Universitat Autònoma de Barcelona

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Que Manuel Gutiérrez Gimeno, licenciado en medicina y especialista en radiodiagnóstico, ha trabajado bajo nuestra dirección en la elaboración del proyecto de tesis doctoral titulado:

«Análisis de nuevas estrategias cardioprotectoras destinadas a modular el remodelado ventricular tras infarto agudo de miocardio: estudio preclínico y seguimiento mediante técnicas de imagen no invasiva de alta resolución»

Estudio que termina en el día de la fecha con todo aprovechamiento, habiéndolo revisado los que suscriben y estando conformes con su presentación para ser juzgado como tesis doctoral.

Y para que conste a todos los efectos, firmamos la presente en

Barcelona, a 28 de junio de 2019.

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Abreviaturas

5HT-2A serotonina
8-SPT 8-p-sulfofenil-teofilina
AAS ácido acetilsalicílico
Ad-R A1/2b/3 receptores de adenosina
Akt proteína cinasa-B
AMPK AMP cinasa
ATP trifosfato de adenosina
b-SSFP *balanced steady state free precession*
Bradi-R receptor de bradicinina
CalDAG-GEFI *calcium DAG-regulated guanine nucleotide exchange factor*
Cox2 cicloxigenasa 2
CytP450 citocromo P450
DAG diacilglicerol
EGE *early gadolinium enhancement*
EGF *epidermal growth factor* (factor de crecimiento epidérmico)
eNOS sintasa endotelial de óxido nítrico
ENT-1 *equilibrative nucleotid transporter*
ERK *extracellular signal–regulated kinase*
FcγR cadena gamma del receptor Fc
FEVI fracción de eyección del ventrículo izquierdo
FT factor tisular
GC guanilil-ciclasa.
GP glucoproteína
GPCR *G-protein coupled receptors*
GSK3 *glycogen synthase kinase 3*
GSK3β cinasa de la glucógeno sintetasa beta
HB-EGF-R receptor del *heparin-binding EGF like growth factor*
IAM infarto agudo de miocardio
ITAM *immune receptor tyrosine based activation motif*
JAK Janus cinasa
KATP canal de potasio dependiente de ATP
LGE *late gadolinium enhancement*
MAPK *mitogen-activated protein kinase*

MEK MAP cinasa
MMP *metaloproteinasa matricial*
mPTP poro de transición de permeabilidad mitocondrial
MVO obstrucción microvascular
NO óxido nítrico
NOX NADPH oxidasa
Op-R receptor de opiáceos
P2Y12 receptor de ADP P2Y12
p70S6K cinasa del p70S6
PAR *protease-activated receptor*
PDK1 *phosphoinositide-dependent protein kinase 1*
PDK1/2 cinasa dependiente de fosfoinosítidos
PI2/PI3 fosfatidil-inositol di/trifosfato
PI3K PI3 cinasa
PKC proteína cinasa C
PKG proteína cinasa dependiente de GMPc
PKG proteína cinasa G
PLC fosfolipasa C
PLC/PLD *fosfolipasa C y D*
Pro *pro-HB-EGF*
Rap1 *Ras-related protein 1*
RISK *reperfusion injury salvage kinase*
RMC resonancia magnética cardíaca
ROS *reactive oxygen species*
S1P-R receptor de esfingosina-1-fosfato
SAFE *survivor activating factor enhancement*
SCACEST síndrome coronario agudo con elevación del ST
SFK *Src family protein kinases*
STAT *signal transducer and activator of transcription*
STIR *short tau inversion recovery*
TNFα factor de necrosis tumoral alfa
TNFα-R receptor del factor de necrosis tumoral
TP receptor de tromboxano
TXA2 tromboxano A2
VASP *vasodilator stimulated phosphoprotein*
VTD volumen telediastólico
VTS volumen telesistólico
vWf factor de von Willebrand

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Resumen

Análisis de nuevas estrategias cardioprotectoras destinadas a modular el remodelado ventricular tras infarto-agudo de miocardio: estudio preclínico y seguimiento mediante técnicas de imagen no invasiva de alta resolución

Introducción: la cardiopatía isquémica es una de las principales causas de morbimortalidad en todo el mundo. En las últimas décadas se ha logrado disminuir considerablemente la mortalidad de los pacientes con infarto agudo de miocardio, a costa de un aumento de los casos de insuficiencia cardíaca crónica. En consecuencia, es necesario idear nuevos enfoques terapéuticos para prevenir el remodelado cardíaco y la aparición de insuficiencia cardíaca. El área total de necrosis se debe a la combinación de la lesión isquémica y el daño por reperfusión (desencadenado por la restauración repentina del flujo sanguíneo en el territorio infartado). Éste último juega un papel clave en el desarrollo del remodelado cardíaco e insuficiencia cardíaca. Los bloqueadores del receptor $P2Y_{12}$ son un grupo de agentes antiplaquetarios de uso habitual en el infarto agudo de miocardio, en el que existen diferentes subtipos con diferentes perfiles farmacocinéticos y farmacodinámicos (ej. el agente tradicional clopidogrel frente al de nueva generación ticagrelor). Se cree que ticagrelor tiene efectos sobre el metabolismo de la adenosina, una molécula con efectos cardioprotectores. En esta tesis queremos demostrar que ticagrelor ejerce un efecto cardioprotector sobre el corazón infartado mayor que el ejercido por clopidogrel y que este efecto es independiente de su efecto antiplaquetario.

Material y métodos: se diseñó un modelo animal (porcino) de infarto agudo de miocardio compuesto por dos estudios con un mismo diseño. Los animales fueron aleatorizados a grupos de igual tamaño que recibieron diferentes intervenciones (placebo, clopidogrel y ticagrelor). Des-

pués de la dosis de carga inicial se indujo un infarto en todos los animales y, en los días posteriores, fueron llevados a una instalación de resonancia magnética (RM) donde se estudiaron parámetros cardiacos funcionales y estructurales (motilidad global y segmentaria, edema, obstrucción microvascular y necrosis). Finalmente, los animales fueron sacrificados para para realizar análisis histológicos y moleculares en los que se evaluó la expresión y activación de moléculas relacionadas con la formación de edema y supervivencia celular (ej. acuaporina-4, AMPK). Mientras que el primer estudio se centró en los efectos durante las primeras 24 horas post infarto, el segundo estaba destinado a la evaluación de los efectos crónicos (3d y 42d después del infarto).

Resultados: El uso de cualquier agente antiplaquetario reduce el daño miocárdico en comparación con placebo (menos edema y necrosis miocárdica, mejores parámetros funcionales en RM) pero ticagrelor lo hace en mayor medida que clopidogrel. La reducción observada, tanto del edema como de la necrosis, es estadísticamente significativa y concuerda con los resultados de los análisis moleculares. La expresión de acuaporina 4 se reduce y la expresión y activación de AMPK aumenta en animales tratados con ticagrelor. Acuaporina 4 es un canal transportador que facilita la entrada de agua en la célula en condiciones de estrés. Por otro lado, AMPK actúa disminuyendo el edema extracelular y promoviendo la supervivencia celular. Ticagrelor también muestra un impacto positivo sobre los parámetros funcionales en las fases aguda y crónica después del infarto. Tanto la fracción de eyección del ventrículo izquierdo como la motilidad regional se ven menos afectadas en los animales tratados con ticagrelor a día 3 y día 42. Es importante remarcar que todos los beneficios descritos con ticagrelor se anulan al añadir un antagonista del receptor de adenosina. Este hecho muestra que los efectos cardioprotectores de ticagrelor están mediados por adenosina.

Conclusión: En esta tesis demostramos que ticagrelor, un inhibidor de nueva generación del receptor $P2Y_{12}$, presenta efectos cardioprotectores adicionales en comparación con clopidogrel y que estos efectos están mediados por adenosina. El impacto positivo se observa en las fases aguda y crónica después del infarto de miocardio y está presente tanto en parámetros estructurales como funcionales.

Abstract

Analysis of new cardioprotective strategies intended to modulate ventricular remodeling after myocardial infarction: pre-clinical study and follow-up by non-invasive, high resolution imaging techniques

Introduction: ischemic heart disease is one of the leading causes of morbidity and mortality worldwide. A sharp decline in the mortality rate of patients suffering from acute myocardial infarction has been achieved, at the cost of rising cases of chronic heart failure. In consequence, it is necessary to devise new therapeutic approaches to prevent cardiac remodeling and the ensuing heart failure. In this regard, cardioprotection appears as a foreseeable mainstay. We now know the total necrotic area derives from a combination of the ischemic injury and an added damage produced by the sudden restoration of blood flow in the obstructed coronary artery's territory, the reperfusion injury. Reperfusion injury plays a key role in the development of cardiac remodeling and chronic heart failure. $P2Y_{12}$ blockers are a group of antiplatelet agents that are standard care in the setting of acute myocardial infarction. However, within the group there are different subtypes with different pharmacokinetic and pharmacodynamic profiles (e.g., the standard of care clopidogrel and the new generation $P2Y_{12}$ receptor blocker ticagrelor). Ticagrelor is believed to have effects on adenosine metabolism, molecule with cardioprotective implications. In this thesis we mean to prove that ticagrelor exerts a higher cardioprotective impact as compared to clopidogrel on the infarcted heart, independently of their antiplatelet effect.

