by Dr. Bruno Rivas Santiago from Centro de Investigación Medica Zacatecas, Instituto Mexicano del Seguro Social (IMSS), Zacatecas, México. EAhy926 cells were routinely cultured in *Dulbecco's Modified Eagle Medium* (DMEM) (Gibco), supplemented with Fetal Bovine Serum (FBS) (10% v/v), (Gibco), penicillin (100 U/ml), streptomycin (100 µg/ml), and Amikacin Sulphate (0.1 µg/ml) (Thermo Fisher Scientific) and Vascular Endothelial Grow Factor (VEGF) supplement form bovine pituitary (3 mg/ml) (Sigma).

Both cell lines were maintained in 25 cm^2 tissue culture-treated flasks in an atmosphere of 5% CO_2 at 37°C. Medium renewal was performed every 2 days and cells were subculture when they became 80% confluent. All the experiments were performed using cells between passages 5 and 8 and done by triplicate.

2.3. Inflammatory and thrombotic stimulus

The following proteins were used as control inflammatory stimulus: Tumor Necrosis Factor (TNF-α) from (Invitrogen Thermo Fisher, KHC3011), Interleukin 6 (IL-6) from (Novex Lifetech), and Lipopolysaccharide (LPS) was obtained from Gibco, Thermo Fisher Scientific (*Waltham, MA USA*). Vascular Endothelial Grow Factor (VEGF) (Sigma) was

used as an endothelial cell activation control, and von Willebrand Factor (vWF) from (IMUBIND Biomedics Diagnosis), Adenosine Di Phosphate (ADP), and epinephrine (EPI) (Chrono-log), were used as prothrombotic internal stimulus.

Dengue Virus serotype 2 Non-structural protein 1 (DENV2 NS1) was generously donated by the Oxford University and used as internal viral protein control. Production and purification of DENV-2 and Asian lineage ZIKV NS1 proteins were carried out by standard Polyethyleneimine (PEI) transfection at 37 °C as previously described [24].

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2.4. EAhy926 and HUVEC stimulation assays

Briefly, cells (10 x 10^3 cell/well) were seeded and grown in a 96 cell-treated plate for 24 h at conditions previously mentioned. Further, 100 µL of fresh medium was added. In individual assays, cells were incubated directly with SARS-CoV-2 full-length spike protein and RBD protein for different concentrations (0.25, 0.5, 1.0, and 2.0 µg/ml) and different times (30, 60 and 120 min) in order to determine the best conditions of endothelial cell activation. At the same time, endothelial cells, in independent assays, were stimulated with TNF-α (20 ng/µl), IL-6 (20 ng/µl), LPS (100 ng/µl) and DENV2 NS1 (2.5 µg/ml), VEGF (10 ng/µl), vWF (10 µg/µl), ADP (20 µM) and EPI (100 µM) as a thrombotic factor. Different stimuli were assessed in order to determinate the differential endothelial cells response and compare them to the Spike and the RBD domain induce response. Concentrations of inflammatory and procoagulant controls were based on previous reports [24–28]. All experiments were performed at 5% CO_2 humidified incubator at 37°C,

After the incubation, the supernatant of the endothelial cells was obtained and stored at −80 ◦C until use for immunothrombotic biomarker determination.

2.5. Assessment of Immunothrombotic Biomarkers

The following immunothrombotic biomarkers were analyzed by flow cytometry: D dimer, type 1 plasminogen activator inhibitor (PAI-1), tissue factor (TF), tissue plasminogen (tPA), coagulation factor IX (F IX), Interleukin 6 (IL-6), Interleukin 8 (IL-8), P-selectin (P-sel), P-selectin glycoprotein ligand-1 (PSGL-1), soluble CD40 Ligand (sCD40L) and *monocyte chemoattractant protein-1 (MCP-1)*. Cell culture supernatant assessment of biomarkers was performed by flow cytometry using the LEGENDplex Kit™

Human Thrombosis Panel Standard and the LEGENDplex Kit™ Human Inflammation Panel 1 both from BioLegend® following the instructions suggested by the supplier. The samples were read on a CytoFLEX equipment, BECKMAN COULTER®.

Briefly, sample was incubated with beads that are differentiated by size. Each bead is conjugated with specific antibodies on its surface that acts as the capture bead for that particular analyte. After washing, a biotinylated detection antibody cocktail is added, and each detection antibody in the cocktail will bind to its specific analyte bound on the capture beads, forming a capture bead-analyte-detection antibody sandwich. Streptavidinphycoerythrin (SA-PE) is subsequently added and samples were taken to the CytoFLEX equipment for analysis.

2.6. von Willebrand Factor determination

von Willebrand Factor was assessed in cell culture supernatant samples using the ab223864 Human Von Willebrand Factor SimpleStep ELISA® Kit. This assay employs a tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the analyte in the sample. The complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. After incubation. TMB Development Solution is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is measured at 450 nm. All assays were performed following specifications and recommendations issued by the supplier.

2.7. Molecular docking assays

The structures of the following molecules: Spike full-length ancestral lineage, ACE-II, Thrombomodulin (CD-141), Basigin (CD-147), Neuropilin 1, Interleukin 6 receptor (IL-6R), Integrin α5β1 and Toll Like Receptor (TLR) 2, 4 and 7 were obtained from RCSB Protein Data Bank (https://www.pdb.org) and imported into AutoDockTools 1.5.6 for docking. We used AutoDockTools to process the proteins by removing ligands, correcting the protein structure, and eliminating the water molecules associated with the protein structure. The PDBQT format was used to perform the docking by AutoDock-Vina and the

blank screen CMD of the system. Structures were visualized using PyMol (version 1.7.2.1).

2.8. Statistical Analysis

All experiments were independently repeated at least three times. Results of multiple experiments are expressed as mean ± standard error (S.E). Multiple group comparisons were made using a one-way analysis of variance (ANOVA). Tukey's test was used as a *post hoc* test for pairwise comparisons. $p<0.05$ values were considered statistically significant in all cases. Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, Inc, San Diego, CA, USA).

2.9. Ethics

Since this study does not involve humans, human data or animals, an ethics committee approval was not required.

3. Results

Acute exposure to the full-length Spike protein and to its RBD increases the expression of endothelial cell activation markers, favoring an immunothrombotic phenotype characterized by the release of proinflammatory, prothrombotic and antifibrinolytic molecules.

3.1 EAhy926 and HUVEC ECs activation its Spike and RBD concentration and time dependent

In order to assess whether the Full-length Spike protein and the RBD domain could induce endothelial cell activation *in vitro*, different concentrations of these proteins (0.25, 0.5, 1, 2 µg/ml) and different times (30, 60, 120 min) were used.

To determinate the protein concentration and time at which endothelial cells release the highest amount of soluble factors indicative of cellular activity, we performed endothelial cell stimulation kinetics and evaluated the supernatant concentrations of IL-6, PAI-1 and tPA, proteins biologically produced by the endothelium. According to our results, it is possible to determine that protein S at a concentration of 0.25 µg/ml for a

stimulation time of 60 min is able to induce the highest amount of IL-6, PAI-1 and tPA, suggesting activity of EAhy926 cells. On the other hand, the RBD domain requires a concentration of 1.0 µg/ml at a stimulation time of 60 min to achieve this response when compared to other conditions $p<0.05$ (Fig. 1 A-C).

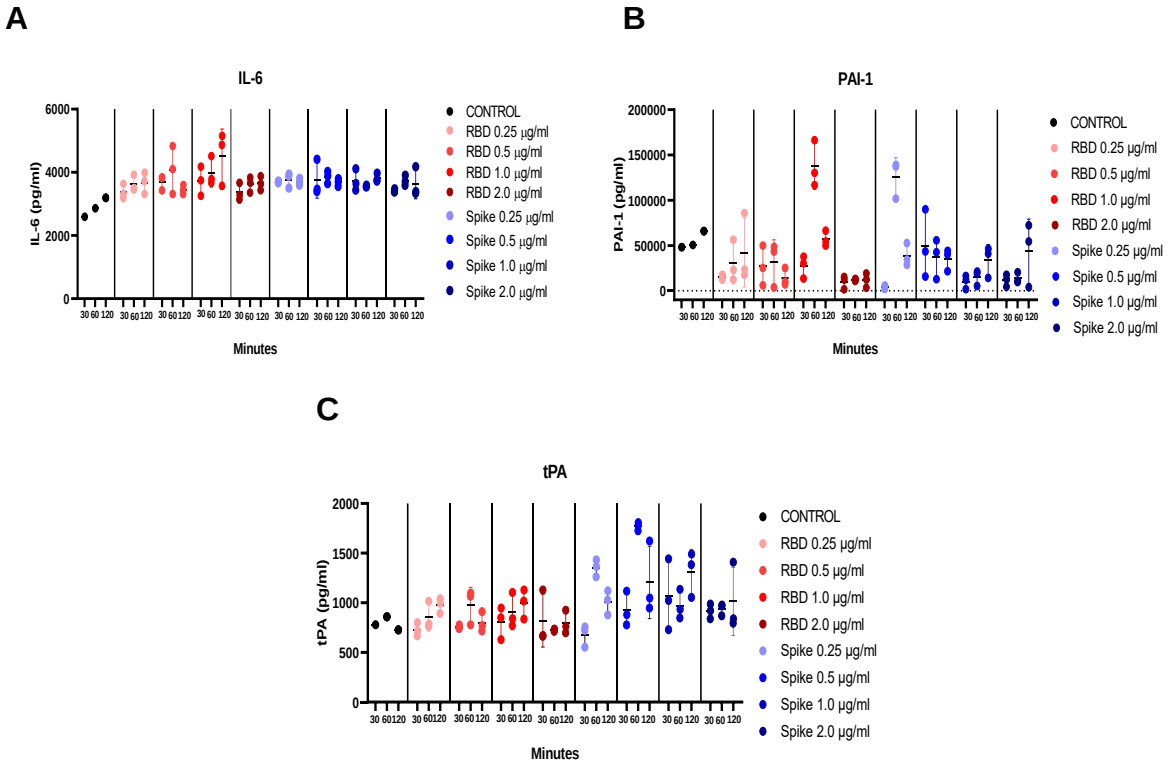


Figure 1. Stimulation of EAhy926 endothelial cells with full-length spike protein and RBD domain. Endothelial cells were incubated with different protein concentrations 0.25, 0.5, 1.0, 2.0 µg/ml for 30, 60 and 120 min at 37℃, 5% CO_2 with spike protein and RBD domain. (A) Release of IL-6 from endothelial cell after incubation with both proteins under different incubation conditions. (B) Concentration of PAI-1 assessed in the supernatant of endothelial cell after concentration and time. (C) Concentration of tPA in the supernatant determined after stimulation with SARS-CoV-2 viral proteins. ($p<0.05$).

In addition, we decided to assess a non-transformed endothelial cell line in order to investigate whether there were differences in the response to SARS-CoV-2 viral structural proteins. Similar results were observed when we evaluated reactivity in the HUVEC cell line. Based on our results, it is possible to determine that the highest inflammatory activity induced by Spike protein in the HUVEC cell line is observed at 60

min at a final concentration of 0.25 µg/ml. On the other hand, the RBD domain requires concentrations of 1.0 µg/ml at 60 min to induce the highest IL-6 release by HUVEC cells (Fig 2A). Similarly, at these concentrations the highest release of tPA and PAI-1 were determined, suggesting that these are the conditions at which the highest endothelial activity is observed (Fig 2B-C) ($p<0.05$).

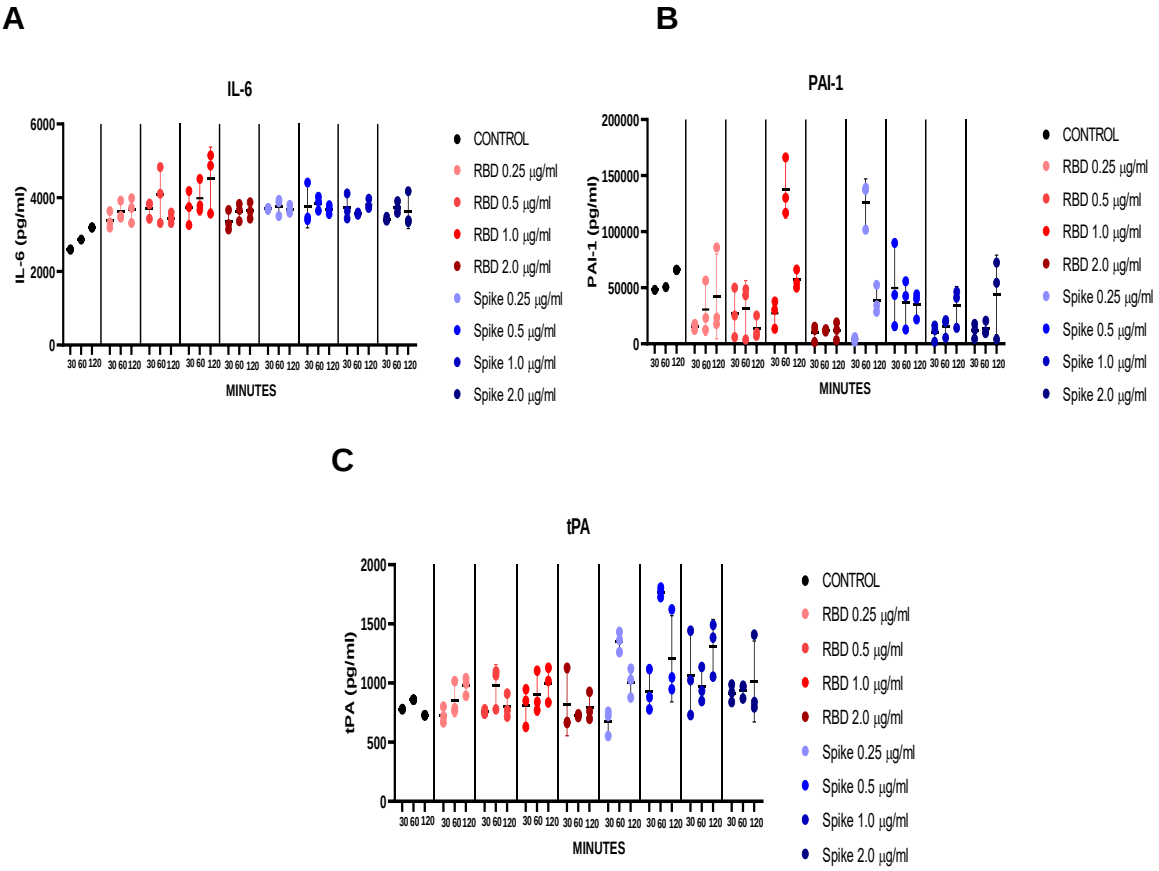
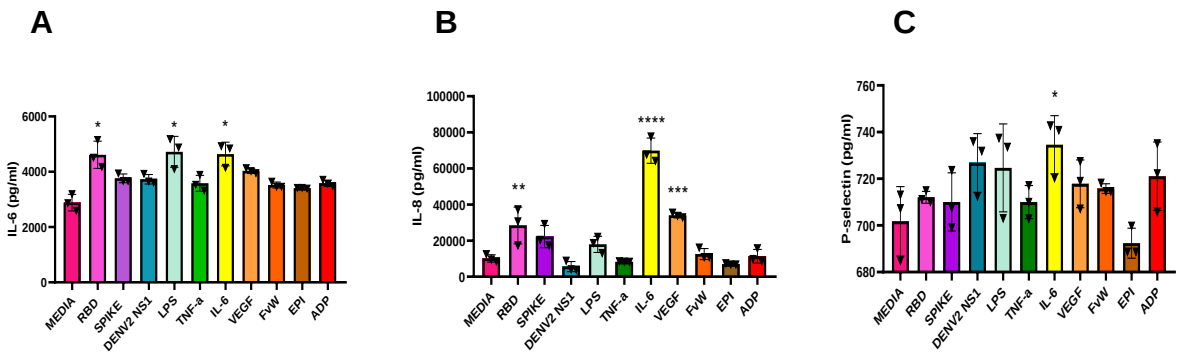


Figure 2. HUVEC endothelial cells time and concentration-dependent activation against full-length spike protein and RBD domain. HUVEC cells were incubated with different protein concentrations 0.25, 0.5, 1.0, 2.0 µg/ml for 30, 60 and 120 min at 37°C, 5% CO_2 with spike protein and RBD domain. (A) Release of IL-6 from HUVEC cell line after incubation with both proteins under different protein concentrations and times. (B) Concentration of PAI-1 assessed in the supernatant of endothelial cell after concentration and time. (C) tPA concentrations in the supernatant determined after stimulation with Spike protein and its RBD domain ($p<0.05$).