Material and methods: we designed an animal (swine) myocardial infarction model composed by two studies with a common layout. Animals were randomly allocated to equally sized groups receiving different interventions (placebo, clopidogrel and ticagrelor). An infarction was induced

in all animals after the initial loading dose and, in the following days, they were brought to a magnetic resonance (MR) facility where functional and structural cardiac parameters were studied. Global and segmental motility as well as cardiac oedema, microvascular obstruction and late necrosis were assessed. Finally, the animals were sacrificed and their heart sliced to perform histologic and molecular analysis that assessed the expression and activation of molecules related to oedema formation and cell survival (i.e. aquaporin-4, AMPK molecules). Whereas the first study was centred in the effects in the first 24h after myocardial infarction, the second study was intended for evaluation of the chronic effects (3d and 42d after MI).

Results: The use of any antiplatelet agent reduces myocardial damage as compared to placebo (less myocardial oedema and necrosis, better functional parameters in MR). However, ticagrelor does so to a greater extent than clopidogrel. The observed reduction is statistically significant in both oedema and necrosis and concurs with the results of molecular analyses. The expression of acuaporine-4 is reduced and the expression and activation of AMPK increased in animals treated with ticagrelor. Acuaporine-4 is a channel transporter that eases the entrance of water in the cell under stress conditions. On the other hand, AMPK acts by diminishing extracellular oedema and promoting cell survival. Ticagrelor also shows a positive impact on functional parameters in both, acute and chronic phases after myocardial infarction. Left ventricle ejection fraction as well as regional wall motion are less impaired in animals treated with ticagrelor than clopidogrel at day 3 and day 42. Remarkably, all the positive effects described with ticagrelor are reversed when an adenosine receptor blocker is added. This fact shows the cardioprotective potential of ticagrelor is mediated by adenosine.

Conclusion: In this thesis we demonstrate ticagrelor, a new generation of $P2Y_{12}$ inhibitor, shows additional cardioprotective effects as compared to clopidogrel and that these effects are mediated by adenosine. The positive impact is observed in the acute and chronic phases after myocardial infarction and is present in both, structural and functional parameters.

Introducción

1.1 Introducción

Importancia socioeconómica del infarto agudo de miocardio

Las enfermedades cardiovasculares han sido la principal causa de muerte a nivel global durante los últimos 15 años. En el año 2015 produjeron la muerte de 17,9 millones de personas, de las cuales aproximadamente un 50 % (8,9 millones) fueron debidas a cardiopatía isquémica (1). Del resto, aproximadamente el 40 % (6,3 millones) fueron causadas por enfermedades cerebrovasculares (ictus isquémico y hemorrágico). Es remarcable que del total de muertes 3/4 partes se produjeron en países en vías de desarrollo. Las razones principales son tres (2):

- Ausencia de planes de prevención, detección y tratamiento precoz.
- Acceso limitado y desigual a los centros sanitarios.
- Coste excesivo de medicamentos en relación a los ingresos (3).

En Europa, las enfermedades cardiovasculares son responsables de aproximadamente cuatro millones de muertes al año, que equivalen a un 45 % de todas las muertes (4). Al igual que en el resto del mundo, la principal patología responsable es la cardiopatía isquémica, y dentro de esta, el síndrome coronario agudo con elevación del segmento ST (SCACEST), la forma más grave de infarto agudo de miocardio (IAM). Según los datos del Instituto Nacional de Estadística, en España el 2017 la cardiopatía isquémica fue responsable de más de 30 000 muertes (5).

El SCACEST se produce a causa de una obstrucción completa de una arteria coronaria, que si se mantiene en el tiempo termina con la muerte celular de todo el tejido miocárdico irrigado por la arteria (miocardio en riesgo). El desarrollo de técnicas de revascularización coronaria ha

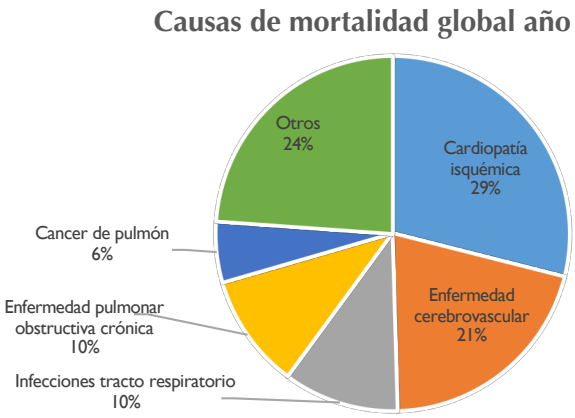

Figura 1. Adaptado de: WHO | Cardiovascular diseases (CVDs). Fact sheet 317

sido clave a la hora de disminuir la mortalidad por infarto. Entre los años 1985 y 2008 su implementación redujo la mortalidad intrahospitalaria de un 30 % a aproximadamente un 5,5 % entre los pacientes con SCACEST, quedando estabilizada posteriormente (6). Paradójicamente, la marcada disminución en la mortalidad aguda por infarto ha condicionado un notable aumento en la prevalencia de insuficiencia cardíaca crónica postinfarto. Esto se debe a que pacientes con infartos extensos, que hubieran muerto antes de la introducción de las estrategias de revascularización, sobreviven tras la revascularización y soporte farmacológico posterior, pero con un corazón dañado (tamaños de infarto considerables) y una función ventricular deprimida. Cabe remarcar que el tamaño final del infarto es un factor pronóstico de supervivencia postinfarto y de desarrollo de insuficiencia cardíaca (7).

En consecuencia, la cardiopatía isquémica condiciona una gran disminución en el grado de salud de una población (8) y su coste económico derivado es muy alto. Los pacientes supervivientes a un SCACEST requieren de costosas intervenciones farmacológicas para intentar prevenir un segundo episodio isquémico, así como el desarrollo de remodelado ventricular postinfarto. En los casos más graves son necesarios dispositivos médicos avanzados para mejorar su supervivencia (desfibrilador autoimplantable, resincronización cardíaca). Por esta razón, una vez conseguido el descenso de la mortalidad por infarto, el nuevo reto para los sistemas de salud es conseguir disminuir su principal secuela, la insuficiencia cardíaca crónica.

1.2
Introducción
Daño por isquemia/reperfusión

Como se ha explicado anteriormente, el SCACEST es la forma más grave de cardiopatía isquémica y la que se asocia a una mayor pérdida de salud. Desde hace décadas se sabe que éste se produce tras la oclusión de una arteria coronaria por fenómenos trombóticos desencadenados tras la ruptura de una placa ateroesclerótica (9). La revascularización de la arteria ocluida es determinante para parar detener el infarto y evitar la muerte del tejido miocárdico (10, 11). Sin embargo, en los últimos años se ha demostrado la existencia de un daño celular añadido producido tras la revascularización, el conocido como daño por reperfusión. La reperfusión miocárdica induce una serie de fenómenos vasculares y celulares que dañan el tejido infartado (12, 13). Por lo tanto, el total de tejido perdido tras el infarto es la suma del daño producido durante la isquemia y el desencadenado durante la reperfusión (daño por isquemia/ reperfusión).

Los fenómenos implicados en el daño por reperfusión que se producen en la microvascularización coronaria (red vascular que nutre los miocardiocitos) quedan englobados bajo el concepto de obstrucción microvascular (MVO[1]). Su importancia se debe al hecho que, independientemente de que exista revascularización de la arteria coronaria proximal, si existe MVO no va a existir una reperfusión efectiva de la zona isquémica. La sangre no va a penetrar en los capilares distales debido a que estos se encuentran obstruidos o dañados (tablas 1 y 2; figura 2).

En los estudios angiográficos esto se pone de manifiesto como un relleno incompleto del lecho miocárdico distal irrigado por la arteria reper-

1 MVO: *microvascular obstruction* (obstrucción microvascular).

Tabla 1. Fenómenos vasculares implicados en el daño isquemia-reperfusión

Vasoconstricción secundaria a liberación de factores vasoconstrictores tras la rotura de la placa de ateroma.
Edema secundario a aumento de la permeabilidad vascular.
Microémbolos de *debris* de la placa de ateroma
Respuesta inflamatoria. Reclutamiento neutrófilos y macrófagos (inmunidad innata) y formación de microémbolos de agregados plaquetarios y leucocitarios.
Disrupción de la pared vascular y hemorragia.

Tabla 2. Fenómenos celulares implicados en el daño isquemia-reperfusión

Hipercontractilidad secundaria a una sobrecarga de calcio intracelular.
Formación de radicales libres de oxígeno.
Colapso de las mitocondrias secundario a la apertura de los canales mPTP*
Muerte celular mediada por activación de enzimas (caspasas) que degradan el citoesqueleto y sarcolema y autofagia (lisosomas).

* mPTP: *mitochondrial permeability transition pore* (poro de transición de permeabilidad mitocondrial).

fundida, razón por la cual también se conoce como fenómeno de «*no reflow*». La MVO/«*no reflow*», implicada en el daño por reperfusión, es determinante en el tamaño final del infarto y la morbilidad/ mortalidad posterior (14).

En la clínica habitual se objetiva MVO en un 10-30 % de pacientes, la cual se desarrolla minutos después de la revascularización de la arteria ocluida y persiste al menos una semana (12). Su incidencia aumenta con el retraso en el tiempo para alcanzar una reperfusión exitosa del miocardio infartado (15).

A nivel celular es básico entender las alteraciones moleculares y estructurales que se producen durante la isquemia para comprender las razones por las cuales el daño celular aumenta durante la reperfusión temprana (16).

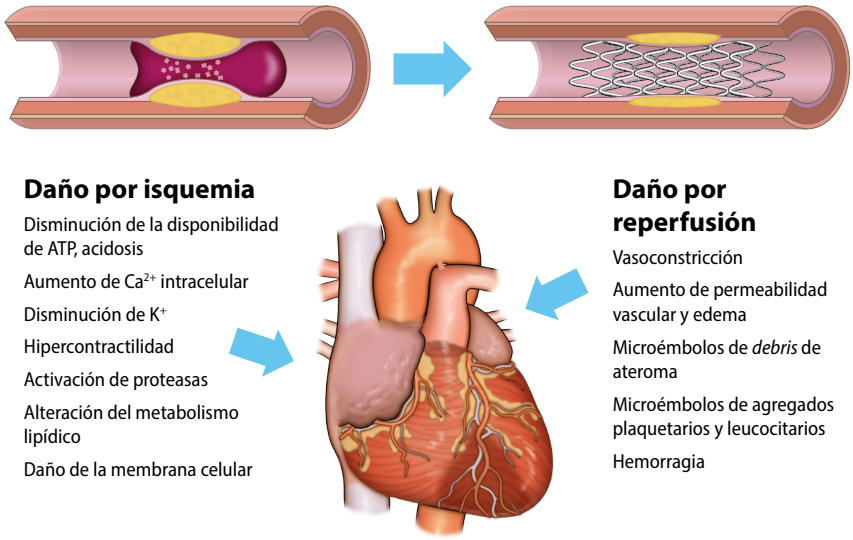

Figura 2. Diagrama que ilustra los mecanismos implicados en el daño por isquemia-reperfusión. La vasoconstricción, edema, microémbolos y hemorragia desencadenados por la reperfusión del tejido isquémico empeoran el daño miocárdico.

Al no haber oxígeno disponible la célula cambia de un metabolismo aerobio a uno anaerobio:

- Menor producción de ATP.
- Acúmulo de lactato y H^+ con la consecuente disminución del pH (acidosis).

La menor disponibilidad de ATP altera los transportadores de membrana que mantienen los gradientes electrolíticos celulares:

- Aumenta el Na^+ intracelular.
- Aumenta el Ca^{2+} intracelular.
- Aumenta el agua intracelular.

Las alteraciones en el balance hidroelectrolítico conducen a una serie de efectos deletéreos que terminan con la desorganización/destrucción de la membrana celular.

- Activación de proteasas intracelulares (pérdida de capacidad de contracción muscular y alteración del citoesqueleto).
- Oxidación del lípidos y alteración del metabolismo lipídico.

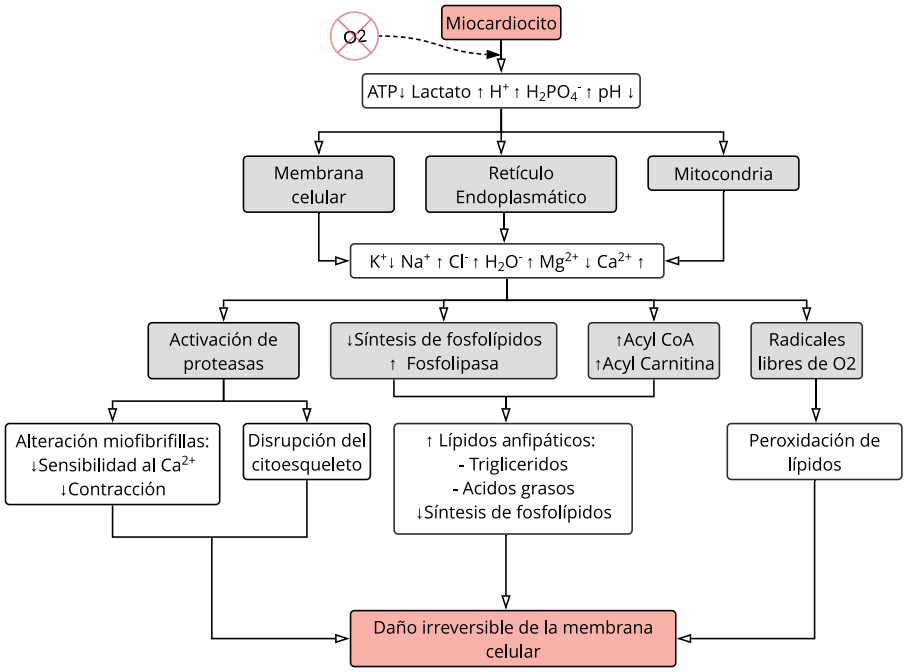

Figura 3. Alteraciones en el metabolismo celular desencadenadas por la isquemia que terminan con la destrucción de la membrana y muerte celular. Adaptado de: Buja LM. Myocardial ischemia and reperfusion injury. Cardiovascular Pathology. 2005;14:170-5

La figura 3 refleja las principales alteraciones celulares producidas por el cese en el aporte de oxígeno.

Durante la reperfusión temprana el restablecimiento brusco del flujo sanguíneo, aporte de oxígeno y «lavado» de H^+ y electrolitos del espacio intersticial determinan tres fenómenos clave en el daño por reperfusión (17):

1. Metabolismo aerobio y recuperación brusca de los niveles de ATP.
    a. La recuperación energética en un entorno celular con exceso de calcio conduce a hipercontractilidad celular.
    b. La hipercontractilidad y un citoesqueleto deteriorado contribuyen al daño celular irreversible.
2. Desaparición rápida de la acidosis intracelular.
    a. Durante la isquemia la acidosis protege frente la hipercontractilidad y la formación de radicales libres e oxígeno.

   b. Una recuperación demasiado rápida del pH puede producir un efecto paradójico dañino.

3. Hiperosmolaridad intracelular relativa y edema intracelular.

   a. Al restablecerse el flujo cardíaco desaparece el exceso de electrolitos en el espacio intersticial. Esto produce una hiperosmolaridad relativa del espacio intracelular con la consecuente entrada de agua y edema intracelular. Este edema participa en la debilidad/daño de la membrana celular.

Además, el restablecimiento del metabolismo aerobio y la normalización de la acidosis favorecen la formación de gran cantidad de radicales libres de oxígeno. Estos, a su vez, condicionan la destrucción de las mitocondrias por la apertura del mPTP (18). Las vías metabólicas relacionadas con la formación de radicales libres de oxígeno y apertura de mPTP son básicas en los mecanismos de cardioprotección (apartado 1.4).

1.3
Introducción
Remodelado cardíaco postinfarto

El remodelado cardíaco se define como el conjunto de modificaciones (moleculares, celulares e intersticiales) que se producen en el corazón después de un daño miocárdico severo, y que clínicamente se manifiestan como cambios en el tamaño, la forma y la función cardíacas (19). Aunque no es exclusivo del infarto (ej., valvulopatías, hipertensión, miocarditis), en el caso de éste es fundamental y va a determinar las secuelas a largo plazo una vez superada la fase aguda.

El remodelado ventricular es un proceso complejo y todavía no del todo bien descrito. Afecta fundamentalmente a los miocardiocitos pero también implica la participación del intersticio (fibroblastos y matriz extracelular), factores hormonales y paracrinos (19). De forma resumida, el proceso se puede dividir en tres fases solapadas entre sí (20):

- Fase hiperaguda (horas): se inicia con la isquemia y posterior reperfusión. La muerte de miocitos libera sustancias que activan al sistema de inmunidad innata (neutrófilos y macrófagos) cuya finalidad es reparar y cicatrizar el corazón dañado. Se inicia la respuesta inflamatoria.
- Fase aguda (horas-días): durante la respuesta inflamatoria abundantes monocitos son atraídos a la región infartada y promueven, una vez convertidos en macrófagos, la reabsorción del tejido muerto y su sustitución por tejido de fibroso/de granulación. El proceso se acompaña de una importante neovascularización para irrigar el tejido de granulación. Tanto los macrófagos como la nueva red de vasos favorecen la migración de abundantes fibroblastos que se diferencian a

miofibroblastos y producen grandes cantidades de colágeno. Se trata de un proceso de fibrosis reparativa (protectora), esencial para mantener la integridad de la pared infartada y evitar una rotura miocárdica. En esta fase el miocardio infartado se adelgaza y se dilata (expansión del infarto) (21).

- Fase crónica (semanas-meses): en infartos severos, la dilatación y ausencia de contracción del área infartada condiciona una redistribución de flujos y presiones en el resto de segmentos cardíacos. Este hecho, asociado a una disminución del número de miocitos por el infarto, promueve la elongación e hipertrofia de los miocitos supervivientes para preservar la función global del corazón. Aunque inicialmente tiene una función compensadora, la dilatación continuada termina con una caída de la capacidad contráctil y evolución hacia la insuficiencia cardíaca. Además, la activación hormonal y paracrina responsable de la cicatrización del infarto también va a favorecer el depósito de colágeno y otras proteínas en el intersticio de zonas no infartadas. Este proceso de fibrosis reactiva o intersticial(dañina) aumenta la rigidez global del miocardio, altera la relajación ventricular y promueve la disfunción diastólica (remodelado ventricular adverso).

Dentro de los factores hormonales comentados, dos de los más importantes son la activación sostenida del sistema renina-angiotensina-aldosterona y el aumento de niveles plasmáticos de noradrenalina. Citoquinas como el TNF-α y la formación de radicales libres de oxígeno también juegan un papel importante en el desarrollo y mantenimiento del remodelado ventricular.