3.2 Spike and RBD stimulates the in vitro proinflammatory phenotype of EAhy926 ECs

Once we determined that spike protein and its RBD domain were able to induced EAhy926 release of inflammatory cytokines and antifibrinolytic proteins suggesting cell activation, we decided to look further and try to decipher the immunothrombotic phenotype present in these cells. We assessed different inflammatory biomarkers in the supernatant of the endothelial cell after stimulated with each SARS-CoV-2 protein.

Our results show an increase in IL-6 secretion in EC incubated with the RBD domain, when compared to the basal state ($p<0.05$). Not statistical differences were found when ECs were treated with spike protein. Interestingly, ECs stimulated with LPS and IL-6 also showed an increase secretion of IL-6 ($p<0.05$), similar to that observed when incubated with the RBD domain (Figure 3A). RBD domain was able to induce EC release of IL-8 ($p<0.01$), this was not observed when ECs were observed with spike protein. VEGF and IL-6 induced higher release of IL-8 when compared to basal state or with another inflammatory to thrombotic stimulus ($p<0.001$) (Figure 3B). When assessing P-sel, its ligand PSGL-1 and sCD40L, SARS-CoV-2 viral proteins did not induce significant differential release of these proteins, only ECs treated with IL-6 showed higher concentrations of these proteins ($p<0.05$) (Figure 3C-E). Finally, only VEGF, and EC know activation protein, induce the release of MCP-1 by EA-hy296 ($p<0.05$) (Figure 3F).



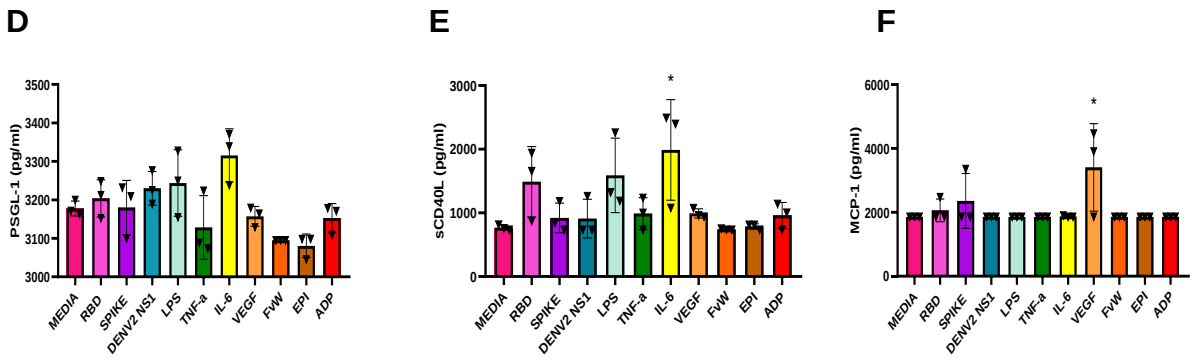


Figure 3. EAhy926 were incubated for 60 min in the absence (media) or in the presence of 0.25 µg/ml of SARS-CoV-2 spike protein and 1.0 µg/ml of RBD, and known inflammatory and thrombotic agonists: DENV NS1 (2.5 µg/ml), LPS (100 ng/µl) TNF-α (20 ng/µl), IL-6 (20 ng/µl), VEGF (10 ng/µl), vWF (10 µg/µl), Epinephrine (100 nM) and ADP (20 nM) for 60 min. The amount of the indicated cytokines and soluble proteins were measured by a bead based immunoenzymatic assay, as described in Materials and Methods. Data are means ± S.D of three independent determinations, each represented by the different dots. Differences were considered significant when compared against control * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ANOVA *pos hoc* Tukey.

3.3 Spike and RBD induces the expression of procoagulant and antifibrinolytic factors in EAhy926 ECs

Next, to address the role of EAhy926 ECs in the pro-thrombotic state observed in COVID-19, we assessed concentrations of D-dimer, coagulation factor IX, PAI-1, tPA, TF and vWF (Figure 4A-F). The amount of D-dimer was significantly increased upon exposure to LPS and IL-6. Viral proteins induce an increase of D-dimer concentrations, but was not statistically different. No differences in FIX concentrations were observed, this result can be considered an internal experimental control, since ECs do not contain or produce FIX. Under the same conditions, the concentration of PAI-1 and tPA show a very characteristic pattern of behavior. Viral proteins, and inflammatory stimuli such as TNF-α, as well as VEGF induced an increase in PAI-1 release ($p<0.001$). On the other hand, tPA concentrations behaved inversely proportional to that observed in PAI-1 determination. These results confirm an antifibrinolytic state induced by viral proteins or ECs activations proteins. No differences were observed when comparing TF concentrations. Finally, vWF

was also higher when ECs were stimulated with TNF-α ($p<0.05$).

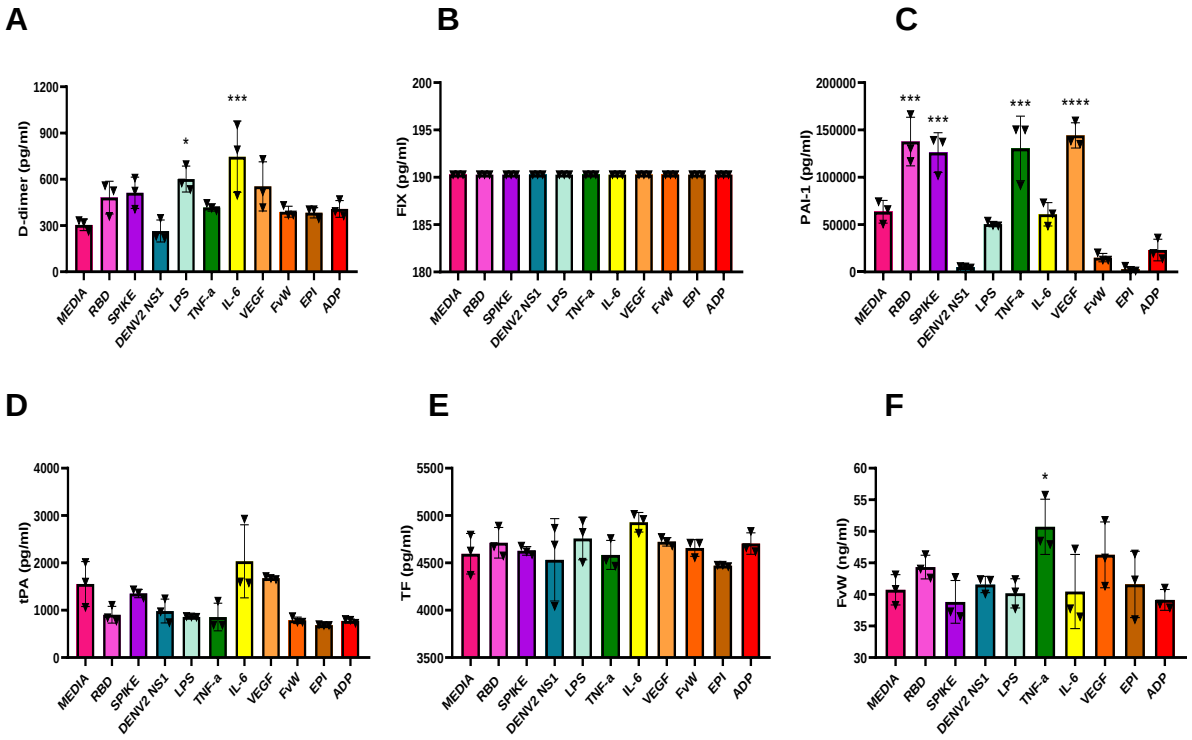


Figure 4. S-protein and RBD induce the expression and release of procoagulant and antifibrinolytic proteins in EAhy926. ECs were treated with 0.25 µg/ml of SARS-CoV-2 spike protein and 0.1 µg/ml of RBD and know activation agonists DENV NS1 (2.5 µg/ml), LPS (100 ng/µl) TNF-α (20 ng/µl), IL-6 (20 ng/µl), VEGF (10 ng/µl), vWF (10 µg/µl), Epinephrine (100 nM) and ADP (20 nM) for 60 min. Results were compared against basal behavior of ECs (media). Levels of D-dimer, FIX, PAI-1, tPA, TF in culture supernatants were quantified by LEGENDPlex assays and vWF was assessed by ELISA. Data presented as mean ± S.D. * $p<0.05$ ***; $p<0.001$; **** $p<0.0001$. ANOVA *pos hoc* Tukey.

3.4 HUVEC ECs release inflammatory cytokines when stimulated with SARS-CoV-2 proteins

Since we observed that protein S and its RBD induced the release of proinflammatory cytokines in the EAhy926 cell line, we proceeded to assess the inflammatory response of HUVEC cells to viral proteins and different immune and prothrombotic insults.

Based on our results, it is possible to observe that SARS-CoV-2 proteins promote IL-6

generation in HUVECs. These results are similar to those observed when treated with other inflammatory and procoagulant stimuli (Fig 5A). On the other hand, protein S enhanced IL-8 release, a result not observed when cells were treated with RBD. The concentrations of IL-8 induced by protein S were similar to those observed when HUVECs were treated with LPS and DENV2 NS1; however, they were lower than those generated by IL-6 and VEGF, agonists of endothelial activation (Fig 5B). Figure 5C-D shows the concentrations of P-sel and the soluble fraction of its ligand (PSGL1) released by HUVECs treated with the different proteins in contrast to the basal state. It is possible to observe that SARS-CoV-2 proteins promoted the generation of these proteins. In the case of P-sel they induce the generation of a higher concentration when compared to other inflammatory, viral and procoagulant stimuli. On the other hand, the PSGL1 released when cells are treated with virus proteins are similar to those observed when HUVECs are stimulated with another viral protein such as DENV2 NS1. Only the RBD domain and VEGF stimulated a significant production of MCP-1 (Fig 5E). Finally, when measuring the concentrations of sCD40L, figure 5F shows that only the RBD domain and the proteins LPS, TNF- α and IL-6 promoted its generation.

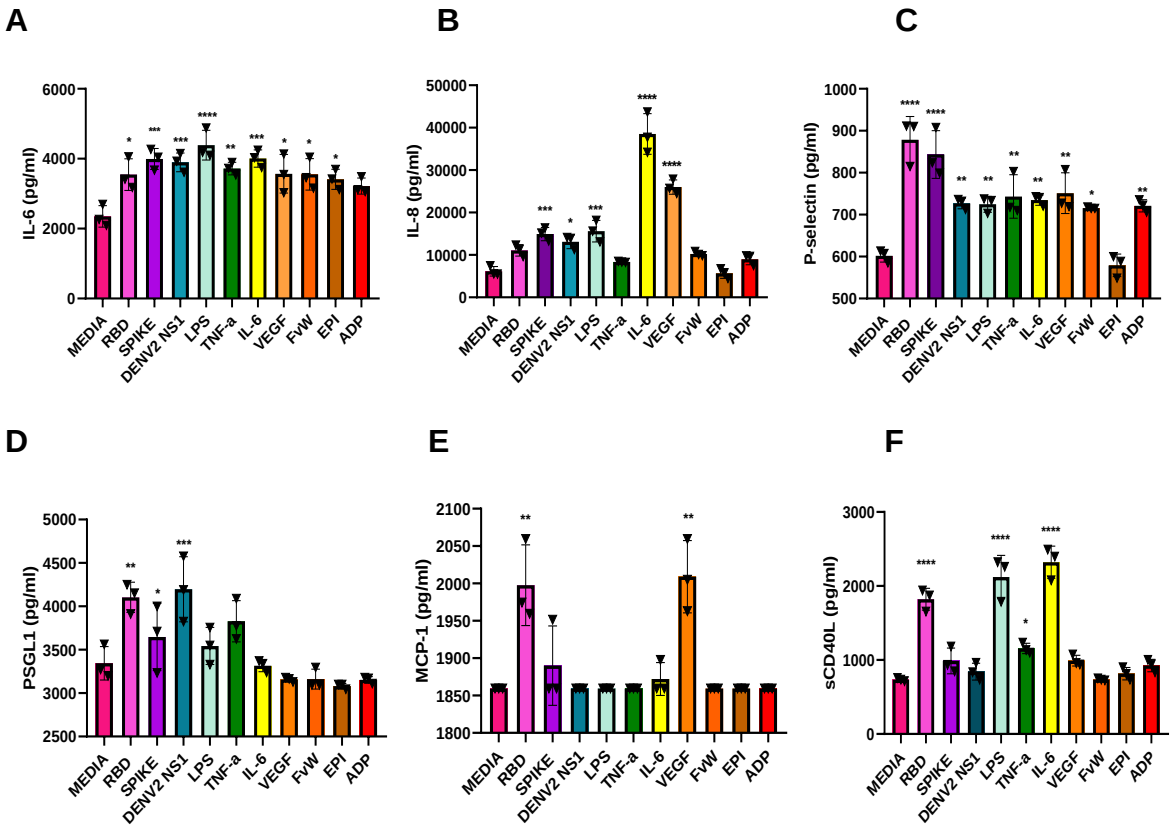


Figure 5. SARS-CoV-2 spike protein and its RBD domain induces HUVEC inflammation. HUVEC were incubated for 60 min with a concentration of 0.25 µg/ml of spike protein and 1.0 µg/ml of RBD domain for 60 min and compared against untreated (media) and treated cells with DENV NS1 (2.5 µg/ml), LPS (100 ng/µl) TNF-α (20 ng/µl), IL-6 (20 ng/µl), VEGF (10 ng/µl), vWF (10 µg/µl), Epinephrine (100 nM) and ADP (20 nM). Figures A-F represents different inflammatory proteins release when treated with the viral proteins and compared against ECs untreated (media). Inflammatory and procoagulant stimuli were included in order to assess differential endothelial cell response. Data are expressed as media ± S.D; p Differences were considered significant when compared against control * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ANOVA *pos hoc* Tukey.

3.5 SARS-CoV-2 proteins induce a prothrombotic and antifibrinolytic profile in HUVEC ECs

Once the inflammatory response in HUVECs by SARS-CoV-2 proteins was established, we decided to explore whether thrombotic and fibrinolytic response was affected. Based on our results it is possible to determine that there is a procoagulant phenotype and a dysregulation of the fibrinolytic response in HUVECs after treatment with inflammatory stimuli.

In figure 6A it is possible to observe that proteins S and RBD did not promote the generation of d-dimer; however, inflammatory stimuli such as LPS, IL-6 and VEGF did enhance their genesis. No changes were observed in the concentrations of coagulation FIX (Figure 6B), since it is not a protein contained or produced by the vascular endothelium. Interestingly, Figures 6C-D demonstrate a dysfunction in the fibrinolytic mechanisms, based on the differences in the concentration of proteins implicated in these processes. It is possible to determine that SARS-CoV-2 proteins favor the release of PAI-1, similar to that observed with other proinflammatory proteins. When assessing tPA, the protein on which PAI-1 acts, it is possible to observe decreases in the concentrations released by HUVECs when contrasted against untreated cells. Neither the S protein nor the RBD domain induced the expression of TF or vWF. Only the dengue NS1 protein, bacterial LPS, and IL-6 favored the release of TF (Fig 6E). No substantial differences were observed in the determined concentrations of vWF (Fig 6F).