La relación entre remodelado ventricular y riesgo de muerte o insuficiencia cardíaca quedó establecida a finales de la década de los 80 del siglo pasado. En 1987 White *et al.* (22) describieron el aumento del volumen telesistólico (VTS) como principal factor pronóstico tras infarto de miocardio. El aumento del volumen telediastólico (VTD) y la disminución de la fracción de eyección del ventrículo izquierdo (FEVI) también se asociaban a peor pronóstico, pero con un valor predictivo menor. Desde entonces, existe abundante evidencia científica confirmando esos resultados. Migrino y col. (23) demostraron la correlación entre el VTS y un mayor riesgo de insuficiencia cardíaca o muerte. Solomon y col. (24) constataron la importancia en el pronóstico de los valores basales tras infarto del VTS, VTD y FEVI.

El tamaño del infarto se considera el mayor determinante de remodelado ventricular. Esta afirmación se probó en los años noventa del siglo pasado (25) y ha sido confirmada de forma repetida mediante el uso de nuevas y más modernas técnicas de imagen (26-28). Es decir, se puede utilizar el tamaño final del infarto como factor predictivo de mortalidad y morbilidad (evolución a insuficiencia cardíaca) en pacientes que han sobrevivido a un SCACEST (28).

1.4 Introducción **Cardioprotección**

Debido a la importancia del tamaño del infarto como determinante del remodelado cardíaco y morbi/mortalidad post-IAM, disminuir el tamaño del infarto es fundamental.

Cuando se ocluye una arteria coronaria, el área total isquémica se conoce como área en riesgo. Esta área morirá de forma invariable y progresiva si no se abre la arteria ocluida y la zona en riesgo se reperfunde. Sin embargo, si la vascularización se restablece (reperfusión) parte de esta área sobrevivirá y volverá a ser funcional, lo que se conoce como miocardio salvado o rescatado. El beneficio es máximo en los primeros 60-90 min y disminuye progresivamente con el tiempo (29). Transcurridas 4-6 h un 30-50 % del área en riesgo permanece viable (12). Un estudio multicéntrico demostró que incluso 12 h después de la oclusión coronaria todavía existe miocardio que puede ser salvado, aunque éste es muy bajo (30).

Desafortunadamente, el tiempo de isquemia se relaciona íntimamente con el daño por reperfusión que aumenta a medida que aumenta el tiempo de isquemia (15). Desde un punto de vista clínico el daño por reperfusión se define como miocardio potencialmente viable que muere tras la abertura de la arteria, a consecuencia directa de la reperfusión del área en riesgo (12). El daño por reperfusión es debido a la presencia brusca de oxígeno en la zona isquémica (estrés oxidativo), a cambios bruscos en el pH (de ácido a básico), a la perpetuación e incremento de la respuesta inflamatoria y al fenómeno de MVO/*no reflow*. El daño por reperfusión se asocia a infartos más grandes, mayores índices de remodelado adverso y peor pronóstico a largo plazo (31).

Aunque el daño por reperfusión ha sido un concepto polémico y cuestionado, la descripción del fenómeno de condicionamiento isquémico ha sido determinante para demostrar su existencia. Diferenciamos tres tipos de condicionamiento:

- **Pre-condicionamiento:** en 1986 Murry y col. (32) disminuyeron el tamaño del infarto en perros aplicando ciclos breves de isquemia/ reperfusión a nivel de la arteria coronaria antes de la inducción de isquemia coronaria. El tiempo de isquemia fue el mismo en el grupo control y el grupo con intervención, lo que sugería la existencia de factores adicionales además del tiempo de isquemia para determinar el tamaño final del infarto.
- **Post-condicionamiento:** en 2003, Zhao y col. (33) consiguieron disminuir el tamaño del infarto en perros aplicando episodios breves de isquemia/ reperfusión en el momento de la revascularización. En este contexto, la disminución del tamaño del infarto sólo podía ser atribuible a la intervención aplicada durante la reperfusión. En 2005, Staat y col. (34) obtuvieron resultados similares en humanos utilizando los niveles de creatinina cinasa como marcador subrogado del tamaño del infarto.
- **Condicionamiento remoto:** el más reciente. Se basa en aplicar ciclos de isquemia/ reperfusión en un territorio vascular diferente del corazón (ej., una extremidad) después del inicio de los síntomas de isquemia, pero antes de la revascularización coronaria. Los resultados fueron prometedores en los primeros estudios en humanos, donde demostró disminuir el tamaño del infarto (13). Sin embargo, dos ensayos clínicos fase III más recientes han resultado neutros en pacientes sometidos a cirugía cardíaca (35, 36).

En los tres casos el concepto de fondo es el mismo. Ciclos breves de isquemia/ reperfusión liberan «factores protectores» que condicionan al corazón y por ello contribuyen a disminuir el tamaño del infarto una vez restablecido el flujo coronario.

Los mecanismos paracrinos e intracelulares que actúan como estos «factores protectores» son complejos y no del todo bien conocidos. Se sabe que los miocardiocitos presentan múltiples receptores de membrana, la mayoría ligados a proteínas G, que activan vías metabólicas relacionadas con el daño por isquemia-reperfusión.

Paradójicamente, estas mismas vías están implicadas en los fenómenos de condicionamiento/cardioprotección (18, 37). Algunos de los principales factores implicados son:

- Opioides
- Bradiquinina
- Esfingosina
- Adenosina
- TNF-α

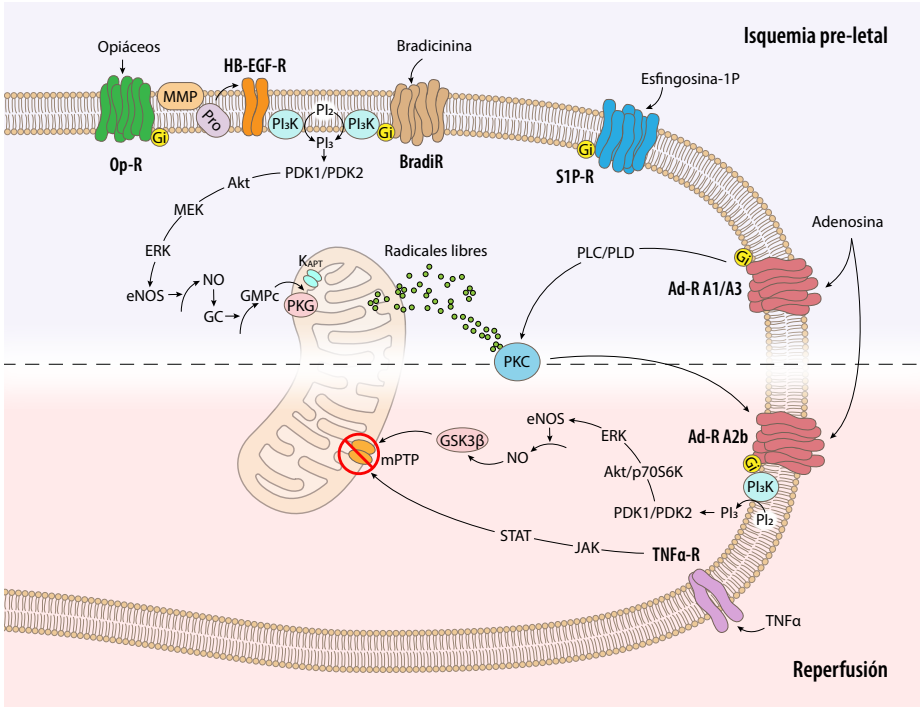

Figura 4. Mapa de las principales vías de señalización involucradas en los fenómenos de pre y post condicionamiento. Op-R: receptor de opiáceos. MMP: metaloproteinasa matricial. EGF: epidermal growth factor. HB-EGF-R: receptor del *heparin-binding EGF like growth factor*. Pro: pro-HB-EGF. PI_2/PI_3: fosfatidil-inositol di/trifosfato. PI_3K: PI_3 cinasa. PDK1/2: cinasa dependiente de fosfoinosítidos. Akt: proteína cinasa-B. MEK: MAP cinasa. ERK: *extracellular signal–regulated kinase*. eNOS: sintasa endotelial de óxido nítrico (NO). GC: guanilil-ciclasa. PKG: proteína cinasa dependiente de GMPc. K_{ATP}: canal de potasio dependiente de ATP. Bradi-R: receptor de bradicinina. S1P-R: receptor de esfongosina-1-fosfato. Ad-R A1/2b/3: receptores de adenosina. PLC/PLD: fosfolipasa C y D. PKC: proteína cinasa C. p70S6K: cinasa del p70S6. GSK3β: cinasa de la glucógeno-sintetasa beta. TNFα-R: receptor del factor de necrosis tumoral. JAK: Janus cinasa. STAT: *signal transducer and activator of transcription*. mPTP: *mitochondrial permeability transition pore*. Modificado de Hausenloy y col. Ischaemic conditioning and targeting reperfusion injury: a 30 year voyage of discovery. Basic Res Cardiol. 2016;111(6):70.

Durante la isquemia se liberan opioides, bradiquinina, esfingosina y adenosina que al unirse a sus receptores correspondientes conducen a la activación de la proteína PKC mediante dos vías intracelulares diferentes: una mediada por AKT-eNOS-PKG y otra mediada por PLC/PLD. El óxido nítrico (NO) y la PKG condicionan la apertura de canales de K^+ mitocondriales que permiten la entrada de potasio en la mitocondria y, tras la reperfusión (aporte de O_2), la salida de radicales libres. Si la reperfusión es abrupta y prolongada se produce una gran cantidad de radicales libres que conduce a la apertura de los mPTP, pérdida de la función mitocondrial y muerte celular. Sin embargo, los ciclos breves de isquemia-reperfusión durante el condicionamiento producen una liberación menor de radicales libres, dado que el aporte de oxígeno en estos ciclos es limitado. Esta es la clave del mecanismo cardioprotector. Una cantidad pequeña de radicales activa la isoforma PKCε que, a su vez, activa las vías metabólicas RISK y SAFE y éstas inhiben la apertura de los mPTP. En el momento de la reperfusión definitiva los mPTP no se abrirán aunque exista una gran liberación de radicales. En este sentido, es importante mencionar que las moléculas de adenosina y TNF-α son capaces de activar directamente las vías RISK/ SAFE. Las vías descritas en este párrafo se encuentran resumidas en la figura 4 (página anterior).

Tomando todos estos datos en conjunto se puede establecer que el tratamiento actual del SCA (revascularización precoz) ha alcanzado una «meseta» en los resultados de mortalidad y morbilidad que es difícil de mejorar. Para disminuir la morbilidad crónica al largo plazo (insuficiencia cardíaca) la estrategia a seguir es garantizar un mayor nivel de cardioprotección al proporcionado únicamente por la revascularización. En este sentido, son de particular interés las estrategias destinadas a disminuir el daño por reperfusión, disminuir el tamaño final del infarto y mejorar el remodelado ventricular (previniendo así el desarrollo de insuficiencia cardíaca).

1.5
Introducción
Antagonistas del receptor plaquetario $P2Y_{12}$

1.5.1 El papel de las plaquetas en la enfermedad isquémica coronaria

Las plaquetas son el principal componente celular en la formación de un trombo tras la rotura de la placa aterosclerótica. De ahí, la relevancia de los fármacos antiplaquetarios en la prevención y tratamiento de las enfermedades isquémicas cardiovasculares.

La exposición al torrente circulatorio de matriz vascular induce una rápida adhesión, activación y agregación de las plaquetas sobre la superficie lesionada (formación de un tapón plaquetario o trombo blanco). Las plaquetas agregadas reclutan otras células (eritrocitos, neutrófilos y ocasionalmente monocitos) para la formación de un trombo mixto. De forma paralela se activa la cascada de la coagulación que produce una red de fibrina que estabiliza el tapón inicial.

La formación del trombo implica a múltiples proteínas, moléculas y receptores (38, 39). Para una información detallada referirse a la referencia número 32. Se puede resumir en los siguientes pasos:

1. Rotura de la placa de ateroma con la consiguiente exposición de componentes de la placa aterosclerótica y de la matriz extracelular a la luz arterial.
2. Adhesión plaquetaria: comprende la migración de las plaquetas hacia la superficie vascular lesionada y la unión de los receptores de la membrana plaquetaria con los ligandos expuestos por la pared vascular.

Los principales ligandos son proteínas de la matriz extracelular (colágeno, fibronectina) y el factor de von Willebrand (vWf) circulante que queda atrapado en la pared. Las principales proteínas de membrana plaquetaria son la glicoproteína Ib/V/IX y la glicoproteína VI.

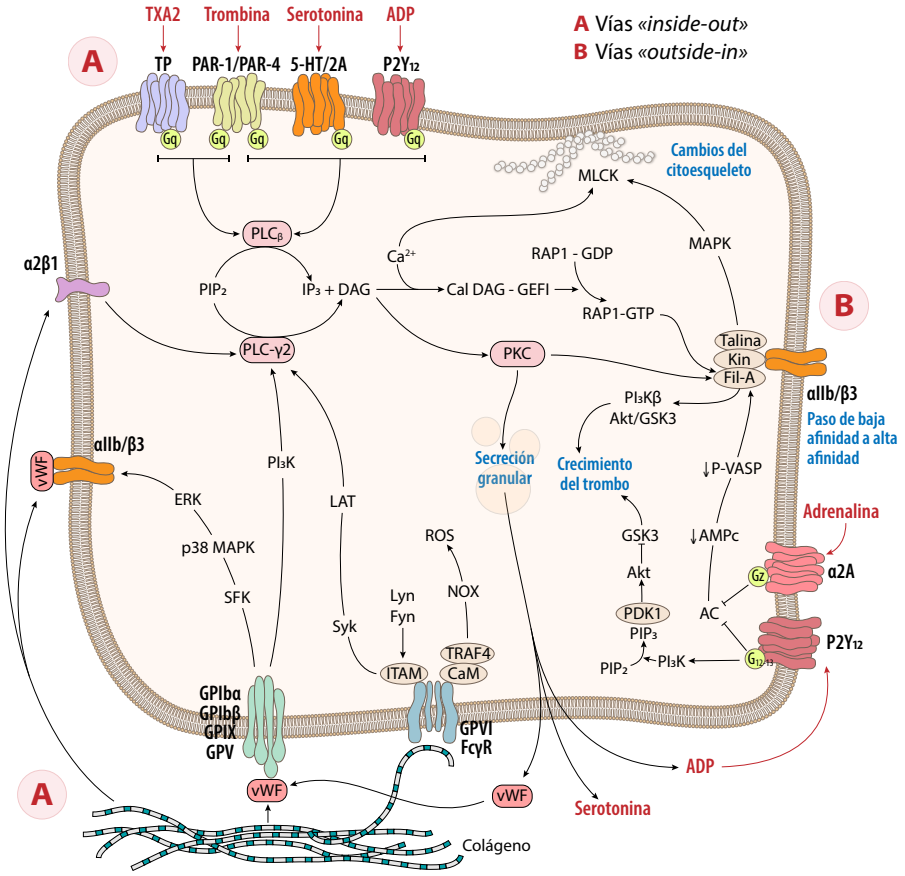

Figura 5. Vías de señalización implicadas en la activación y agregación plaquetaria. Las vías *inside-out* se activan cuando diferentes moléculas en el plasma sanguíneo interaccionan con los receptores de la membrana plaquetaria y acaban con la activación de la glicoproteína IIb/ IIIa. Al cambiar de conformación, la GP IIb/IIIa activa las vías *outside-in*. GP: glucoproteína; vWF: factor von Willebrand; FcγR: cadena gamma del receptor Fc; SFK: Src *family protein kinases*; PLC: fosfolipasa C; DAG: diacilglicerol; MAPK: *mitogen-activated protein kinase*; ITAM: *immune receptor tyrosine-based activation motif*; ROS: *reactive oxygen species;* NOX: NADPH oxidasa; PKG: proteína cinasa G; GPCR: *G-protein coupled receptors*; ADP: adenosin-difosfato; TXA2: tromboxano A2; TP: receptor de tromboxano; 5HT-2A: receptor de serotonina; PAR: *protease-activated receptor*; IP3: inositol trifosfato; CalDAG-GEFI: *calcium DAG-regulated guanine nucleotide exchange factor*; Rap1: *Ras-related protein* 1; GSK3: *Glycogen Synthase Kinase 3*; PIP2/PIP3: fosfatidil inositol di/trifosfato; Akt: proteína cinasa B; PDK1: *phosphoinositide-dependent protein kinase 1*; P2Y12: receptor de ADP; VASP: *vasodilator stimulated phosphoprotein*. Modificado de Vilahur G, Gutiérrez M, *et al*. Intracellular platelet signalling as a target for drug development. Vascul Pharmacol, 2018.

3. Activación plaquetaria: se desencadena por múltiples mecanismos que terminan con el cambio conformacional y consiguiente activación de la proteína de superficie GPIIb/IIIa y un cambio drástico de la morfología plaquetaria de discoide a esfera espinosa; dos procesos fundamentales para la formación del trombo intraarterial. Las plaquetas activadas liberan gránulos plaquetarios que contienen sustancias pro-agregantes que estimulan la activación y agregación de nuevas plaquetas.

4. Agregación plaquetaria: desencadenada por la activación de la proteína GPIIb/IIIa. El cambio conformacional conlleva que ésta exponga los lugares de enlace para el fibrinógeno y el vWf, favoreciendo así la interacción plaqueta-plaqueta. Se produce una acumulación local de plaquetas que forman una masa expandible de tamaño creciente que continúa reclutando más plaquetas a medida que éstas alcanzan el microambiente protrombótico.

5. Cascada de la coagulación: la rotura de la placa ateroesclerótica condiciona la exposición al torrente sanguíneo del factor tisular (TF) presente en las células espumosas que forman la lesión aterosclerótica. Se trata de una glicoproteína de bajo peso molecular capaz de iniciar la cascada extrínseca de la coagulación activando el factor VII. El factor VII activado (VIIa) activa el factor X y éste activa la vía común, en la que la trombina (factor IIa) pasa el fibrinógeno a fibrina (factor Ia). A su vez, pequeñas cantidades de trombina son capaces de producir la activación plaquetaria por lo que ambos procesos (activación plaquetaria y cascada de la coagulación) quedan interrelacionados.

1.5.2 Importancia terapéutica de los antagonistas del receptor $P2Y_{12}$

El ácido acetilsalicílico (AAS) inhibe de forma irreversible la enzima ciclooxigenasa 2 y evita la formación de tromboxano A2. Es el primer fármaco que probó disminuir la mortalidad en pacientes con SCA (40) y continúa siendo uno de los pilares de la terapia antiagregante, tanto en eventos agudos como en prevención secundaria (41). Sin embargo, su uso sólo reduce en un 25 % el riesgo de sufrir un evento cardiovascular. Este hecho pone de manifiesto la complejidad de las vías de señalización que convergen en la formación del trombo plaquetario, y la necesidad

de bloquear de forma concomitante varias de estas vías para optimizar el efecto antiagregante.