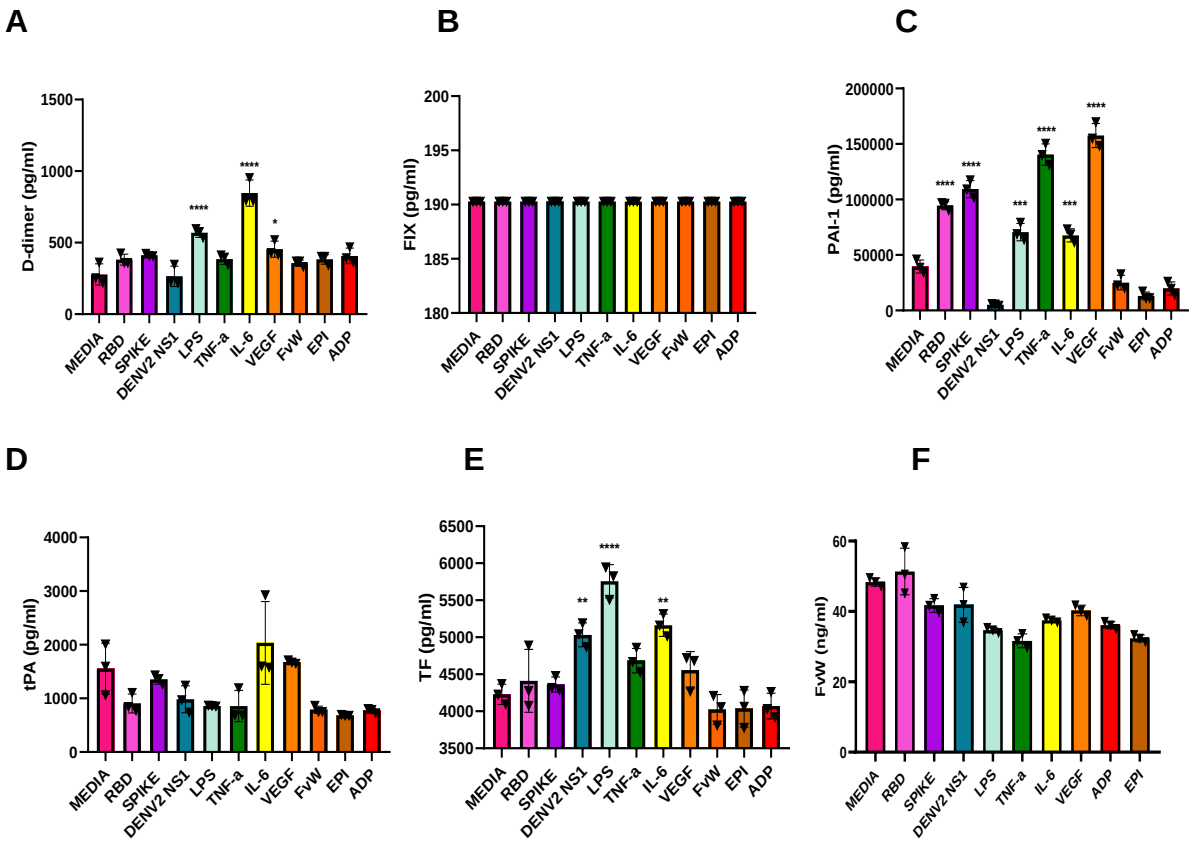


Figure 6. SARS-CoV-2 spike protein and RBD exacerbate the expression and release of procoagulant and antifibrinolytic proteins in HUVEC cell line. ECs were treated with 0.25 µg/ml of SARS-CoV-2 spike protein and 0.1 µg/ml of RBD and compared against untreated cells and know activation agonists: DENV NS1 (2.5 µg/ml), LPS (100 ng/µl) TNF-α (20 ng/µl), IL-6 (20 ng/µl), VEGF (10 ng/µl), vWF (10 µg/µl), Epinephrine (100 nM) and ADP (20 nM). Levels of D-dimer, FIX, PAI-1, tPA, TF and vWF produced by HUVEC are shown. Data presented as mean ± S.D. * p<0.05 ***; p<0.001; **** p<0.0001. ANOVA *pos hoc* Tukey.

3.6 Full-length Spike protein and its RBD domain could interact with different endothelial cell receptors in addition to ACE-II

According to our results, Spike protein could match with alternative endothelial cell receptors besides the ACE-II. In the present work we assessed the possible Spike protein interactions with different endothelial cell receptors. The docking score determines the possible interaction between molecules. A more negative value means a significant

probability of interaction between molecules. Another relevant value in molecular docking assays is the confidence score, which, values close to 1 suggest that the process occurs under the *in-silico* conditions used. We used ACE-II as docking internal control. CD-141, CD-147, Interleukin 6 receptor and TLR´s 2,4, and 7 showed values of docking score with the Spike protein even better than those reported with ACE-II reporting also good confidence scores, all above 0.9. On the other hand, interaction between the viral protein with Neuropilin 1 and Integrin α5β1 showed values similar to those found when matching with ACE-II. Likewise, the confidence scores showed results higher than 0.86 (Table 1). Possible interactions sites between Spike protein and endothelial cell receptors are shown (Figure 7).

Table 2

Spike-endothelial cell affinity obtained from molecular docking

Endothelial cell receptor	**Docking score**	**Confidence score**
ACE-II	- 291.62	0.9444
Thrombomodulin (CD-141)	- 335.25	0.9760
Basignin (CD-147)	- 302.45	0.9547
Neuropilin 1	- 250.09	0.8810
Integrin α5β1	- 241.22	0.8611
IL-6R	- 328.49	0.9726
TLR-2	- 340.98	0.9785
TLR-4	- 332.87	0.9748
TLR-7	- 398.33	0.9931

Figure 6

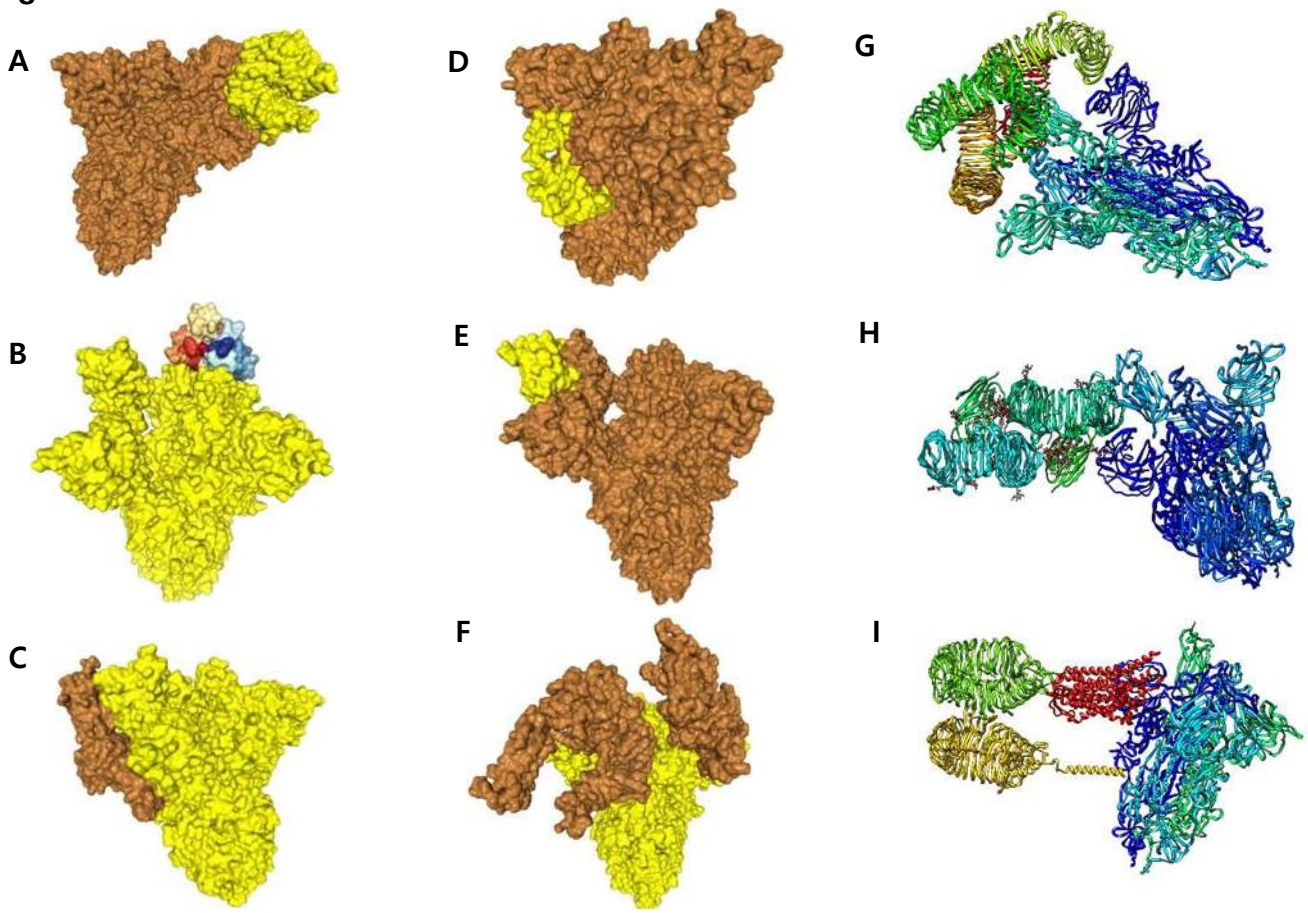


Figure 7. Molecular docking representation of SARS-CoV-2 spike protein docked with A) ACE-II, B) CD-141, C) CD-147, D) Neuropilin 1, E) Integrin α5β1, F) IL-6R, G) TLR-2, H) TLR-4 and H) TLR-7.

4. Discussion

Endothelial cell activation is recognized as important driver of the severe COVID-19 pathogenesis, this vascular endothelial damage involves multiorgan damage, characterized by severe inflammatory processes, active thrombosis, and fibrinolytic dysfunction [29–32]. The detailed mechanism underlying the SARS-CoV-2 infection still has mechanisms to describe. Here, we investigate the hypothesis that the Spike protein and the RBD domain of SARS-CoV-2 virus can activate endothelial cells and lead to the generation of an immunothrombotic phenotype in these cells.

Endothelial cells are a rich source of angiotensin-converting enzyme 2 (ACE2), known

as the main viral receptor widely distributed in many tissues [17]. This places the endothelial cells as targets for entry into the organism. It has been suggested that Spike protein may promote endothelial cell activation [33].

In this work we demonstrate that both the Spike protein and the RBD domain can induce endothelial cell activation in a protein concentration and time of stimulation dependent manner. Our results are in agreement with other studies, which report the ability of Spike protein to induce endothelial activity and dysfunction [34,35].

Based on our results, it is possible to determine that viral proteins enhance IL-6 generation in ECs. These results are similar to those observed when cells are treated with proinflammatory and infectious agonists such as IL-6 and LPS. Our results are in agreement with reports suggesting that Spike protein favors the release of this cytokine and places IL-6 as a driver of endothelial inflammation [28,36]. There are reports that propose the activation of the JAK/STAT3 and PI3K/AKT pathway and the NF-κβ factor, associating inflammation and endothelial dysfunction [36,37]. Similarly, IL-6 expression by ECs has been linked to increased release of MCP-1 in these cells [34,37,38]. In our results it is possible to observe that although the viral proteins increase the concentrations of this chemotactic protein, there are no significant differences with respect to the control. The only stimulus that by itself increases MCP-1 concentrations is VEGF, a potent activator of ECs [26].

On the other hand, we found that, in both cell lines, viral proteins induced the release of IL-8. This cytokine is associated with an acute inflammatory process and is stored in the Weibel Palade bodies (WPB) of endothelial cells [39]. The expression of this cytokine indicates endothelial activation, increasing neutrophil chemotaxis and cell adhesion. Similarly, stimulation with IL-6, LPS, VEGF and dengue NS1 viral protein, all proinflammatory insults, favored endothelial IL-8 release. These results suggest that SARS-CoV-2 viral proteins are potent inducers of the inflammatory phenotype in the endothelium. Our results are in agreement with those reported by Haffke and cols who observed elevated concentrations of IL-8 released by injured endothelium [40]. Similarly, it has been reported that endothelial cells stimulated with SARS-CoV-2 virus induce the expression of this chemotactic cytokine [41].

Another marker of endothelial activation that we studied was the expression of P-sel, a protein associated with a proadhesive endothelial phenotype for leukocytes, promoting

the local inflammatory state [42]. We also determined values of the soluble fraction of its ligand, the PSGL-1 protein, which is expressed mainly by leukocytes and endothelial cells and is directly related to leukocyte-endothelial cell interaction [43]. We found that in the EAhy926 cell line, coronavirus proteins did not induce a significant expression of this endothelial activation biomarker. Only IL-6 stimulation led to the expression of this protein, these results are consistent with previous reports linking their endothelial expression to inflammatory challenges [39]. On the other hand, in the HUVEC cell line it was possible to determine increases in the amounts of P-sel with both viral proteins, and with the immunological insults tested. This difference in the expression of this protein may be associated with the differential cellular metabolism between a transformed line and a basal cell line. An alternative pathway for P-sel expression independent of the classical WPB degranulation pathway has been reported, which proposes increases in cAMP and intracellular calcium concentrations [44]. When PSGL-1 concentrations were assessed, we also observed a differential expression, where only HUVEC cells showed a high concentration of this protein upon stimulation with viral proteins S and RBD and with NS1 DENV protein. Our results agree with those reported by Bhargavan and cols who reported the association of these proteins with coagulopathy and NETosis [45]

Similar results we observed when determining sCD40-L, only stimulation with IL-6 achieved the expression of this protein in the EAhy296 line. Proinflammatory stimuli (IL-6 and TNF-α), viral stimuli such as RBD peptide, bacterial stimuli such as LPS favored the elevation in the concentrations of this protein in the HUVEC cell line. sCD40-L is highly expressed in antigen presenting cells and endothelial cells and is associated with lymphocyte activation, favoring the activation of the adaptive immune response [46]. On the other hand, this protein has been associated with a poor prognosis in subjects affected by COVID-19 [47]. High concentrations of this factor have also been correlated with a state of immunosuppression [48].

These results confirm that the exacerbated inflammatory response observed in COVID-19 is being fueled in part by ECs that are activated directly by the virus or its proteins. And that this endothelial inflammation provides positive feedback, perpetuating endothelial activation, favoring the adhesion and activation of leukocytes and inducing the release of more inflammatory factors by the endothelium. Similarly, this inflammatory

response to the Spike protein can elucidate the thrombogenic response observed in some cases of VIITT where Spike is the immunogen used in vaccination platforms. More studies need to be carried out to confirm these hypotheses.

Once the inflammatory response of the endothelium was determined, we decided to evaluate the thrombotic profile and the fibrinolytic response in these cells. Initially, it was possible to observe that endothelial activation with strong inflammatory stimuli such as IL-6, LPS and VEGF favor the generation of D-dimer in the conditioned medium. D-dimer is a product of fibrin proteolysis and has been found to be elevated in patients with severe COVID-19, positioning it as a prognostic and treatment marker in the disease [49,50]. Finding D-dimer in the supernatant of cells treated with these stimuli indicates the generation of fibrin and its subsequent proteolysis as a consequence of fibrinolytic activity by the endothelium. It is possible that the fibrin is a consequence of the conversion of fibrinogen by thrombin found in the culture medium. When we assessed the fibrinolytic state, we observed that both SARS-CoV-2 proteins and activating and inflammatory stimuli increase PAI-1 expression in both cell lines. On the other hand, we studied the concentrations of tPA, substrate of PAI-1, and we observed that only IL-6 favors its expression and, curiously, an inverse correlation is evident between an increase in PAI-1 and a decrease in tPA in the stimuli with viral proteins, indicating a dysfunctional fibrinolytic process [51]. Our results are in agreement with those reported by Han and Pandey who observed a high expression of PAI-1 and associated the metalopeptide ZMPSTE24 as a regulator of this pathway [52]. Abnormalities in the fibrinolytic status of patients affected by covid-19 have been previously reported [47,53]

Based on these results it is possible to suggest that coronavirus proteins alone do not induce D-D genesis in endothelial culture, this D-D production is totally linked to the inflammatory process and the activation of the coagulation systems; however, S and RBD proteins lead to a dysfunction in the fibrinolytic process which perpetuates the endothelial injury and the procoagulant environment.

Finally, when assessing the concentrations of coagulation modulating proteins, we observed that stimulation with TNF-a induced the expression of vWF in the EAhy926 cell line. Even though it was possible to observe an increase in the concentrations of this protein when HUVEC and EAhy926 cells were treated with viral and procoagulant stimuli,

they were not significant when compared to the control. vWF is a protein stored in WPBs, highly procoagulant and released upon endothelial activation and injury [39]. Our results agree with Guo and Kanamarlapudi who reported that protein S induces an increase in vascular permeability, accompanied by vWF release, in an ACE-2 receptor-dependent pathway. [54]. High vWF concentrations have been directly associated with disease severity and active immunothrombotic states in patients affected by COVID-19 [47,53].

Other proteins that we evaluated were coagulation factor IX, in which we found no differences in its expression in any cell line in response to any stimulus. This is explained by the fact that this factor is not stored nor generated by endothelial cells [55] TF expression suggests endothelial cell activation and injury. This protein is procoagulant and activates the proteolytic cascade of coagulation [56]. SARS-CoV-2 proteins did not favor the expression of this protein, only inflammatory insults in HUVECs. TF expression by endothelial cells have been reported after exposure to the RBD domain to endothelial cells, generating coagulopathy [57]. Similarly, this expression of TF has been associated with activation of the inflammasome through endothelial activation via the NRL3 pathway [58].