Por ello, se desarrollaron nuevos fármacos antiagregantes destinados a complementar al AAS y reducir el riesgo de sufrir eventos trombóticos. Uno de los grupos más importantes son los antagonistas del receptor de ADP $P2Y_{12}$. Estos se pueden clasificar en las siguientes clases:

a. **Tienopiridinas:**

Aunque inicialmente ideados para pacientes intolerantes al AAS, el uso conjunto de AAS y antagonistas del receptor $P2Y_{12}$ demostró ser útil en pacientes sometidos a intervencionismo coronario (implantación de *stent*). La asociación de AAS y ticlopidina (tienopiridina de primera generación, inhibidora irreversible del receptor $P2Y_{12}$) consiguió disminuir en un 75 % los eventos cardiovasculares (muerte, infarto, *bypass*, nueva angioplastia) respecto al tratamiento combinado de AAS y anticoagulantes orales (42). Sin embargo, los efectos secundarios hematológicos (neutropenia, púrpura trombótica trombocitopénica) y gastrointestinales de la ticlopidina limitaron su utilización.

El desarrollo de clopidogrel, tienopiridina de segunda generación sin los efectos secundarios de la ticlopidina, permitió superar esta limitación y el uso de antagonistas del receptor $P2Y_{12}$ se generalizó en la práctica clínica (43, 44). Sin embargo, clopidogrel tiene dos importantes limitaciones: un inicio de acción lento y una falta de respuesta adecuada en algunos pacientes.

Clopidogrel es un profármaco que ha de ser absorbido intestinalmente y metabolizado por el sistema citocromo P450 hepático (CytP450) para obtener el metabolito activo. Este hecho condiciona un inicio de respuesta lento que conlleva bajas tasas de antiagregación en las primeras horas tras la administración del fármaco (45). Es necesaria una dosis alta de clopidogrel (600 mg), al menos 4 h antes de la implantación de un *stent* para conseguir un nivel adecuado de antiagregación y disminuir de forma óptima el número de eventos cardiovasculares post procedimiento (46, 47). Además, un número no despreciable de pacientes son «no-respondedores» al tratamiento con clopidogrel en el contexto de un SCACEST. Entre un 24-46 % de pacientes tratados con una dosis de carga de 300 mg de clopidogrel y aproximadamente un 15 % de los tratados con 600 mg no responden (45, 48). Posibles causas de esta ausencia de respuesta son

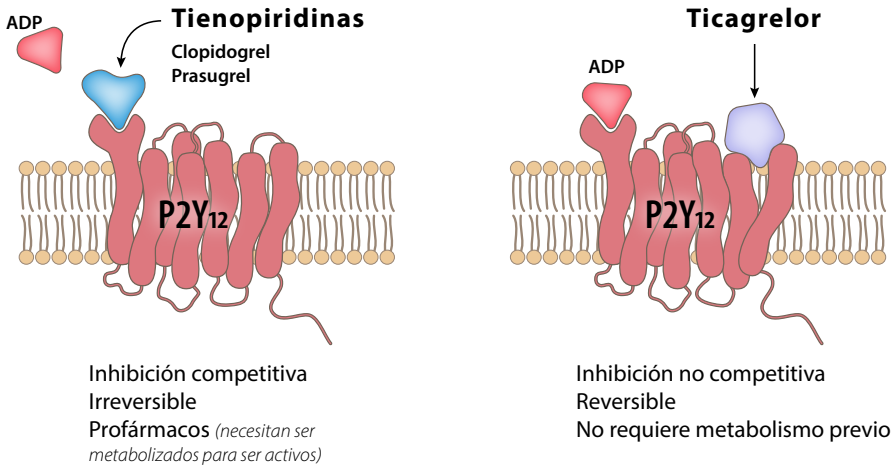

Figura 6. Diagrama que representa las diferencias en el mecanismo de acción de las tienopiridinas y ticagrelor.

una alteración de la absorción intestinal y/o del metabolismo hepático del fármaco debido a la presencia de polimorfismos genéticos en las enzimas que metabolizan el fármaco (especialmente en la enzima CytP450), así como la presencia de un estado de «hiperactividad plaquetaria» que se produce durante un SCA.

A pesar de que clopidogrel sigue siendo un fármaco ampliamente extendido en la práctica clínica, se han continuado desarrollando fármacos para superar sus limitaciones. Por ejemplo, prasugrel, una tienopiridina de tercera generación, presenta un metabolismo independiente del sistema CytP450 hepático, lo que le confiere un inicio de acción más rápido y un efecto más potente que clopidogrel (49, 50). Como contrapartida, prasugrel se asocia a mayor riesgo de complicaciones hemorrágicas graves (incluida hemorragia intracerebral, comúnmente fatal) (51, 52).

b. **Fármacos no tienopiridínicos:**

Por otro lado, se han desarrollado fármacos no tienopiridínicos que actúan de forma diferente sobre el receptor $P2Y_{12}$. Ticagrelor es una triazolopirimidina que, a diferencia de las tienopiridinas, inhibe de forma no competitiva, directa y reversible el receptor $P2Y_{12}$.

Al igual que prasugel, ticagrelor consigue una inhibición plaquetaria más rápida y efectiva que clopidogrel. En el estudio ONSET/ OFFSET una dosis de carga de 180 mg de ticagrelor produjo una inhibición plaqueta-

ria a las dos horas del 88 %, en comparación con un 38 % de clopidogrel (53). La inhibición plaquetaria máxima durante las primeras 8 h fue mayor (93 % vs. 58 %) y más rápida (2 h vs. 7,8 h) con ticagrelor. Previamente, el estudio PLATO (*Platelet Inhibition and Patient Outcomes*), ya había demostrado una ventaja terapéutica de ticagrelor frente a clopidogrel (54). En pacientes que habían sufrido un SCA, ticagrelor se asoció a una menor mortalidad global (causas cardiovasculares y no cardiovasculares) y a un menor número de reinfartos durante los 12 meses que duró el estudio. Aunque las complicaciones hemorrágicas mayores no difirieron de manera estadísticamente significativa entre ambos grupos, el número de hemorragias intracerebrales si fue significativamente mayor en el grupo tratado con ticagrelor. Sin embargo, más recientemente, el estudio PEGASUS ha confirmado los efectos positivos de ticagrelor en pacientes estables con antecedentes de infarto, sin que haya incrementado el riesgo de hemorragia intracraneal (55).

La disminución de la mortalidad global (independientemente del número de infartos) en el grupo tratado con ticagrelor respecto al tratado con clopidogrel fue especialmente llamativa, y despertó un gran interés sobre un potencial efecto beneficioso independiente de la capacidad antiagregante (mediada por bloqueo del receptor $P2Y_{12}$).

Ticagrelor posee beneficios exclusivos asociados a un aumento en la biodisponibilidad local de adenosina, replicando los fenómenos de condicionamiento isquémico. Este aumento de la biodisponibilidad se realiza a través de dos mecanismos básicos: 1) ticagrelor induce la liberación de ATP por parte de los eritrocitos. El ATP liberado se convierte a adenosina por ecto-ADPasas (CD39 y CD73) presentes en el endotelio vascular (56); y 2) ticagrelor inhibe el receptor ENT-1 [2], responsable de la captación de adenosina por los eritrocitos (57).

El efecto final de adenosina en la célula es la inhibición de la apertura de los mPTP en la reperfusión temprana, evitando muerte celular posterior.

Sin embargo, se desconoce si las propiedades intrínsecas de ticagrelor le confieren un mayor potencial cardioprotector en comparación con otros antagonistas del receptor $P2Y_{12}$.

2 ENT-1: *Equilibrative nucleotid transporter*.

1.6
Introducción
Resonancia magnética como herramienta de investigación en el contexto de Infarto agudo de miocardio

1.6.1 Conceptos básicos (58, 59)

La resonancia magnética cardíaca (RMC) es una técnica de imagen avanzada que integra en un único estudio la valoración anatómica y funcional del corazón. Permite obtener imágenes con un alto contraste entre tejidos, alta resolución espacial[3] y alta resolución temporal en cualquier plano del espacio.

Se fundamenta en la respuesta de los átomos de hidrogeno al ser sometidos a:

- Un campo magnético principal estático (B0).
- Gradientes de campos magnéticos que se superponen al principal.
- Ondas de radiofrecuencia.

Las bases físicas detrás de la RM son complejas y su explicación exhaustiva queda fuera del alcance de esta tesis. De forma resumida, se puede afirmar que la RM permite la obtención de una gran cantidad de «secuencias». Cada secuencia da como resultado imágenes que aportan información específica sobre la región anatómica estudiada.

En resonancia magnética cardíaca distinguimos dos grupos principales de secuencias: las secuencias morfológicas o de caracterización tisular y las secuencias funcionales.

3 Resolución espacial: capacidad para diferenciar estructuras pequeñas, cercanas una de otra. Resolución temporal: capacidad para adquirir imágenes consecutivas en pequeños periodos de tiempo.

Las secuencias de caracterización tisular aportan información sobre la composición de la región anatómica estudiada. Distinguimos.