Furthermore, different endothelial surface receptors alternative to the ACE-2 protein have been proposed for SARS-CoV-2 [59,60]. For this reason, we decided to evaluate by bioinformatic processing the possible interactions and affinities that exist between the Spike protein and the proposed receptors, using ACE-2 as an internal molecular docking control. Based on our results it is possible to confirm that there are alternative endothelial receptors to ACE-2 which may serve as pathways of virus infection and may be associated with the infectious capacity of SARS-CoV-2. We report that thrombomodulin (TM) (CD-141) has a high affinity for protein S, as does bisignin or EMMPRIM (CD-147). These results support the hypothesis that inhibition of the thrombomodulin-thrombin-activated protein C pathway axis is associated with the endothelial dysfunction observed in COVID-19 [61].TM is a membrane glycoprotein whose ligand is thrombin, acting as a natural anticoagulant by neutralizing the proteolytic capacity of this molecule. This protein also naturally regulates the inflammatory response in ECs, so its blockade influences the immunothrombotic response of the endothelium to proinflammatory stimuli [62]. On the other hand, our results support Wang et al, who proposed CD-147 as an alternative route of

infection of endothelial cells by SARS-CoV-2 virus [63]. This receptor has even been proposed as a therapeutic target in COVID-19 [64].

Other receptors shown to be targets of protein S were neuropilin 1, a protein associated with angiogenic processes in response to VEGF. This protein has been linked to the maintenance of endothelial adhesion and has been reported to participate in endothelial activation in SARS-CoV-2 virus infection [65–67]. Glycoprotein α5β1 is another endothelial receptor with good affinity for protein S. Robles and cols confirmed that the virus can infect endothelium through this receptor [28].

On the other hand, since ECs express a wide variety of innate immune receptors, among which the Toll-like receptor (TLR) family stands out [68–70]. We decided to assess the affinity of TLR-2, TLR-4 and TLR-7. All 3 receptors showed an affinity even higher than that observed with the natural virus receptor. Suggesting that these membrane and intracellular receptors, respectively, are not a gateway for the virus. TLR activation initiates NF-κβ and MAPK signaling through MyD88 and/or RIG-1 favoring the establishment of an inflammatory phenotype in the endothelium [70,71]. Normally, TLR7 are absent in resting ECs, but are inducible under inflammatory activation [71]. It is well known that these TLRs are associated as receptors for PAMPs in other infectious processes that cause vascular injury such as COVID-19 [72]. Different authors have shown that these receptors can be used by the virus to infect human cells, including platelets, kidney, neurons and endothelial cells [73–77]. Our results confirm this and support the imperativeness of testing them as therapeutic targets.

Finally, observing the exacerbated inflammatory state of COVID-19 and based on our results where we observed that IL-6 favors endothelial activation. We decided to evaluate whether the IL-6 receptor (IL-6R) could act as a receptor for protein S. Our results show that there is a high affinity between this IL-6R and the Spike protein. Receptor blocking treatments such as tocilizumab have shown benefits in COVID-19 patients [78,79]. This suggests that the IL-6 receptor plays an important role in perpetuating the inflammatory state and endothelial dysfunction observed in SARS-CoV-2 infection.

5. Conclusion

SARS-CoV-2 Spike protein and its RBD domain activate and induce an

immunothrombotic phenotype in HUVEC and EA.hy926 endothelial cells. This response is characterized by the released of inflammatory cytokines, prothrombotic and anti-fibrinolytic soluble proteins that favor the establishment of an immunothrombotic environment. The activation of ECs can be associated with different endothelial receptors in addition to ACE-2.

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VIII.III. *Capítulo 3:* Los resultados correspondientes a este capítulo se incluyen en el manuscrito: *"Immunothrombosis, The Pathological Triad Between Endothelium, Innate Immune System and Platelets: Role in COVID-19"*

Immunothrombosis, The Pathological Triad Between Endothelium, Innate Immune System and Platelets: Role in COVID-19.

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Abstract

COVID-19 is a disease caused by the SARS-CoV-2 virus. This disease primarily affects the respiratory tract; however, it is severe disease states COVID-19 has been associated with activation of the innate immune system and the coagulation system. This immunothrombotic response has been correlated with worse disease outcomes. Immunothrombosis is defined as the activation of the coagulation system as a consequence of an innate immune response. The pathophysiology of immunothrombosis involves different cells such as platelets, vascular endothelium and cells of the innate immune system such as neutrophils and monocytes. This immunothrombotic process results in the perpetuation of the inflammatory state and a process of clot generation at the vascular level that can put the patient's life at risk. In this article we review the participation of the different cells involved in the immunothrombotic response in COVID-19 and its correlation with the clinical manifestations of affected patients.

Key words: COVID-19, immunothrombosis, platelet, endothelium, immune system.

1. Introduction

The vascular system is formed by an extensive network of vessels including arteries, veins and capillaries, intimately linked by endothelial cells from the heart to the smallest capillaries. In basal state, these cells confer numerous properties that allow blood to circulate fluidly and prevent its outflow [1,2]. This system is largely related to hemostasis, a process that maintains the integrity of the circulatory system and allows the blood flowing through it [3]. Vascular hemostasis is regulated by the coagulation system, which under normal conditions displays anticoagulant properties. However, when this system presents a pathological deviation, uncontrolled activation can occur generating thrombosis [4]. Thrombosis involves a complex process in which platelets, endothelium and various coagulation proteins participate [5,6].

The relationship between thrombosis and the innate immune system, mainly in the inflammatory process, has recently begun to be studied. In 2004, the interaction between platelets and immune system cells mediated by P-selectin and P-selectin ligand (PSGL1) in coronary stents was described for the first time [7]. A couple years later, the capacity of platelets to be activated by the Toll-Like Receptor 2 (TLR-2), a receptor classically associated with the immune system, was described [8]. Finally, in 2013 Engelmann and Massberg introduced the term immunothrombosis to describe the relationship between the innate immune system and thrombosis, in the control of pathogenic microorganisms, first described in bacteria [9]. Immunothrombosis is then a pathological state that involves different cells such as platelets, endothelial cells and immunological response cells.

The immunothrombotic mechanism has been widely studied in septic processes, where the infection has been associated with a prothrombotic state [10–12]. However, in viral infections the subject has not been studied in depth. The viral family *Coronaviridae* have been reported causing hematological and coagulation alterations. Some of these can affect humans, like the coronavirus causing Middle East respiratory syndrome (MERS-CoV), the coronavirus causing severe acute respiratory syndrome (SARS-CoV) and the coronavirus causing severe acute respiratory syndrome 2 (SARS-CoV-2)[13,14].

Coronaviruses are single-stranded RNA enveloped viruses with genetic material of around 30 Kb [15]. SARS-CoV-2, first described in late 2019, uses ACE-2 (angiotensin-converting enzyme 2) recognized by the Spike (S) glycoprotein, exposed on its envelope,

as a receptor to infect cells. ACE-2 receptor is widely expressed on different tissues in human anatomy [16,17].

The infection has an incubation period of 5 to 14 days, and the spectrum of infection provides 4 different scenarios: 1% of asymptomatic patients, 80% of patients with mild symptoms, 14% of severe cases and 5% of critical cases [18]. Among the physiological alterations observed in COVID-19, those involving the coagulation and immune systems have been described [19–21]. In this review, we highlight these alterations and endothelium and platelets involvement one this viral infection.

2. Hypercoagulable state in COVID-19

Complications in severe SARS-CoV-2 disease states have been associated with hypercoagulability and an active process of immunothrombosis. In February 2020, Tang et al. reported the first coagulation parameters determinations, finding abnormalities in D-dimer and fibrin degradation products (FDP) levels, as well as prolongations in prothrombin time and activated partial thromboplastin time, in patients who survived the disease in contrast to deaths [19]. Han et al. corroborated the findings of Tang et al. and observed that fibrinogen levels were also elevated in patients with severe COVID-19. They also reported alterations in the natural anticoagulant processes by observing decreased antithrombin concentrations in these patients [20].

Di Micco et al. later confirmed the previous results in a cross-sectional study and concluded that serum fibrinogen levels above 617 mg/dL in COVID-19 patients could serve as an early marker of those patients who would become complicated with the course of the disease [22]. Presumably elevated fibrinogen concentrations are product of hepatocyte activation by interleukin-6 (IL-6), a cytokine elevated in exacerbated inflammatory responses [23]. These alterations are indicative of a hypercoagulable process.

When coagulation process is activated, the system also induces its fibrinolytic activity to maintain homeostasis. D-dimer is a consequence of these process and has been positioned as an early biomarker of an active coagulation state in COVID-19 [24]. Several groups have reported statistically significant elevations in patients with severe COVID-19 disease [20,25,26]. Hai-Han et al. reported in particular that concentrations above 0.5 µg/ml were associated with severe SARS-CoV-2 infection [27]. Also, Soni et al. reported that

plasma D-dimer concentrations above 2.01 µg/ml were directly associated with a high mortality rate in patients with COVID-19 [28]. This positions D-dimer as an excellent prognostic biomarker in these patients.

Recent studies have shown that even months after SARS-Cov-2 virus infection, convalescent patients can maintain elevated D-dimer levels (>500 ng/mL), this information indicates to clinicians the need to maintain anticoagulant treatments in recovered patients who were hospitalized [29].Other groups have reported the disparity in natural anticoagulant systems. Zhang et al. reported in 12 patients with a diagnosis of COVID-19 and recorded thrombotic events, values below the normal range for protein C, protein S and antithrombin [30]. In December 2020, Zuo et al. Observed in a cohort of 118 patients, elevation of tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI-1) in plasma that correlated with a high mortality rate [31].

In the same way, other groups have studied the processes of secondary hemostasis and the relationship between hemostasis abnormalities and prognosis in COVID-19 positive patients. Wang et al. evaluated 213 patients in a retrospective study and concluded that in addition to elevated concentrations of fibrin degradation products, prolongations in prothrombin times (PT) which evaluate the extrinsic coagulation pathway, are predictive factors of a poor prognosis in COVID-19 [32]. Baranovskii et al. in another study enrolling 82 patients with COVID-19 concluded that prolongations in PT and elevated levels of C-reactive protein (CRP) a hepatic biomarker of inflammation, can be used as prognostic markers of severe pneumonia in COVID-19 [33].

Within the broad range of biomarkers that present alterations in severe stages of COVID-19, von Willebrand factor (vWF) has also been reported. Von Willebrand factor is a plasmatic multimeric glycoprotein, produced in endothelial cells and megakaryocytes, the largest protein that can circulate in the bloodstream (500-20000 kD). It can also be found in the endothelial matrix. It is mainly stored in the Weibel-Palade (WP) corpuscles in endothelial cells and in the alpha granules of platelets. As the endothelial cell is the main storage center for von Willebrand factor, this protein is recognized as a dysfunction biomarker and high concentrations of this protein in plasma suggest endothelial damage[34]. This factor is expressed when endothelial cells are activated [35]. The main role of vFW is to mediate adhesion and aggregation of platelets, in case of endothelial injury

and protect coagulation factor VIII of proteolytic degradation [36,37].

The biological activity of vWF depends on the size of its multimers, when the vWF is secreted, it is shown in its complete conformation with high molecular weight multimers (HMWMs), that consists of several hundred vWF multimers, each multimer has binding sites for coagulation factor VIII, platelets glycoproteins (Ib/IX and Ib/IIIa), collagen and ADAMTS-13 [38], HMWMs are most efficient in hemostasis and are highly prothrombotic; the enzyme in charge of regulated the multimer size by cleaving vFW is ADAMST13 (a desintegrin and metalloproteinase with thrombospondin-like motifs, member 13) [39,40].

Ultralarge vWF multimers (ULvWF) enhance platelets aggregation by inducing its activation [41,42]. Structural, functional or autoimmune alterations of this enzyme are the etiology of thrombotic thrombocytopenic purpura, a disease that occurs with high concentrations of ULvWF [43]. Helms and collaborators reported elevations in the concentrations of vFW and in its activity in 87.7% of the patients studied [26]. Similarly, there are reports of high plasma concentrations of factor VIII, with this parity contributing to the procoagulant state observed in patients in severe COVID-19 and as predictors of oxygen assistance requirements [21,25,26,30,44].

Other research groups have studied the ADAMST13:vFW ratio, observing a decrease in ADAMST13 enzyme concentrations and plasma elevations of the vWF antigen, associating this imbalance with liver damage, where ADAMST13 is produced, in patients with COVID-19, moreover, this effect; correlates with the presence of thrombotic microangiopathy and with a poor prognosis in patients [45]. The low concentration of this enzyme has also been associated with the presence of a high number of neutrophils and the process of netosis in these immune cells triggered by stimuli mainly of infectious origin [46]. Because of the absence or low concentrations of ADAMST13 and the endothelial lesion observed in COVID-19, studies have reported the presence of circulating ULvWF and its relationship with mortality in hospitalized patients [47].

Alterations in these biomarkers associated with secondary hemostasis processes allow us to suggest an active state of hypercoagulability and decreased fibrinolytic activity, with continuous formation of microthrombi that contribute to the pathology of the viral agent.

3. Platelets Beyond hemostasis

Originated at the megakaryocyte, a cell located mainly in the bone marrow, platelets are cells whose most important and studied physiological function is their participation in the processes of hemostasis and thrombosis [48,49]. Platelets circulate in the bloodstream in an inactive state. They are stimulated when exposed to elements expressed by the extracellular matrix of an injured or activated endothelium, soluble agonist molecules secreted by the platelets themselves or other cells of the circulatory microenvironment, during infectious processes caused by bacteria or viruses, or when innate immune system is activated involving tissue factor expression by monocytes or netosis process by neutrophils [50–52].

The involvement of platelets in immunity is suggested by their unique structural and generation characteristics. Activated platelets acquire a characteristic morphological and phenotypic structure. This response allows them to increase their adhesiveness and aggregation capacity by expressing ligands and receptors to interact with other cells present in circulation and with injured endothelium. Another characteristic is the increase in their contact surface and, in turn, they release a variety of mediators capable of intervening in coagulation, inflammation and chemotaxis of the injured site, affecting hemostasis and homeostasis of the vascular system [53–55]. Diverse viral infections have been reported to present with thrombocytopenia, either by cell lysis or by inhibition of their synthesis; in addition, viruses such as human immunodeficiency, influenza, hepatitis C and dengue have been found to be internalized on these cells [50].

In SARS-CoV-2 infection, no similarity has been observed in findings reported by different research groups. Initially, it was believed that in COVID-19 there was a process of disseminated intravascular coagulation (DIC), a process observed in sepsis, and characterized by a hypercoagulant state where, in parallel and due to the consumption of platelets in thrombosis, hemorrhages occurred. This state is not observed in COVID-19 and differences between DIC and COVID-19 coagulopathy have been extensively discussed [56–59].

Distinct pathways have been reported by which platelets may be activated in the pathological process of SARS-CoV-2. Through direct recognition of the virus, by means of damage-associated molecular patterns (DAMPs) caused by the infection, such as some

proinflammatory cytokines or tissue factor expressed by the injured endothelium.

Participation of platelets in the immune response mediating interactions with monocytes or neutrophils and releasing their granular content, rich in immunological chemotactic factors has also been report [60]. Younes et al. demonstrated the presence of internalized viral RNA in platelets, as well as a hyper-reactive state of these cells in patients with the disease [61]. Zhang et al. described the expression of the ACE2 receptor in the platelet membrane and the activation of these cells in response to stimulation with the SARS-CoV-2 virus and the spike protein. Suggesting the virus capacity to directly activate platelets [62].

Some groups have reported thrombocytopenia in patients with severe states of COVID-19 and have associated it with coagulation disorders and mortality in patients. In a study performed by Bao et al. where they evaluated the platelet count of 178 patients, subjects classified with severe COVID-19 presented low platelet counts [63]. Henry et al. performed a meta-analysis including more than 3,000 patients from 21 studies, reporting a difference in platelet counts between severe and non-severe COVID-19 patients [64]. Similarly, levels of platelet counts reported on patient's admission to hospital suggest that it may be used as predictor of oxygen requirements [25]. Besides these reports, there are other works where platelet counts are found to be within the normal range [26]. But not only platelet counts have been studied, Bongiovanni and coworkers demonstrated a procoagulant phenotype in platelets from patients with COVID-19, with expression of classic proteins of an activated cell, such as glycoprotein IIb/IIIa or P-selectin [65]. It is important to continue with the study of these sentinel cells of the innate immune response and their response in SARS-CoV-2 infection; as well as their participation in immunothrombosis in COVID-19.

4. Endothelial duality in COVID-19

Endothelial tissue is one of the most extensive in the human anatomy. Lining arteries, veins and small vessels. Endothelium is responsible for maintaining the separation between blood tissue and other organs. In addition, it has diverse activities such as homeostatic maintenance of the circulatory microenvironment, collaborates modulating the coagulation process, blood vessel tone, modulates immune diapedesis, regulates

oxidative and vascular processes such as vasoconstriction and vasodilatation [23].

Endothelial dysfunction (ED) is characterized by activation of the endothelial cell by either a physical or a biological mechanism, and results in phenotypic changes that include the expression of various adhesion molecules such as: vFW, E-Selectin, P-Selectin and vascular adhesion molecule (VCAM), inducing a pro-adhesive state in the tissue. ED is also characterized by release of proinflammatory cytokines such as interleukin 8 (IL-8), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) and induction of an anticoagulant state by inhibition of natural regulatory mechanisms, mediated by the protein C and S system, as well as antithrombin [23,66,67].

Endothelium is one of the multiple cells that express the ACE2 receptor. SARS-CoV-2 infection has been described in pulmonary endothelium, causing pulmonary endothelitis, activating the endothelium and inducing the activation of the coagulation system [68,69].

Another proposed mechanism by which ED occurs is mediated by the interaction of SARS-CoV-2 and its receptor ACE2. The physiological function of this enzyme is the conversion of angiotensin II into fragments: angiotensin 1-7 and 1-9, which play an essential role in the renin-angiotensin system, regulator of cardiovascular homeostasis. When there is an alteration in the renin angiotensin system due to angiotensin II accumulation hypertension states may occur, which contribute to ED [70]. It is known that diabetic, elderly, hypertensive or obese individuals present a state of ED characteristic of these pathologies, which may be exacerbated in COVID-19 and correlate with a worse prognosis.

It has also been described that in COVID-19 the state of hypoxia observed in critically ill patients favors the expression of hypoxia-induced factor 1 (HIF-1) in endothelial cells. This factor may inhibit fibrinolytic processes and enhance the coagulation pathway [71].

In post mortem histopathological samples of COVID patients reported findings indicates lung endothelial disfunction[72]. Biomarkers that indicate endothelial injury, such as vWF, have also been found to be elevated in these patients suggesting endothelial activity [24,34,47]. Nizzoli and collaborators reported in a series of 30 patients the elevated presence of circulating endothelial cells, another indicative of endothelial injury [73]. These reports confirm the ED in SARS-CoV-2 infection, a situation that contributes to a severe state of the disease.

5. Exacerbated innate immune response, adversary in COVID-19

The main function of the innate immune response is to respond to stimuli that challenge the homeostasis of the organism. This important funtion is played by a wide range of cells with the capacity to recognize these agents and release biomolecules that potentiate and modulate the response, as well as a series of proteins that participate in the same way.

In some patients with COVID-19 the immune response activated by the viral infections far from acting as a protector, neutralizing and eliminating the virus; it becomes an aggravator of the disease. The innate immune system responds in an exacerbated form by a mechanism known as cytokine storm. This term was initially introduced in 1993 and described the exacerbated inflammatory response caused by the uncontrolled activation of cells of the innate immune system and the production, in high concentrations, of cytokines such as: interferons (IFNs), interleukins (IL), chemokines. This process can present localized or systemic damage [74,75].

The pathophysiological mechanism by which this cytokine storm is triggered in COVID-19 is similar to the one observed in bacterial sepsis states. It responds to the expression of proinflammatory cytokines such as interleukin-1 (IL-1) or TNF-α, that stimulate other immune cells in the microenvironment to enhance production of other proinflammatory cytokines via the NF-κB transcription factor signaling pathway [76]. This uncontrolled production of inflammatory cytokines triggers the activation of the NLRP3 inflammasome with the production of IL-1β as a result, and stimulation of IL-6 synthesis. One of the main functions of IL-6 is to activate the hepatocyte to induce the production of other inflammatory proteins such as C-reactive protein (CRP), a marker of the inflammatory state of an organism [23].

There is reported evidence of this inflammatory state in patients with COVID-19. Petrey et al. observed elevations in IL-6, IL-8, IL-1α, TNFα, and some chemokines concentrations in patients with COVID-19 when compared to non-COVID-19 patients [77]. In another study, Liu and colleagues found in a cohort of 147 patient's plasmatic elevations of IL-6 and IL-8, as well as high concentrations of ferritin, a hepatic protein expressed in states of acute inflammation. Relating the hyperinflammatory process with prominent coagulation disorders [78]. Similarly, in two meta-analyses performed by Henry and

collaborators, which included 21 studies, and Wang and collaborators who included a total of 3,939 patients, observed elevations in IL-6, IL-1β, TNF, and Interleukin 10 (IL-10); as well as high concentrations of ferritin and elevations in neutrophil counts, suggesting the participation of these cells presumably through the process of netosis [64,74]. The neutrophil: dimer-D ratio has even been proposed as a biomarker to differentiate patients with severe disease [79].

Netosis is a process orchestrated by neutrophils, phagocytic cells of the innate immune system, responsible for host defense against diverse pathogens, mainly bacteria [52]. During this highly regulated mechanism, neutrophils expel extracellular DNA traps (NETS), composed of DNA, histones, enzymatic granules and bactericidal molecules. This process is employed as a control response to the etiologic agent of a disease [80]. Among the various functions of this mechanism, one is to capture the pathogen preventing its dissemination and invasion to other tissues outside the circulatory system. NET´s can also neutralize microorganisms favoring its elimination by the innate immune system and its bactericidal proteases, as well as the chemoattraction of other immune cells [81]. As mentioned above, neutrophil netosis can be stimulated by inflammatory cytokines; however, platelets can also induce this process by direct interaction with these cells through P-Selectin [9,67]. But netosis is a prothrombotic mechanism by nature. Histones H3 and H4, expressed during this process, are platelet activators, vWF can interact with these traps and favor platelet recruitment, and coagulation factor XII is activated, presumably, by the negative charges inherent to the genetic material, favoring the generation of a procoagulant state [9,80–82].

The convergence of activation of these mechanisms and cells has a protective function as purpose for the organism; however, exacerbated activation is pathological and counterproductive for patients. Immunothrombosis is a relatively recently described process and could be observed in individuals with a severe course of COVID-19 disease (Figure 1).

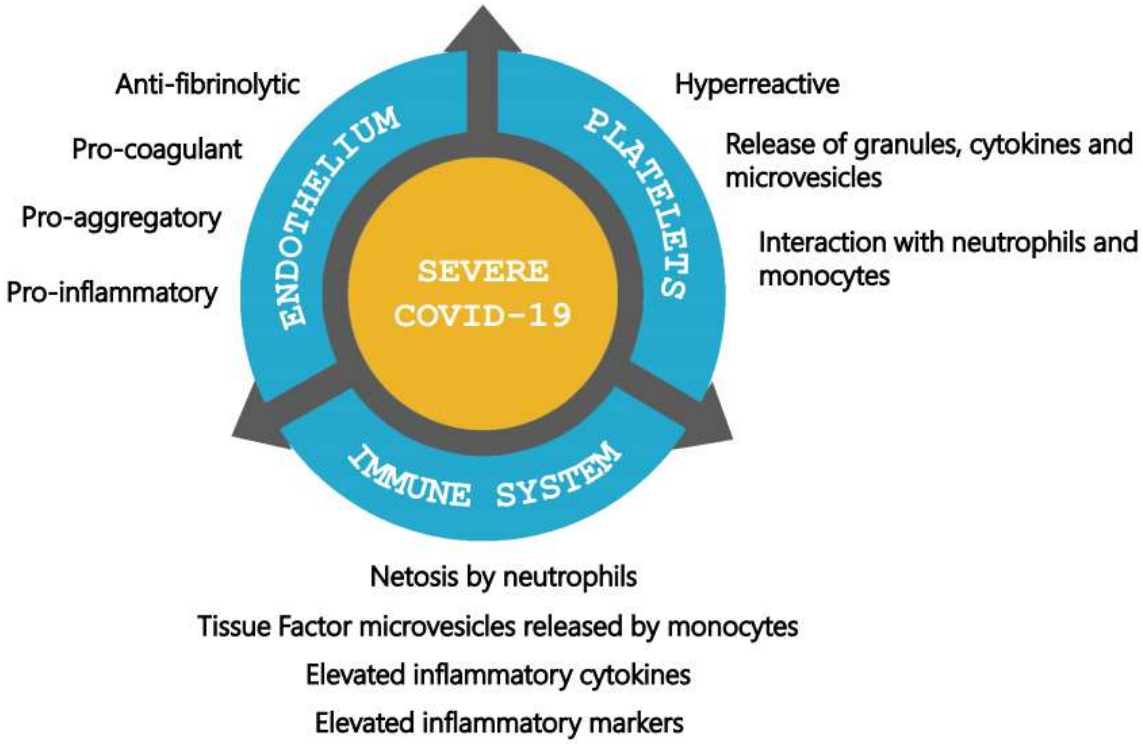

Figure 1: Immunothrombosis in COVID-19. During the process of severe COVID-19, there is a state of active immunothrombosis. Platelet activation characterized by cell degranulation and inflammatory cytokine production has been described, as well as their interaction with neutrophils and monocytes. On the other hand, the innate immune system is activated favoring the establishment of immunothrombosis through netosis by neutrophils, the release of microvesicles rich in tissue factor by monocytes and the release of soluble inflammatory factors. The vascular endothelium has anti-fibrinolytic, pro-coagulant, proaggregating and proinflammatory characteristics.

6. Conclusions

Severe COVID-19 is characterized by a procoagulant state associated with a hyperacute inflammatory state and involvement of processes such as netosis, platelet hyperreactivity and endothelial dysfunction. The immunothrombosis observed in critically ill patients due to COVID-19 correlates with patient's poor prognosis and oxygen requirement. This state should be considered by the healthcare team as a clinical marker in these patients and represents a potential target for pharmacological therapies.

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VIII.IV. *Capítulo 4:* Los resultados correspondientes a este capítulo se incluyen en el manuscrito: *"Differential thromboinflammatory response to circulating SARS-CoV-2 vaccine platforms in a cohort of immunized subjects in Mexico"*

Differential thromboinflammatory response to circulating SARS-CoV-2 vaccine platforms in a cohort of Immunized subjects in Mexico.

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Abstract

Vaccines are the best public health strategies against microbiological agents affecting humanity, this is the case of SARS-CoV-2. Few cases of thrombosis after the ChAdOx1 vaccination have been reported. This study aimed to assess different inmunothrombotic biomarkers by flow cytometry and ELISA assays in samples from subjects after the first dose of vaccine with one of the different biologics used in México. Data from 44 subjects distributed according to the biologic applied in BNT162b2, AZD1222 (ChAdOx1), CoronaVac, and Ad5-nCoV-Covidecia and 14 healthy donors were included. Levels of inflammatory cytokines IL-6 and IL-8, P-selectin, PSGL-1, and CD40L are increased in viral vector-based and attenuated virus vaccinated groups ($p<0.001$). Thrombotic-associated markers PAI-1 and tPA were increased only in ChAdOx1 immunized group ($p<0.05$). Viral vector vaccines lead to an increase of plasmatic inflammatory markers but only the ChAdOx1 vaccine show differences in markers linked to endothelial activity.

Keywords: SARS-CoV-2, COVID-19, COVID-19 vaccines, thrombosis, vaccination.

1. Introduction

Over four years have passed since coronavirus disease 2019 (COVID-19) was first reported at the end of 2019 [1,2]. Vaccines have been positioned as the best public health strategy to combat the SARS-CoV-2 pandemic and its variants. Since 2021, different types of vaccines have been authorized for emergency use around the world in an attempt to curb the increasing number of SARS-CoV-2 infections and reduce mortality[3]. The majority of vaccines aim to develop antibodies against the Spike (S) protein[4].

Among the different approved vaccination platforms, some of them use the inactivated virus technology (BBV152 Covaxin by CanSino Biologics Inc and CoronaVac by Sinovac Research and Delevope Co), others use messenger RNA (mRNA) technology encoding for protein S (BNT162b2 manufactured by Pfizer Inc./BioNTech and Spikevax from Moderna) and others use nonreplicating adenoviral vector coding for glycoprotein S (AZD1222 Covishield from AstraZeneca, Gam-COVID-Vac from Gamaleya National Center, Ad5-nCoV Covidecia from CanSino Biologics Inc and Ad26. COV2-S from Janssen-Cilag) [5,6].

Randomized clinical trials focused on analyzing the side effects of COVID-19 vaccines have reported mostly mild effects (pain, redness, swelling) and systemic effects (fatigue, headache, muscle or joint pain). However, among the serious adverse events associated with vaccination against SARS-CoV-2, the appearance of a rare state of thrombosis has been reported. This process has been named Vaccine Induced Immune Thrombotic Thrombocytopenia (VIITT) and has been mainly associated with the ChAdOx1 vaccine [7]. Several pathophysiological mechanisms have been proposed by which VIITT occurs [8]; however, according to several published studies, the formation of platelet Factor 4 - immunoglobulin type G anti PF4 complex induces activation and subsequent platelet aggregation through the FCIIIR receptor, is responsible development of a prothrombotic state [9]. Although the incidence of VIITT worldwide is very low, ranging from 0.5 to 6.8 cases per 100,000 vaccines approximately [10], there is limited information on factors associated with susceptibility to this phenomenon and whether there is a correlation between adenovirus-dependent platforms and the development of this pro-thrombotic state. The aim of this work was

to evaluate a series of thrombotic-inflammatory biomarkers in subjects vaccinated against SARS-CoV-2 with the different vaccine platforms approved in Mexico.

2. Materials and Methods

2.1. Samples

A total of 55 subjects were included in the study distributed as follows. 41 samples corresponded to subjects who received the first dose of SARS-CoV-2 vaccine distributed in 4 study groups according to the biologic applied: BNT162b2, AZD1222 Covishield, CoronaVac, and Ad5-nCoV-Covidecia. All samples were collected between days 5 and 20 after the first dose. Samples from 14 healthy donors were included as a control group. Samples from this group were obtained during the period from August to December 2019 and preserved in a biobank, ensuring that none have been exposed to SARS-CoV-2 or had previous immunization against the virus. All participants were over 18 years of age and signed an informed consent letter before entering the study.

2.2. Blood sample collection

Whole blood was obtained from the participants by venipuncture after signing the informed consent form. Blood for plasma samples was obtained in vacutainer tubes with 3.2% sodium citrate anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA) and processed within 2 hours after collection. Plasma was obtained by centrifugation at 1,000 x g for 15 minutes at room temperature and preserved at -70°C until biomarkers determination.

2.3. Determination of immunothrombotic biomarkers

The following thrombotic-inflammatory biomarkers were analyzed: Interleukin 6 (IL-6), Interleukin 8 (IL-8), P-selectin, P-selectin ligand 1 (PSGL-1), sCD40L, D-dimer, tissue plasminogen activator (tPA), tissue plasminogen activator inhibitor 1 (PAI-1), tissue factor and coagulation factor IX. Plasma determination of these biomarkers was performed by flow cytometry using the BioLegend® LEGENDplex Kit TM Human Thrombosis Panel Standard following the instructions suggested by the supplier.

Samples were read in a CytoFLEX, BECKMAN COULTER®. Briefly, samples were incubated with beads that differ in size and fluorescence intensity. Each bead group is conjugated with specific antibodies on its surface that capture a specific analyte. After washing, a cocktail of biotinylated detection antibodies is added, forming a detection complex between the capture bead-analyte-detection antibody. Finally, Streptavidin-phycoerythrin (SA-PE) is added and incubated for 30 minutes. Samples are taken to the CytoFLEX cytometer for analysis.

2.4. von Willebrand Factor

von Willebrand Factor plasma (vWF) concentrations were assessed by using the BIOMEDICA DIAGNOSTICS IMUBIND ® vWF ELISA kit. This is a “sandwich” ELISA that uses a goat polyclonal antibody as capture antibody. Samples were incubated on a pre-coated plate and subsequently, a polyclonal antibody conjugated to rabbit peroxidase (HRP) was added to detect the immunocomplex formed. The addition of perbonate/3,3,5,5-tetra-methylbenzidine (TMB) as a substrate and its reaction with HRP creates a blue coloration that is stopped by pH change. All determinations were performed according to the supplier's specifications and recommendations. The plate was read using a Thermo Scientific Multiskan FC reader® at a wavelength of 450 nm.

2.5. Statistical analysis

Sociodemographic and categorical variables were expressed as frequencies and percentages. Continuous variables are expressed using mean, standard deviation, median, and minimum and maximum values. The results obtained were expressed as averages and standard deviations. Kolmogorov Smirnov test was used to evaluate normality. A test of variance with a Tukey post hoc test was used to evaluate differences between groups. A p value <0.05 was considered statistically significant. GraphPad 8.1 software was used to perform the analysis (San Diego, CA).