- Secuencias T1: reflejan las diferencias en el tiempo de «relajación longitudinal» (T1) de los átomos de hidrógeno de cada tejido. Muy útiles para delimitar la anatomía normal del organismo y en estudios donde se administra contraste intravenoso (se explican más adelante en este mismo apartado).
- Secuencias T2: reflejan las diferencias en el tiempo de «relajación transversal» (T2) de los átomos de hidrógeno. Distinguimos dos subgrupos, secuencias T2 y T2*. En las secuencias T2 el tiempo de relajación transversal depende únicamente de las interacciones entre átomos de hidrógeno. Sin embargo, en las secuencias T2* se ve acelerado por inhomogeneidades en el campo magnético principal.
    - Las secuencias T2 son muy buenas para detectar agua libre y edema tisular. En estas secuencias, es común aplicar técnicas para anular la señal de la grasa (hacer que se vea hipointensa) y así potenciar la detección del edema. En imagen cardíaca, la más común es la T2-STIR.
    - Las secuencias T2* detectan los depósitos de hierro en los tejidos (como los producidos por la degradación de la hemoglobina tras una hemorragia) ya que inducen pequeñas inhomogeneidades en el campo magnético.

Las secuencias funcionales permiten la valoración de procesos dinámicos. En resonancia magnética cardíaca distinguimos:

- Secuencias de estado estacionario (b-SSFP) o «de cine»: secuencia muy rápida que permite estudiar uno o múltiples niveles anatómicos a lo largo de múltiples fases del ciclo cardíaco. Las imágenes se muestran en «paquetes» que contienen los niveles estudiados a lo largo de 20-24 fases. Al reproducirlas de forma continua dan como resultado pequeños clips o videos. Mediante *software* especializados se usan para calcular con precisión los volúmenes cardíacos, grosor parietal y fracción de eyección.
- Secuencias de contraste de fase (*phase-contrast*): producen imágenes de escaso valor anatómico pero muy útiles para estudiar flujos. Aunque su precisión en el cálculo de velocidades es menor que la de la ecografía, a diferencia de esta, permiten calcular volúmenes además

de flujos. Son muy útiles en el estudio de valvulopatías, especialmente cuando existe una mala ventana ecográfica.

- Secuencia de perfusión de primer paso: secuencia post contraste, rápida y potenciada en T1 que permite valorar la vascularización miocárdica. Se definen tres cortes anatómicos (basal, medio y apical) que se estudian de forma continua a lo largo de múltiples latidos. A diferencia de las secuencias de «cine» todas las imágenes se adquieren en la misma fase del ciclo cardíaco. Se obtienen imágenes estáticas en las que es posible observar como la sangre llega a las cavidades cardíacas y posteriormente se distribuye por el miocardio correctamente perfundido.

Además de la secuencia de perfusión de primer paso, existen dos otras secuencias post contraste de gran relevancia en resonancia magnética cardíaca.

- Secuencia de realce tardío (LGE, *late gadolinium enhancement*).
- Secuencia de realce precoz (EGE, *early gadolinium enhancement*).

Ambas son secuencias de caracterización tisular que estudian la distribución del gadolinio (medio de contraste IV utilizado en RM) en el tejido miocárdico. El gadolinio es un elemento químico que presenta como característica principal la capacidad para disminuir el tiempo T1. En la práctica esto se traduce en que es capaz de aumentar la señal T1 de los tejidos en los que se localiza de forma que se ven más hiperintensos («brillantes»). La forma libre es tóxica, por lo que se administra en forma de quelatos que evitan su absorción celular y hacen que se distribuya únicamente en el espacio extracelular. En el miocardio normal los miocardiocitos ocupan el 75 % del volumen y el espacio extracelular únicamente un 25 % (60). Este hecho se asocia a una cinética determinada del gadolinio, que presenta una fase de «captación» y otra de posterior «lavado» dentro del miocardio.

- Secuencia de realce tardío (LGE o *late gadolinium enhancement*): todas las patologías que lesionan las membranas celulares (infarto agudo) o aumentan el volumen de tejido extracelular (infarto crónico, miocardiopatías no isquémicas) retrasan el «lavado» del gadolinio miocárdico. En condiciones normales, a los 10 min después de la inyección del contraste ya no queda gadolinio en el tejido miocárdico normal, pero si en las zonas con espacio extracelular aumentado. La secuencia se basa en la aplicación de un pulso de inversión al inicio que anula la

señal del miocardio normal (se ve negro) pero no la del tejido que todavía contiene gadolinio. Como resultado, las áreas patológicas se ven hiperintensas sobre un fondo hipointenso. El patrón de distribución del gadolinio es diferente según el tipo de patología, por lo que esta secuencia es fundamental a la hora de hacer el diagnóstico etiológico de las miocardiopatías (61) .

- Secuencia de realce precoz (EGE o *early gadolinium enhancement*): Las diferencias con el LGE son dos: 1) aunque se aplica un pulso de inversión el miocardio normal se ve «gris» y no negro. 2) en lugar de a los 10 min post inyección de contraste la secuencia se adquiere después de 1 min y sirve para estudiar el estado de la micro-vascularización miocárdica. El tejido con una micro-vascularización conservada presentará una señal intermedia (gris) mientras que las zonas con obstrucción micro vascular se verán hipointensas (negras) debido a que el contraste no es capaz de penetrar en ellas. Esta secuencia también es útil para detectar trombos en la luz ventricular (se ven hipointensos y se diferencian muy bien del miocardio y luz ventricular).

1.6.2 Utilidad clínica e implicación pronóstica en el infarto de miocardio.

Aunque la RM ha demostrado su valor diagnóstico y pronóstico en múltiples patologías cardíacas (62), nosotros nos centraremos en su utilidad en el infarto de miocardio.

La tabla 3 resume los fenómenos fisiopatológicos que se pueden estudiar mediante RM en la fase aguda y crónica después de un infarto.

a. **Función cardíaca (b-SSFP)**

La RM actualmente es el *gold standard* en la evaluación de la función cardíaca (63, 64). Es una técnica exacta y reproducible que permite muestrear todo el ventrículo, evitando la asunción de suposiciones geométricas. Las modernas secuencias de cine aportan una gran resolución espacial con buen contraste entre el miocardio y la sangre ventricular. De forma manual o semiautomática se pueden delimitar los contornos endocárdico y epicárdico, lo que permite valorar los siguientes parámetros: volumen telediastólico (VTD) y telesistólico (VTS) , fracción de eyección (FE), motilidad regional y masa ventricular.

Secuencia RM	Fenómeno
b-SSFP	Función cardíaca (volúmenes, función global y regional, grosor parietal, masa miocárdica)
LGE	Necrosis (fase aguda) y fibrosis miocárdica (fase crónica)
T2-STIR	Edema tisular y miocardio en riesgo
EGE	Obstrucción microvascular
T2*	Hemorragia

Tabla 3. Principales secuencias de RMC y sus usos.

b. Necrosis / Fibrosis (LGE)

Después de un infarto se altera la cinética normal del gadolinio en el miocardio, de manera que en la secuencia LGE el tejido infartado aparece hiperintenso respecto al miocardio normal. Es importante mencionar que durante la fase aguda esta secuencia puede sobrestimar el volumen de tejido infartado debido a la captación de gadolinio por parte de tejido dañado pero viable en los bordes del núcleo necrótico (65). La secuencia de LGE ha demostrado valor pronóstico tanto en la fase aguda como en la crónica después de un infarto: 1) en la fase aguda se relaciona con la probabilidad de disfunción ventricular a largo plazo (27). 2) en la fase crónica es de gran utilidad para evaluar la viabilidad de un segmento infartado. En determinados pacientes con enfermedad coronaria crónica se plantea la revascularización de una arteria que irriga un segmento ya infartado. En estos casos, se considera que el tejido es viable (y por lo tanto mejorará su función con la revascularización) si la captación de contraste es <50 % del grosor de la pared (66).

c. Edema tisular y miocardio en riesgo (T2-STIR)

Después de la oclusión de una arteria coronaria, una de las primeras consecuencias de las alteraciones metabólicas que se producen es la aparición de edema celular (16). Si el flujo no se restablece, las alteraciones metabólicas progresan conduciendo a alteraciones estructurales y muerte celular. Este proceso se produce de forma progresiva siguiendo una «ola» o «frente isquémico» desde el subendocardio hasta el subepicardio y puede ser detenido si se restablece la circulación coronaria. En este sentido,

la detección del edema es muy útil para determinar el «área en riesgo» (área que evolucionara a necrosis) y evaluar el porcentaje de «miocardio salvado» (diferencia entre el área en riesgo —edema en T2-STIR— y el área de necrosis —realce en LGE—) (65).

d. **Obstrucción microvascular y hemorragia** (67)

Ambas son consecuencia del daño por isquemia-reperfusión y se asocian a mal pronóstico. En este contexto, la hemorragia se considera consecuencia de un daño por isquemia-reperfusión/MVO severo en el que se extravasan eritrocitos fuera del torrente sanguíneo. La MVO queda reflejada en las secuencias de perfusión de primer paso, EGE y LGE. En las dos primeras se habla de MVO temprana, en la tercera de MVO persistente. La hemorragia se pone de manifiesto en las secuencias T2 como un núcleo hipointenso dentro del área de edema. Sin embargo, en algunos casos una MVO severa sin hemorragia puede producir el mismo efecto tanto en las secuencias T2-STIR como LGE. La secuencia T2* es más específica para la caracterización de hemorragia ya que detecta las inhomogeneidades en el campo magnético producida por los productos de degradación de la hemoglobina. Aunque, ambos se asocian con mal pronóstico, la existencia de hemorragia parece conferir peor pronóstico que la MVO (68).