2.6. Ethics

This study was approved by an appropriate Institutional Review Board: The Ethics and Research Committee of the Faculty of Medical and Biological Sciences "Dr.

Ignacio Chávez" with registration number: 004/P/5/2021 (Anexo XIII.II). Written informed consent was obtained from all participants for their anonymized information to be published in this article.

3. Results

A total of 55 participants were included in this clinical study. All were residents of the state of Michoacán, Mexico. The main inclusion criteria were to have received the first dose of immunization against the SARS-CoV-2 virus and to be within 5 - 20 days' post application. 41 participants were classified into 4 groups according to the biological applied. A total of 11 participants with BNT162b2 (26.8%), 10 subjects were given AZD1222 (24.4%), 9 participants immunized with CoronaVac (22.0%) and 11 vaccinated with Ad5-nCoV-Covidecia (26.8%). The control group consisted of 14 samples from apparently healthy volunteers at the time of sampling. The clinical and demographic characteristics of the study population are shown in Table 1. Mean age of the study subjects showed no significant differences ($p = 0.0756$). Hypertension, diabetes mellitus 2, obesity, Body Mass Index, hormonal treatment, and previous SARS-CoV-2 infection were reported as study variables. A difference in prevalence of hypertension and previous COVID-19 infection was found in the group vaccinated with BNT162b2.

3.1. Inflammatory associated biomarkers

Within the panel of biomarkers analyzed in this study, we evaluated the inflammatory profile. Our results show differences IL-6 concentrations significantly elevated in the groups immunized with the vaccines using non-replicating viral vectors AZD1222 (955.8 ± 241.0 pg/ml) Ad5-nCoV-Covidecia (1,111 ± 520.0 pg/ml) and inactivated virus (1,044 ± 278.7 pg/ml), with respect to the control group (389.3 ± 301.5 pg/ml). Not so in the group including subjects vaccinated with mRNA platform ($p = 0.0008$) (Figure 1A). On the other hand, we found increase IL-8 concentrations in all vaccinated groups: BNT162b2 (1,656 ± 1,066 pg/ml), AZD1222 (1,864 ± 597. 0 pg/ml), CoronaVac (2,121 ± 645.3 pg/ml), Ad5-nCoV-Covidecia (1,830 ± 920.1 pg/ml) when compared to the concentrations of the control group (815.1 ± 555.4 pg/ml) ($p = 0.0113$)

(Figure 1B).

On the other hand, significant elevations in plasma concentrations of sCD40L were found in the groups immunized with adenoviral platforms AZD1222 (4,563 ± 2,059 pg/ml), Ad5-nCoV-Covidecia (3,422 ± 1,127 pg/ml) and CoronaVac vaccine (4,653 ± 2,173 pg/ml) comparing with the control group (1,348 ± 891.6 pg/ml) ($p<0.0001$). (Figure 2A). We observed elevated P-selectin concentrations in those samples belonging to the AZD1222 (1,901 ± 961.4 pg/ml) and CoronaVac (1,964 ± 900.1 pg/ml) groups when compared to the control group (588.5 ± 316.1 pg/ml) ($p<0.0001$) (Figure 2B). Likewise, we found differences in plasmatic PSGL-1 concentrations in subjects from the AZD1222 (8,592 ± 2,497 pg/ml), CoronaVac (8,789 ± 586.2 pg/ml) and Ad5-nCoV-Covidecia (7,508 ± 1,637 pg/ml) groups as compared to the control group (5,120 ± 1,110 pg/ml) ($p=0.0001$) (Figure 2C).

3.2. Biomarkers associated with coagulation

We assessed the concentrations of biomarkers associated with the processes of coagulation and fibrinolysis. Our results show increased plasma concentrations of tPA (6,110 ± 2,446 pg/ml) only in the group immunized with the AstraZeneca vaccine when compared to the control group (2,991 ± 1,264 pg/ml) ($p=0.0069$) (Figure 3A). Likewise, this group showed elevated PAI-1 concentrations (46,557 ± 26,423 pg/ml) against the values observed by the control group (26,114 ± 12,153 pg/ml) ($p=0.0137$). (Figure 3B).

When determining plasma concentrations of D-dimer, TF, FIX and vWF we did not find differences between any of the study groups when compared to the control group (Figure 4A, 4B, 4C, 4D).

4. Discussion

Throughout the modern history of medicine vaccination has been one of the most effective public health strategies against various microbiological agents affecting humanity, this is the case of the COVID-19 pandemic. The development of various immunization platforms has contributed to decreasing the mortality rate from COVID-19. However, although most side effects are mild manifestations, there are few reports of vaccination associated with adverse events and prothrombotic features.

In our study, we provided evidence that vaccination platforms using non-replicating adenoviral vectors and inactivated viruses elevate concentrations of inflammatory markers such as IL-6, IL-8, P-selectin and PSGL1, as well as those associated with a fibrinolytic process such as PAI-1 and tPA.

Our results show elevations in the concentration of IL-6 and IL-8 in the groups immunized with vaccines that use viral platforms, this response to a process of local inflammation, recruitment of immune infiltrates, consequence of the activation of the immune system [11]. IL-6 is a proinflammatory cytokine that is expressed as a consequence of an active immune response. Among its functions it participates in the differentiation of B lymphocytes and the consequent production of immunoglobulins [12]. Increases in IL-6 levels have been reported in other vaccination platforms, such as Bacillus Calmette-Guérin [13] , toxoid vaccine [14] and influenza [15,16], Farsakoglu et al, associate this IL-6 increase to an active secretion of this interleukin by dendritic cells [15]. Plasmatic IL-6 increase after vaccination with the ChAdOx1 vaccine has been reported by Willems et al [17]. In addition, IL-6 induces the expression of tissue factor (TF), which plays a key role in the regulation of hemostasis. The elevation in the plasmatic concentrations of this biomarker in the groups that were immunized with adenoviral platforms and with the inactivated virus, which is probably due to the fact that the innate response is activated with the production and release of IL-6, which in turn, can participate in the generation of antibodies [18]. IL-8 is another proinflammatory cytokine expressed in neutrophils, fibroblasts, epithelial cells, hepatocytes, alveolar macrophages, and endothelial cells that participates in the communication and adhesion between immune cells and the vascular endothelium [19]. IL-8 also plays a role in leading to an activated, prothrombotic, neutrophil phenotype characterized by degranulation and NET formation [19]. Increased levels of IL-8 shown in the AZD1222 and CoronaVac group may be related to an enhancement of the immune response against the antigen. This mechanism has been reported in other species after vaccination [20].

P-selectin belongs to a group of adhesion molecules produced by the endothelium and platelets and involved in leukocyte migration and inflammation [21]. Our results showed high concentrations of this biomarker in groups vaccinated with viral vectors

such as adenoviral or attenuated viral biologics, this may be associated with an endothelium activation, as a consequence of the active immune response. Endothelium expresses high concentrations of this molecule on its surface, favoring the activation and adhesion of lymphocytes [22]. This immune insult also plays a role in platelet activation, thrombin production by activated platelets stimulates P-selectin expression inducing a prothrombotic state in acute inflammatory situations.

The uncontrolled activity of immune cells such as neutrophils, the subsequent release of their granular contents, and the production of oxygen free radicals, correlate with an increased endothelial activity that can trigger a procoagulant process. P-selectin ligand is expressed in stimulated endothelium and T lymphocytes, cells that participate in the adaptive response [23,24]. One of the P-selectin main roles is to participate in communication between leukocytes and endothelial cells favoring cell rolling, contributing to innate immune response [24]. CD40 ligand, a protein expressed on activated T lymphocytes, participates in the interaction between this lymphoid cell line with antigen presenting cells (APC), favoring the induction of a cellular adaptive response. Our results may be associated with an active innate response that stimulates the presentation of pathogen-associated molecular patterns (PAMPs) of the platforms used against APCs [25]. On the other hand, this protein promotes the maturation and function of B cells, which are responsible for immunoglobulins production, complementing the objective of vaccination [26]. CD40L is also associated with the activation of endothelial cells, favoring the production of cytokines, chemokines, and ROS by this tissue [25,27].

In the same way, endothelial injury favors the generation of anti-fibrinolytic mediators such as PAI-1, counteracting the effect of tPA, which leads to a state of inadequate fibrinolysis [28]. Our results show that these two markers involved in hemostasis are elevated in the group that was vaccinated with the chimpanzee adenoviral platform. This may be associated with the inflammatory process triggered by the vector itself, together with the immunological insult represented by the encoding of the Spike protein. Also, a trans-signaling of IL-6 correlates with increased levels of PAI-1 from vascular endothelial cells [29] as shown in the AZD1222 group. This hyperinflammatory state may be associated with endothelial activation, which results

in a positive feedback to generate an immunothrombotic state [30]. It has also been reported that elevated levels of PAI-1 have been associated with platelet activation [31]. Activation of these cells induces the production of platelet factor 4 (PF4). Several reports have associated elevations in PF4 concentrations in subjects with TITV. In this process anti-PF4 antibodies are generated, this immunocomplex, in turn, activates more platelets through the platelet Fc receptor, triggering a state of hypercoagulation [32].

It is not known if there are factors that may be associated with an increased risk of presenting this process. None of the subjects studied presented an adverse event linked to a pathological process of hemostasis. This is evidenced by the null difference in D-dimer and vWF concentrations between the study groups. Although it may be endothelial activation, the release of vWF from the Weibel-Palade bodies (WPB) is stimulus-dependent, in this case, possibly due to inflammatory stimuli in response to the vaccine, which also increases endothelial barrier function ends up neutralizing the exocytosis of the WPB and with this the vWF [33]. On the other hand, plasma concentrations of TF did not show significant differences between groups this could be related to the expression of TF in monocytes vesicles and endothelial cells surface but not in soluble form.

This study shows some limitations. First of all, the number of patients in the study groups is small. A larger number of samples and the analysis of other parameters that evaluate the thrombotic-inflammatory state would be necessary to directly associate these alterations with immunization. In addition, as this was a transversal study, the concentrations of these biomarkers in the enrolled subjects were not followed up. It would be interesting to analyze whether the levels determined are maintained during subsequent times.

Our results show increased levels of inflammatory factors: IL6, IL-8, P-selectin, PSGL-1, CD40L, and fibrinolysis-associated markers: PAI-1, tPA, in those groups vaccinated with biologics using viral platforms. This response can be directly attributed to the innate immune response against the vector and a subsequent response against the Spike protein encoded by the adenoviral platforms, with the consequent induction of an adaptive response that induces the generation of memory T cells and

immunoglobulin production. On the other hand, a fibrinolytic disparity may be associated with an exacerbated endothelial activation that under specific situations could be linked to a thrombotic process.

5. References

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Table I. Clinical and Demographic Characteristics of the Study Population

VARIABLE	Healthy donors (n = 14)	BNT162b2 (n = 11)	AZD1222 (ChAdOx1) (n = 10)	CoronaVac (n = 9)	Ad5-nCoV (n = 11)	p value
Age (years, mean ± SD)	34.64 ± 11.61	54.5 ± 3.4	55.30 ± 3.0	46.38 ± 11.61	41.55 ± 10.28	0.0756
Male n (%)	7 (50 %)	7 (64%)	4 (40 %)	1 (13%)	4 (36 %)	0.0001
Diabetes mellitus n (%)	N/A	1 (9%)	1 (10%)	1 (13%)	1 (9%)	0.4411
Hypertension n (%)	N/A	3 (27%)	N/A	1 (13%)	2 (18%)	0.0001
BMI (mean ± SD)	23.15 ± 2.23	25.06 ± 2.76	25.04 ± 3.67	27.53 ± 3.93	24.90 ± 1.83	0.5439
Smoker n (%)	NA	4 (36%)	1 (10%)	N/A	1 (9%)	0.0001
Hormone treatment n (%)	N/A	N/A	N/A	N/A	N/A	NA
Previous COVID-19 n (%)	N/A	4 (36%)	1 (10%)	1 (13%)	2 (18%)	0.0001*

* ANOVA test was used to evaluate the comparison between groups.

** BMI: Body Mass Index.

*** NA: No applied

Figures.

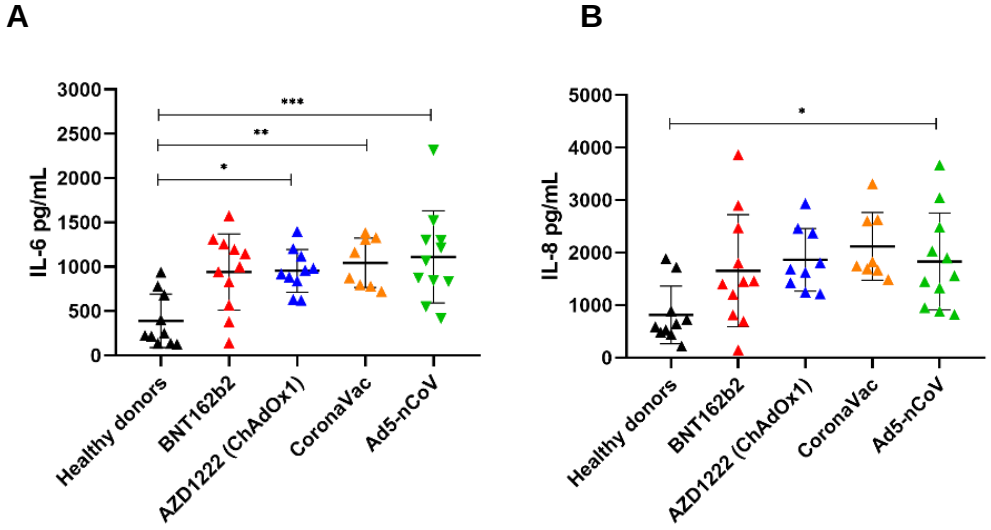

Figure 1. Comparison of plasma concentration of inflammatory associated biomarkers in subjects vaccinated against SARS-CoV-2 according to the biologic applied. **A)** An increase in plasma IL-6 concentrations is observed in subjects vaccinated with adenoviral and inactivated virus vaccines. The Ad5-nCoV and CoronaVac groups showed concentrations above 1,000 pg/ml, while the group immunized with AZD1222 showed a mean of 950 ug/ml. **B)** Plasmatic levels of IL-8 increased in all the groups (> 1,000 pg/mL) are showed when compare with the control group (< 1,000 pg/mL). (ANOVA, ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05). 131 x 110 mm (300 x 300 DPI).

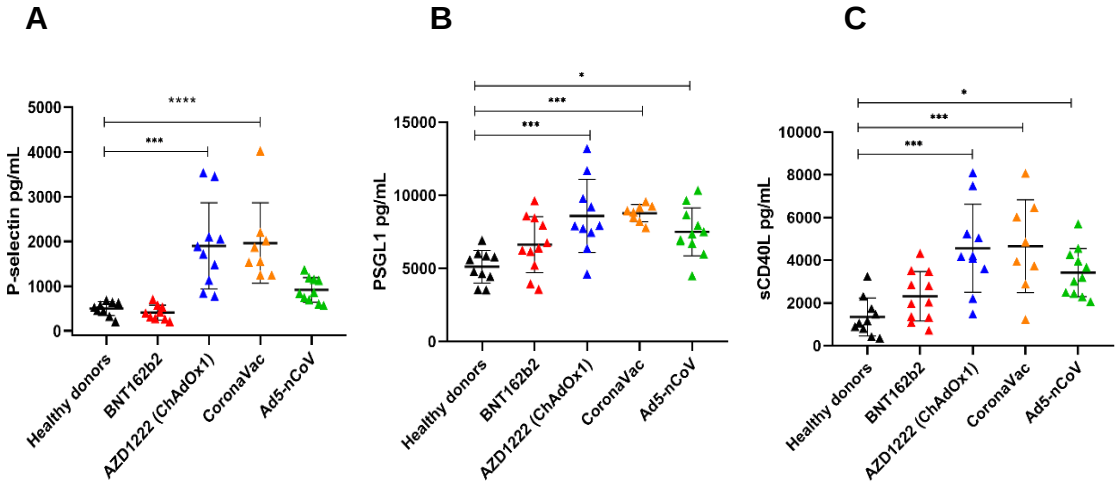

Figure 2. Comparison of plasma concentration of inflammatory associated biomarkers in subjects vaccinated against SARS-CoV-2 according to the biologic applied. **A)** An increase in plasma P-selectin concentrations is observed in subjects vaccinated with AZD1222 and CoronaVac. This groups showed concentrations above 2,000 pg/ml, while the control group showed a mean of 588 ug/ml. **B)** PSGL1 plasma concentrations from all 3 groups of AZD1222, CoronaVac, Ad5-nCoV showed a significant difference when compared with the group of healthy donors. **C)** sCD40L plasma concentrations from all 3 groups of AZD1222, CoronaVac, Ad5-nCoV showed a significant difference when compared with the group of healthy donors. The groups immunized with adenoviral vector AZD1222 and with the viral attenuated CoronaVac showed the highest concentrations > 4,000 pg/mL (ANOVA, ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05). 131 x 110 mm (300 x 300 DPI).

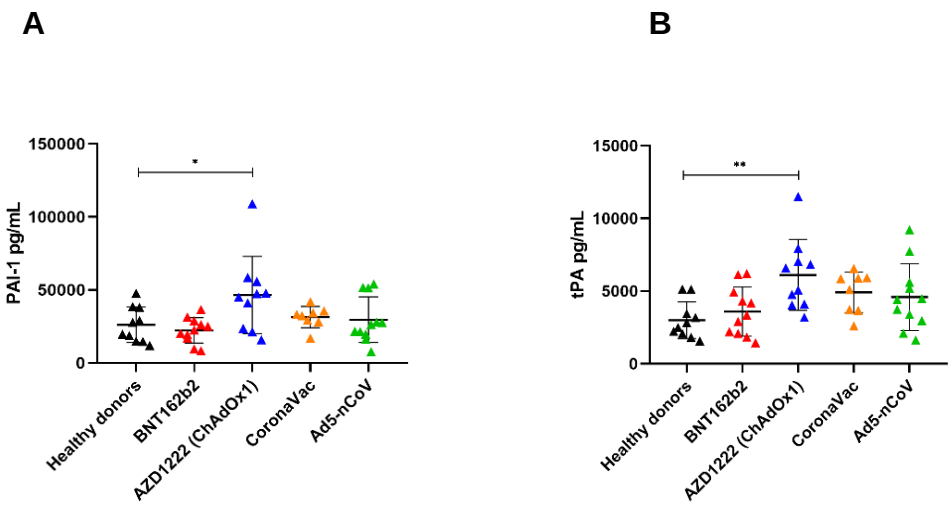

Figure 3. Comparison of plasma concentrations of thrombotic associated biomarkers in subjects vaccinated against SARS-CoV-2 according to the biologic applied. **A)** Plasma concentrations of PAI-1 showed significant difference in AZD1222 group when compared to healthy donors. It is observed that the adenoviral platform increases significantly PAI-1 levels. **B)** Plasma concentrations of tPA showed significant difference in AZD1222 group with a mean above 5,000 pg/mL when compared to healthy donors. (ANOVA ** p < 0.01, *p < 0.05). 131 x 110 mm (300 x 300 DPI).

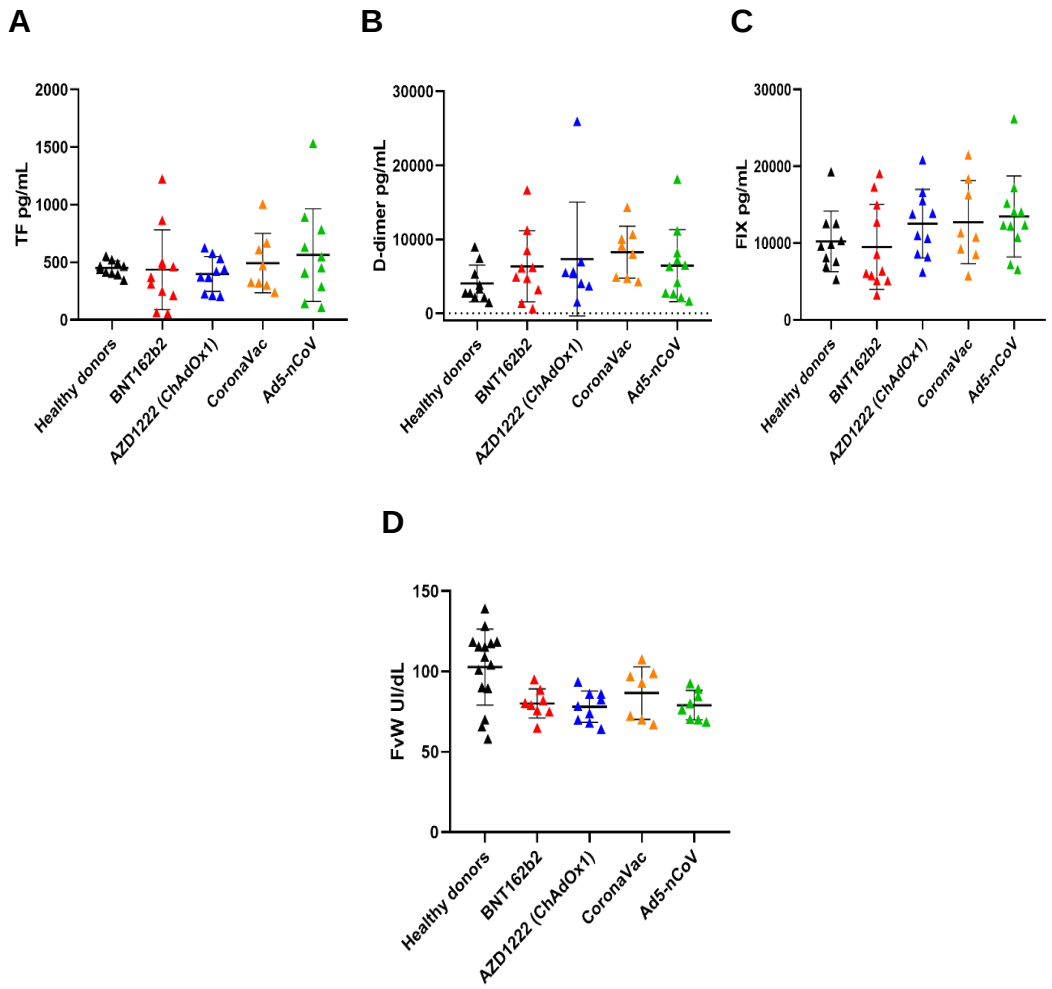

Figure 4. Comparison of plasma concentrations of thrombotic associated biomarkers in subjects vaccinated against SARS-CoV-2 according to the biologic applied. **C)** No differences in plasmatic TF concentrations between vaccinated groups when compare with healthy donors. **D)** No differences between groups in D-dimer plasmatic concentrations were found. **E)** Differences in plasmatic TF concentrations are not found between study groups. **F)** No differences in plasmatic FIX concentrations are found between groups. **G)** Plasma concentrations of FvW showed no differences between groups. (ANOVA ** $p < 0.01$, *$p < 0.05$). 131 x 110 mm (300 x 300 DPI).

IX. DISCUSIÓN GENERAL

El SARS-CoV-2, virus causante de COVID-19, ocasionó la pandemia más reciente en la historia de la humanidad. Esta enfermedad es una patología con afectación respiratoria; sin embargo, la presencia de un estado proinflamatorio y protrombótico se ha correlacionado con la gravedad de la enfermedad y el pronóstico del paciente (26,31). El sistema inmune innato conserva una estrecha relación con el proceso de coagulación. La activación de la coagulación en respuesta a un proceso inflamatorio agudo recibe el nombre de inmunotrombosis. La respuesta inmunotrombótica involucra la actividad de las plaquetas, las células endoteliales y los leucocitos (36).

El SARS-CoV-2 utiliza a la proteína Spike (S) para infectar las células hospederas en el ser humano a través de un dominio proteico denominado Dominio de Unión a Receptor (RBD) (11). Se ha propuesto a la Enzima Convertidora de Angiotensina 2 (ECA-2) como el principal receptor de esta proteína (17). ECA-2 se encuentra altamente expresada en células endoteliales y en plaquetas (17), por lo que este trabajo evaluó la respuesta inmunotrombótica de estos linajes celulares a la proteína S y a el dominio RBD de esta proteína.

De manera inicial se determinó la reactividad plaquetaria ante la proteína Spike y al dominio RBD. Se observó que las proteínas virales inducen la expresión de P-Selectina y de la GP IIbIIIa, la cual es dependiente del tiempo de estimulación y la concentración proteica. Estos resultados coinciden con los reportados por Zhang et al. (2020), quienes observaron una activación plaquetaria utilizando la misma concentración de la proteína Spike y la subunidad 1 (S1) de la proteína, región dónde se localiza el dominio RBD (61).

Posteriormente se evaluó si las proteínas virales inducían la agregación plaquetaria. Se determinó que las proteínas del SARS-CoV-2 no favorecen la agregación de las plaquetas; sin embargo, se observó que las plaquetas que se incubaron con la proteína Spike completa se encuentran en un estado de hiperreactividad, lo que ocasiona que al ser estimuladas con dosis bajas de colágeno se reestablezca el proceso de agregación plaquetaria. Estos resultados coinciden con lo reportado por Zhang et al. (2020), quienes demostraron que las células pre-estimuladas con la proteína Spike o su subunidad 1, pueden agregarse cuando se añaden dosis bajas de agonistas de activación conocidos (61). Este comportamiento plaquetario se ha descrito anteriormente con otras proteínas

virales. En nuestro grupo de trabajo, García-Larragoiti et al. (2021) describieron que la proteína no estructural (NS1) del virus del dengue induce una reactividad plaquetaria que favorece la formación de agregados plaquetarios cuando se estimulan con bajas dosis de epinefrina (62).

Finalmente, al ser las plaquetas una fuente rica de factores solubles que participan en los procesos inmunotrombóticos, se evaluaron las concentraciones de estos biomarcadores liberados por las plaquetas después de ser incubadas con las proteínas virales. Los resultados demuestran que la proteína Spike y el dominio RBD promueven la liberación de factores proinflamatorios y procoagulantes por parte de las plaquetas. Se encontró un aumento en las concentraciones de IL-6, IL-8, P-selectina, sCD40L y MCP-1 en el medio condicionado de las plaquetas tratadas con las proteínas virales. Estas moléculas están implicadas en la respuesta inflamatoria aguda. Se observó que la liberación de estos factores solubles es dependiente del tiempo de exposición plaquetaria a las proteínas virales. Esta respuesta puede estar relacionada con un proceso de degranulación selectivo, respaldando la hipótesis de la presencia de subpoblaciones plaquetarias con diversos contenidos de α-gránulos, y que sugiere que existen plaquetas procoagulantes y plaquetas proinflamatorias las cuales actúan dependiendo del estímulo recibido (63,64). Estos resultados coinciden con lo reportado por van Holten et al. (2014), quienes observaron mediante inmunoensayos y ensayos proteómicos que existe un perfil de liberación del contenido granular heterogéneo que es dependiente de las características del agonista de activación utilizado (65).

Una vez demostrado el fenotipo proinflamatorio inducido en las plaquetas por la proteína S y el dominio RBD, se evaluó la participación de las plaquetas en el establecimiento de un ambiente procoagulante y antifibrinolítico. Se demostró que las plaquetas incubadas con las proteínas virales, secretan PAI-1 y el factor IX de la coagulación, lo que favorece un microambiente procoagulante. Lo anterior se demuestra al observar la generación de los dímeros-D. Estos resultados concuerdan con diversos estudios que reportan un fenotipo plaquetario procoagulante en COVID-19 (66,67).

Paralelamente, se evaluó la respuesta endotelial a estas proteínas virales, dada la relevancia que tienen estas células en la respuesta inmunotrombótica. Se utilizaron dos líneas celulares endoteliales, las EA.hy 926 (provenientes de la fusión de endotelio

venoso de cordón umbilical con la línea A549 de cáncer de pulmón) y las HUVEC (provenientes de vena de cordón umbilical). Ambas líneas conservan las características de las células endoteliales y responden de manera selectiva a estímulos inflamatorios, angiogénicos y procoagulantes (68).

De manera inicial, se observó que la activación endotelial en respuesta a la proteína S y al dominio RBD son dependientes de la concentración proteica y el tiempo de estimulación. Estos resultados concuerdan con lo reportado por otros grupos de trabajo quienes determinaron que la activación endotelial inducida por el SARS-CoV-2 o por sus proteínas estructurales responde a una cinética dependiente de la concentración (69–71).

Posteriormente, se evaluó el fenotipo inmunotrombótico inducido en estas células por las proteínas S y RBD. Se encontró que los retos virales favorecen la expresión y liberación al medio de IL-6, IL-8, P-selectina, sCD40L y MCP-1, y que esta respuesta es similar a la generada por estímulos inflamatorios como la IL-6 y el LPS, así como el factor de crecimiento endotelial vascular (VEGF), factor inductor de la activación endotelial (68,72,73). El fenotipo proinflamatorio observado en estos resultados puede estar asociado a la activación de la vía de señalización intracelular del factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas (NF-KB), la cual favorece la expresión de moléculas inflamatorias (74). Estudios adicionales son necesarios para confirmar esta hipótesis.

También se observó que ambas proteínas virales favorecen la generación de dímeros-D, y una correlación negativa en el eje PAI-1/tPA, lo que está asociado con un mecanismo de coagulación activo y un proceso de fibrinolisis desregulado. Estos resultados concuerdan con lo publicado por otros grupos de trabajo quienes reportan que la proteína S favorece la liberación de PAI-1, tPA y factores inflamatorios en células endoteliales tratadas con la proteína S (75,76).

Una vez determinada la respuesta plaquetaria y endotelial a las proteínas virales, se evaluaron potenciales receptores alternativos a la ECA-2 mediante estudios *in silico* utilizando docking molecular. En este trabajo se reportan 9 diferentes receptores expresados a nivel membranal en plaquetas y células endoteliales con capacidad para ligarse a la proteína S. Se encontró que la basignina (CD-147), el receptor de la IL-6, la

integrina α5β1 y los TLR-2,4 y 7 expresados en ambos linajes celulares, así como la GP IIbIIIa expresada únicamente en plaquetas, la trombomodulina (GP 141) y neuropilina 1 específicas de endotelio, poseen una alta afinidad por la proteína S; similar o incluso mayor a la existente entre la proteína S y la ECA-2. Diversos estudios previos han evaluado a estos receptores y los han propuesto como receptores alternativos a la ECA-2 (60,70,72,77–79).

Con la finalidad de correlacionar los resultados observados en los ensayos *in vitro,* se procedió a evaluar el estado inmunotrombótico en pacientes que cursaban con la enfermedad COVID-19 (Anexo XIII.I). Se observó una leucocitosis con neutrofilia en los pacientes que tenían un peor curso de la enfermedad. Se ha demostrado que la presencia de los neutrófilos es sugestiva de activación endotelial y, por tanto, la producción y de factores procoagulantes como el fibrinógeno, tPA, PAI-1 y FvW (80,81).

Los resultados del presente trabajo muestran un aumento del tPA en pacientes hospitalizados por COVID-19, lo que puede atribuirse a la interacción entre las citocinas proinflamatorias y el endotelio, cuya estimulación favorece la liberación de tPA (82). Las concentraciones de PAI-1 fueron mayores en el grupo de enfermedad grave. Resultados similares fueron reportados por Henry et al, (2021), lo que implica una anomalía en el proceso fibrinolítico, contribuyendo a un mayor estado de hipercoagulabilidad que puede conducir al empeoramiento del estado del paciente (83). Otro biomarcador sugestivo de fibrinolisis activa es el dímero-D. En los resultados de este trabajo se observó un aumento de las concentraciones de dímero-D, a pesar de que en los pacientes se encontró un tPA elevado, es probable que el tPA disminuya en algún momento de la evolución de la enfermedad o que su concentración se vea superada en mayor medida por la del PAI-1, lo que da lugar a una hipofibrinólisis (84).

De igual manera, se encontraron niveles elevados de FvW en los pacientes que cursaban con un estadio grave de la enfermedad. Estos resultados concuerdan con lo que nuestro grupo de trabajo reportó en 2021 (26), y con los comunicados por Ward et al, (2021), quienes compararon las concentraciones de FvW en pacientes con COVID-19 con las de controles sanos (85), así como los reportados por Mancini et al, (2021), quienes observaron disfunción endotelial y niveles elevados de la proteína FvW en sujetos en estadio grave (86). Posteriormente, se evaluó la estructura multimérica del

FvW. Se encontró que los sujetos que cursaban con COVID- 19 grave o que murieron a causa de la enfermedad, presentaban multímeros de alto peso molecular (HMW-vWF) los cuales correlacionan con un mayor estado procoagulante. Esto podría confirmar que existe una desgranulación activa de las plaquetas y de las células endoteliales contribuyendo a la fisiopatología de COVID-19 grave (87).

Con base en estos resultados es posible sugerir que existe un estado hiperinflamatorio que correlaciona con la gravedad de la enfermedad. Estos resultados concuerdan con un estudio realizado por nuestro grupo de trabajo durante la primera oleada de infecciones en 2020 (26). Por otro lado, en trabajos previos se ha descrito que los pacientes COVID-19 con peor pronóstico presentan niveles plasmáticos elevados de citocinas inflamatorias y factores protrombóticos (88).

Los resultados obtenidos permitieron observar que aquellos pacientes con COVID-19 que desarrollaron un peor curso de la enfermedad mostraron un aumento del volumen plaquetario medio (VPM) y una hiperactivación plaquetaria determinada por una expresión elevada de P-selectina y GP IIbIIIa. Leopold et al, (2021), reportaron una hiperreactividad plaquetaria en pacientes con COVID-19 (89). El estudio de plaquetas en COVID-19 ha permitido observar que la trombocitopenia es poco frecuente en pacientes con COVID-19 y se correlaciona con una mayor morbilidad/mortalidad (90).

La integración de los resultados de este trabajo permite sugerir, que en el estado inmunotrombótico presente en COVID-19 participan activamente las plaquetas y las células endoteliales a través de la liberación de marcadores solubles de carácter proinflamatorio y protrombótico. De igual manera es posible observar una marcada disfunción endotelial y desregulación del proceso fibrinolítico, condicionando el pronóstico de los pacientes.

Finalmente, durante el curso de la pandemia, se diseñaron y aprobaron diversas vacunas contra el SARS-CoV-2, las cuales disminuyeron significativamente la morbimortalidad de la enfermedad (91,92). Sin embrago, en algunos sujetos inmunizados se reportaron casos de una nueva entidad patológica denominada Trombosis Trombocitopénica Inmune inducida por la Vacunación (VITT) la cual se asoció directamente con la vacuna ChAdOx1 nCoV-19 (58,93). Por esta razón, se decidió estudiar el perfil inmunotrombótico en muestras de sujetos inmunizados con las diferentes

vacunas circulantes en el estado de Michoacán.

Se observó que la vacuna ChAdOx1 nCoV-19 favorece un proceso inflamatorio agudo caracterizado por el aumento en las concentraciones plasmáticas de citocinas inflamatorias como la IL-6, IL-8, P-selectina, PSGL-1 y el sCD40L. Estos resultados coinciden con lo reportado con otros grupos de investigación, quienes observaron alteraciones en los perfiles de coagulación e inflamación después de la aplicación de esta vacuna (94). Esta respuesta es característica de un proceso inmunológico activo; sin embargo, al determinar las concentraciones de biomarcadores relacionados con el proceso de coagulación y fibrinolisis, fue posible observar que la vacuna ChAdOx1 nCoV-19 genera la desregulación de los mecanismos de degradación de fibrina; así como una actividad endotelial aumentada, demostrada por la expresión de FvW. Estos resultados concuerdan con las características clínicas reportadas en casos de VITT por otros grupos de trabajo alrededor del mundo (58,95,96).

Resulta relevante mencionar que ninguno de los sujetos involucrados en el estudio presentó un evento trombótico. Esto evidencía que los beneficios de la vacunación son mayores a los posibles eventos adversos; sin embargo, obliga al personal sanitario a evaluar y dar seguimiento clínico personalizado a aquellos sujetos inmunizados con esta vacuna que presentan una patología proinflamatoria o trombofílica de base, así como a aquellos que se encuentran bajo tratamientos farmacológicos que pueden asociarse con un proceso procoagulante que puede potenciar una respuesta trombogénica.

El resumen integral de los hallazgos inmunotrombóticos reportados en este trabajo de investigación se desglosan en la Figura 8.

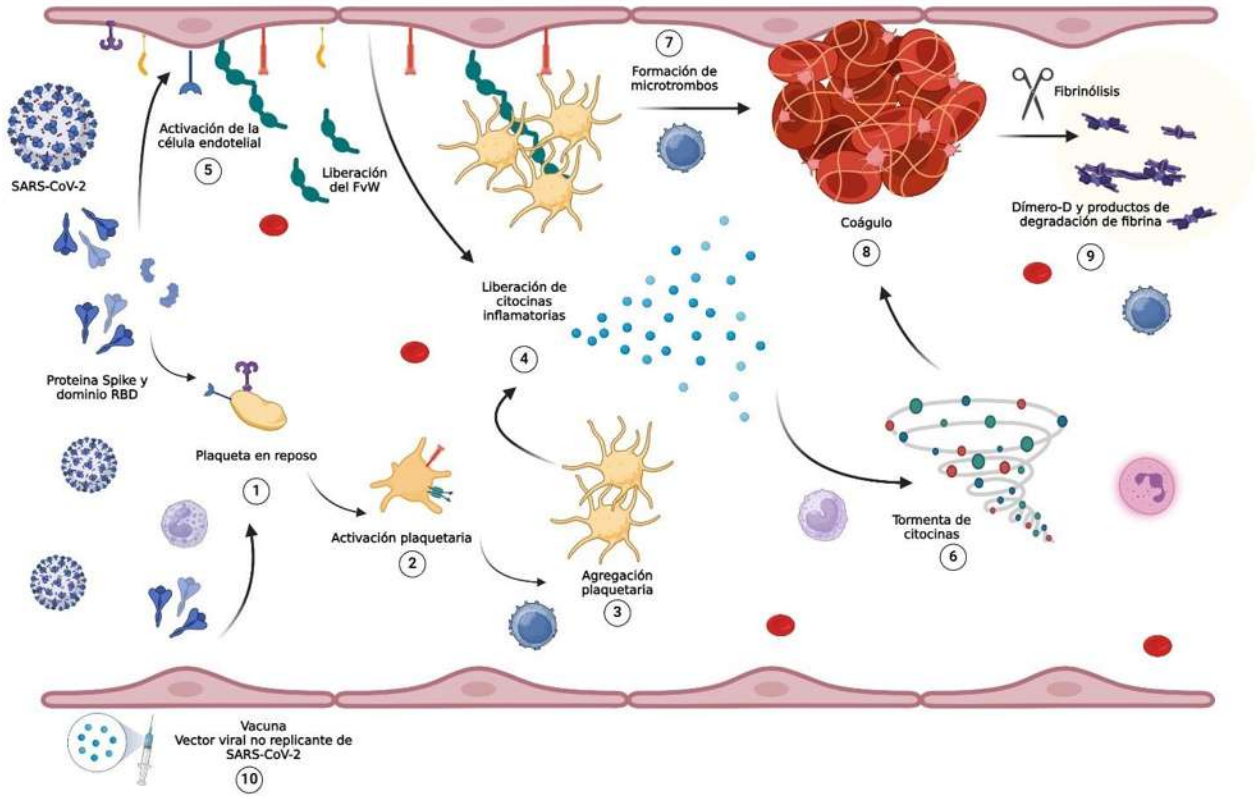

Figura 8. Mecanismos inmunotrombóticos presentes durante la infección por SARS-CoV-2, COVID-19 y posterior a la vacunación contra el virus SARS-CoV-2. La proteína Spike y el dominio RBD inducen la activación plaquetaria **(1)** favoreciendo la desgranulación selectiva de estas células, liberando factores proinflamatorios y procoagulantes **(2)** que potencian la tormenta de citocinas y estimulan el estado inmunotrombótico **(4)**. El fenotipo hiperreactivo plaquetario predispone un estado proagregante en las plaquetas ante estímulos procoagulantes **(3)**. El endotelio vascular es hiperreactivo a las proteínas virales del SARS-CoV-2 induciendo la expresión de moléculas solublesque potencian la inflamación, favorecen la coagulación intravascular y desregulan los procesos fibrinolíticos **(5)**. La activación de estas vías condiciona la formación de microtrombos **(6)** y favorecela formación de coágulos que correlacionan con la gravedad del proceso infeccioso **(7-9)**. Las vacunas diseñadas a base de vectores virales no replicantes desencadenan el proceso inmunotrombótico **(10)**. vWF: Factor de von Willebrand, DPF: Productos de Degradación de la Fibrina.

X. CONCLUSIONES

La proteína Spike y el dominio RBD del virus SARS-CoV-2 activan a las plaquetas generando un fenotipo hipercoagulante que ante la exposición a pequeños estímulos puede inducir la agregación plaquetaria.

De igual manera, la proteína Spike y el dominio RBD pueden activar a las células endoteliales. La activación de las plaquetas y de las células endoteliales induce la liberación de moléculas proinflamatorias, procoagulantes y antifibrinolíticas, favoreciendo el establecimiento de un estado inmunotrombótico.

Clínicamente, los pacientes afectados por COVID-19 muestran un estado inmunotrombótico activo el cual correlaciona con la gravedad de la enfermedad y con el pronóstico del paciente. Asimismo, la vacuna ChAdOx1 nCoV-19, favorece el establecimiento de un estado inmunotrombótico en los sujetos inmunizados.

XI. PERSPECTIVAS Y RECOMENDACIONES

El presente trabajo contribuye al estudio de la participación de las plaquetas y células endoteliales en la fisiopatología de la infección por SARS-CoV-2. Con base en los resultados obtenidos se propone continuar con la línea de investigación de la siguiente manera:

1. Evaluación de la participación de los receptores identificados por docking molecular en la infección del virus SARS-CoV-2 en las células endoteliales y en las plaquetas, a través del uso de inhibidores específicos de cada receptor.

2. Determinación de la generación de Trampas Extracelulares de Neutrófilos (NETs) inducida por las proteínas Spike y el dominio RBD.

3. Determinación de las concentraciones de la proteína Spike circulantes en sangre en pacientes recuperados de COVID-19 y su correlación con la persistencia de síntomas.

4. Identificación de las vías de señalización activadas por las proteínas Spike y el dominio RBD en plaquetas y en células endoteliales.

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XIII. ANEXOS

XIII.I Aprobación ética: Valoración del riesgo trombótico inflamatorio en pacientes afectados por COVID-19 y recomendaciones del tratamiento.

Gobierno del Estado
de Michoacán de Ocampo

Dependencia SECRETARIA DE SALUD DE MICHOACÁN
HOSPITAL GENERAL "DR. MIGUEL SILVA"
Sub-dependencia
Oficina COMITES DE ETICA EN INVESTIGACIÓN E INVEST.
No. de oficio 5009/051/21
Expediente
Asunto:
Autorización de extensión de protocolo de investigación.

"2021, año de la Independencia"

Atapaneo, Michoacán, 13 de septiembre del 2021.

C. DRA. MARTHA EVA VIVEROS SANDOVAL
INVESTIGADORA TITULAR
PRESENTE.

Por este conducto le informamos, que el Comité de Ética en Investigación con número de Registro Conbioética-16-CEI-004-20161212 con fecha de expedición diciembre 13 del 2019 y el Comité de Investigación con número de Registro Conbioética-17-CI-16053153 con fecha de expedición 11 de noviembre del 2017 del Hospital General "Dr. Miguel Silva", **Autorizaron la extensión hasta septiembre de 2022** del protocolo de investigación número 530/01/20 titulado: **Valoración del riesgo trombótico inflamatorio en pacientes afectados por covid-19 y recomendaciones del tratamiento.**

Quedamos a sus órdenes para cualquier duda o comentario, aprovechamos la oportunidad para enviarle un cordial saludo.

ATENTAMENTE

DRA. CLAUDIA AGUSTINA RAMOS OLMOS
PRESIDENTA DEL COMITE DE ETICA EN INVESTIGACION
DEL HOSPITAL GENERAL "DR. MIGUEL SILVA"

COMITE DE ÉTICA EN INVESTIGACIÓN
HOSPITAL GENERAL "DR. MIGUEL SILVA"
SECRETARIA DE SALUD DE MICHOACÁN

DR. JOSÉ FRANCISCO LÓPEZ BELTRÁN
PRESIDENTE DEL COMITÉ DE INVESTIGACIÓN DEL HOSPITAL GENERAL "DR. MIGUEL SILVA".

COMITE DE INVESTIGACIÓN
HOSPITAL GENERAL "DR. MIGUEL SILVA"
SECRETARIA DE SALUD DE MICHOACÁN

C.c.p. Dra. María Patricia Martínez Medina - Subdirectora de Enseñanza e Investigación, Hospital General "Dr. Miguel Silva". PRESENTE
C.c.p. minutario

CARD*JFLB*sev

Michoacán #EstáenTi
*El contenido del presente documento es responsabilidad directa del titular del área Administrativa

XIII.II Aprobación ética: Evaluación del riesgo trombótico inflamatorio en individuos vacunados contra el virus SARS-CoV-2.

Facultad de Ciencias Médicas y Biológicas
"Dr. Ignacio Chávez"

Secretaría Académica
Morelia, Michoacán, 19 de agosto de 2021

M.C. ALAN FABRICIO CANO MENDEZ
ALUMNO DE POSGRADO FACULTAD DE CIENCIAS MÉDICAS Y BIOLÓGICAS "DR. IGNACIO CHÁVEZ"
P R E S E N T E

A S U N TO: DICTAMEN

Le notifico que el protocolo de investigación que Usted presentó ante esta Secretaría Académica a mi cargo, y cuyo título es "***Evaluación del riesgo trombótico inflamatorio en individuos vacunados contra el virus SARS-CoV-2***" y que fue sometido a la evaluación del Comité de Investigación y Ética en Investigación de esta Dependencia Universitaria, quien (es) de acuerdo con las recomendaciones de sus integrantes y de los revisores consideraron que cumple con la calidad metodológica y los requerimientos de ética y de investigación vigentes, por lo que el protocolo fue **AUTORIZADO**, habiéndose asignado el número de registro de la dependencia siguiente:

COMITÉ DE INVESTIGACIÓN Y ETICA EN INVESTIGACIÓN	ÁREA	Nº DE REGISTRO
FACULTAD DE CIENCIAS MÉDICAS Y BIOLÓGICAS "DR. IGNACIO CHÁVEZ"	POSGRADO	004/P/5/2021

Se le solicita informar del grado de avance del mismo acorde al cronograma de actividades así como el título de la revista, volumen, año y páginas una vez que el mismo sea publicado. En caso de que el mismo contemple la graduación de un alumno indicar fecha y grado académico obtenido.

A t e n t a m e n t e:

DRA. PAOLA LÓPEZ HERNÁNDEZ
SECRETARIA ACADÉMICA

FACULTAD DE CIENCIAS MÉDICAS Y BIOLÓGICAS "DR. IGNACIO CHÁVEZ" SECRETARÍA ACADÉMICA

c.c.p. Coordinador de la Investigación Científica UMSNH
c.c.p. Archivo de la Secretaría Académica
c.c.p. Jefatura de Investigación Fac Cs Med y Biol. "Dr. Ignacio Chávez"
PLH/can

Formato de Declaración de Originalidad y Uso de Inteligencia Artificial

Coordinación General de Estudios de Posgrado
Universidad Michoacana de San Nicolás de Hidalgo

A quien corresponda,

Por este medio, quien abajo firma, bajo protesta de decir verdad, declara lo siguiente:

- Que presenta para revisión de originalidad el manuscrito cuyos detalles se especifican abajo.
- Que todas las fuentes consultadas para la elaboración del manuscrito están debidamente identificadas dentro del cuerpo del texto, e incluidas en la lista de referencias.
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- Que conoce la normativa de la Universidad Michoacana de San Nicolás de Hidalgo, en particular los Incisos IX y XII del artículo 85, y los artículos 88 y 101 del Estatuto Universitario de la UMSNH, además del transitorio tercero del Reglamento General para los Estudios de Posgrado de la UMSNH.

Datos del manuscrito que se presenta a revisión		
Programa educativo	Programa Institucional de Doctorado en Ciencias Biológicas	
Título del trabajo	Estudio de la Respuesta Inmunotrombótica a la Proteína Spike del virus SARS-CoV-2	
	Nombre	**Correo electrónico**
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Uso de Inteligencia Artificial		
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Asistencia en la redacción	No	

Formato de Declaración de Originalidad y Uso de Inteligencia Artificial

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Rubro	**Uso (sí/no)**	**Descripción**
Traducción al español	No	
Traducción a otra lengua	No	
Revisión y corrección de estilo	No	
Análisis de datos	Si	Se utilizó el programa GraphPad Prism 7.0 para el análisis de resultados y el programa SPSS para la elaboración de bases de datos.
Búsqueda y organización de información	Si	Se utilizó la base de datos PubMed para la busqueda de artículos científicos.
Formateo de las referencias bibliográficas	Si	Se utilizó el programa Zotero para el formateo de referencias bibliográficas.
Generación de contenido multimedia	No	
Otro		

Datos del solicitante	
Nombre y firma	Alan Fabricio Cano Méndez
Lugar y fecha	Morelia, Michoacán a 30 de octubre de 2024

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