Como ya se ha comentado, se considera el tamaño del infarto como el principal factor de mal pronóstico a largo plazo después de un IAM (remodelado ventricular, insuficiencia cardíaca, muerte). Sin embargo, en los últimos años han aparecido estudios que indican la importancia de la MVO y hemorragia a la hora de obtener una estratificación pronostica más precisa (69).

1.7
Introducción
Modelo porcino de IAM: potencial de traslacionabilidad

Los estudios en modelos animales son esenciales para entender los mecanismos fisiopatológicos y moleculares detrás de los síndromes coronarios, así como para estudiar potenciales estrategias terapéuticas. Sin embargo, no todos los modelos animales son igualmente extrapolables al corazón humano (12).

Los modelos con roedores permiten la cría de múltiples animales con un coste menor que el de otros animales de mayor tamaño. Además, existe disponibilidad de animales transgénicos. En contraposición, presentan amplias diferencias anatómicas y fisiológicas con respecto al corazón humano que impiden su extrapolación directa. Su frecuencia cardíaca es mucho más alta que en humanos y debido al pequeño tamaño, incluso cuando existe una oclusión completa de una arteria coronaria, existe difusión de oxígeno a las capas más internas del miocardio. El grado de circulación colateral (diferente entre especies) y la cepa utilizada (dentro de una misma especie) añaden una notable variabilidad en cuanto a la progresión y tamaño de infarto según el modelo utilizado (72-74).

El paso fundamental antes de los ensayos clínicos en humanos es la investigación con mamíferos de mayor tamaño dado que presentan una fisiopatología cardíaca similar a la humana. En este sentido, los dos modelos más utilizados son perros y cerdos. Los perros presentan un sistema de colaterales bien desarrollado que les confiere una protección innata a la isquemia coronaria. Por otro lado, los cerdos, al igual que los humanos, presentan escasa circulación colateral (73). Este aspecto es determinante dado que permite una mejor extrapolación de parámetros como la pro-

gresión a necrosis del área en riesgo y tamaño final del infarto. Del mismo modo, el corazón de los cerdos presenta un sistema de conducción eléctrica equiparable al humano. Paradójicamente, los primates no-humanos son muy resistentes al infarto por lo que son poco adecuados para estudios preclínicos (75). Teniendo en cuenta las características descritas, el modelo porcino es el que muestra mayor similitud con los humanos (anatomía cardíaca, escasa circulación colateral) y un mayor potencial de traslacionabilidad.

Hipótesis y objetivos

2.1

Hipótesis

En base a todo lo anteriormente expuesto, nuestra hipótesis de trabajo es la siguiente:

La administración de ticagrelor previa a la reperfusión mecánica de un vaso coronario ocluido ejercerá un efecto cardioprotector directo en mayor medida que clopidogrel. Este efecto cardioprotector directo está mediado por adenosina y es independiente de su capacidad de bloquear el receptor plaquetario $P2Y_{12}$. En la práctica, la administración de ticagrelor se traducirá en menor daño miocárdico, mejor función cardíaca y menor remodelado adverso del ventrículo izquierdo en comparación con la administración de clopidogrel (a dosis antiplaquetarias equipotentes).

2.2

Objetivos

El objetivo principal de esta tesis es demostrar que la administración oral de ticagrelor previamente a la inducción de un infarto de miocardio disminuye en mayor medida el daño postinfarto que la administración oral de clopidogrel. Este objetivo principal se divide en los siguientes objetivos secundarios:

1. Analizar el efecto beneficioso de ticagrelor en comparación con clopidogrel en la fase aguda postinfarto (primeras 24h).
2. Analizar el efecto beneficioso de ticagrelor en comparación con clopidogrel en la fase crónica postinfarto (3d y 42d).
3. Demostrar que el efecto cardioprotector añadido de ticagrelor sobre clopidogrel esta mediado por adenosina.

Todos los objetivos se llevarán a cabo utilizando el modelo animal de infarto agudo de miocardio que más similitudes presenta con el humano, el modelo porcino. Se analizarán parámetros cardíacos funcionales y anatómicos mediante RMC (técnica de imagen de referencia en el marco del IAM) que se correlacionarán con parámetros bioquímicos y moleculares.

Materiales y métodos

3

Materiales y métodos

El material y métodos utilizados quedan detallados en los artículos publicados como parte del proyecto de investigación de esta tesis:

- Vilahur G, **Gutiérrez M**, Casani L, *et al.* Protective Effects of Ticagrelor on Myocardial Injury After Infarction. Circulation. 2016;134(22): 1708–1719.
- Vilahur G, **Gutiérrez M**, Casani L, *et al.* $P2Y_{12}$ antagonists and cardiac repair post-myocardial infarction: global and regional heart function analysis and molecular assessments in pigs. Cardiovasc Res. 2018;114(14): 1860–1870.

ORIGINAL RESEARCH ARTICLE

Protective Effects of Ticagrelor on Myocardial Injury After Infarction

Editorial, see p 1720

BACKGROUND: The $P2Y_{12}$ receptor antagonist ticagrelor has been shown to be clinically superior to clopidogrel. Although the underlying mechanisms remain elusive, ticagrelor may exert off-target effects through adenosine-related mechanisms. We aimed to investigate whether ticagrelor reduces myocardial injury to a greater extent than clopidogrel after myocardial infarction (MI) at a similar level of platelet inhibition and to determine the underlying mechanisms.

METHODS: Pigs received the following before MI induction: (1) placebo-control; (2) a loading dose of clopidogrel (600 mg); (3) a loading dose of ticagrelor (180 mg); or (4) a loading dose of ticagrelor followed by an adenosine A1/A2-receptor antagonist [8-(*p*-sulfophenyl)theophylline, 4 mg/kg intravenous] to determine the potential contribution of adenosine in ticagrelor-related cardioprotection. Animals received the corresponding maintenance doses of the antiplatelet agents during the following 24 hours and underwent 3T-cardiac MRI analysis. Platelet inhibition was monitored by ADP-induced platelet aggregation. In the myocardium, we assessed the expression and activation of proteins known to modulate edema formation, including aquaporin-4 and AMP-activated protein kinase and its downstream effectors CD36 and endothelial nitric oxide synthase and cyclooxygenase-2 activity.

RESULTS: Clopidogrel and ticagrelor exerted a high and consistent antiplatelet effect (68.2% and 62.2% of platelet inhibition, respectively, on challenge with 20 μmol/L ADP) that persisted up to 24 hours post-MI ($P<0.05$). All groups showed comparable myocardial area-at-risk and cardiac worsening after MI induction. 3T-Cardiac MRI analysis revealed that clopidogrel- and ticagrelor-treated animals had a significantly smaller extent of MI than placebo-control animals (15.7 g left ventricle and 12.0 g left ventricle versus 22.8 g left ventricle, respectively). Yet, ticagrelor reduced infarct size to a significantly greater extent than clopidogrel (further 23.5% reduction; $P=0.0026$), an effect supported by troponin-I assessment and histopathologic analysis ($P=0.0021$). Furthermore, in comparison with clopidogrel, ticagrelor significantly diminished myocardial edema by 24.5% ($P=0.004$), which correlated with infarct mass ($r=0.73$; $P<0.001$). 8-(*p*-Sulfophenyl)theophylline administration abolished the cardioprotective effects of ticagrelor over clopidogrel. At a molecular level, aquaporin-4 expression decreased and the expression and activation of AMP-activated protein kinase signaling and cyclooxygenase-2 increased in the ischemic myocardium of ticagrelor- versus clopidogrel-treated animals ($P<0.05$). These protein changes were not observed in those animals administered the adenosine receptor blocker 8-(*p*-sulfophenyl)theophylline.

CONCLUSIONS: Ticagrelor, beyond its antiplatelet efficacy, exerts cardioprotective effects by reducing necrotic injury and edema formation via adenosine-dependent mechanisms.

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Sources of Funding, see page 1717

Key Words: cardiac imaging techniques ■ cardioprotection ■ edema ■ myocardial infarction ■ purinergic P2Y receptor antagonists

Clinical Perspective

What Is New?

- We show that ticagrelor reduces cardiac damage post–myocardial infarction (necrosis and edema) to a greater extent than clopidogrel by an adenosine-induced organ-protective response.
- We propose a possible molecular signaling pathway by which ticagrelor might reduce myocardial edema.

What Are the Clinical Implications?

- Our studies provide reliable and mechanistic demonstration (use of a preclinical animal model of closed-chest myocardial infarction in combination with 3T-cardiac MRI, pathological and molecular analysis) that the benefits of ticagrelor exceed the platelet-inhibition effects.
- Ticagrelor cardioprotective effects extend beyond its platelet function inhibition, helping to explain the beneficial effects seen clinically in the PLATO trial (Platelet Inhibition and Patient Outcomes).

For more than a decade dual-antiplatelet therapy with aspirin (cyclooxygenase inhibitor) and clopidogrel ($P2Y_{12}$ receptor antagonist and a second-generation thienopyridine) has remained the cornerstone of treatment for patients with acute coronary syndrome (ACS).[1] Despite the benefits associated with clopidogrel therapy in these high-risk patients, laboratory and clinical experience have led to the identification of several clopidogrel limitations, including delayed onset of action and high interindividual variability,[2] underscoring the need for improved antiplatelet treatment strategies. In this regard, the more recent oral $P2Y_{12}$-receptor antagonist prasugrel (a third-generation thienopyridine) and ticagrelor (a cyclopentyltriazolo-pyrimidine) have been shown to overcome many of the pharmacodynamic limitations associated with clopidogrel treatment, eventually improving outcomes in patients with ACS.[3,4] Ticagrelor, unlike the thienopyridines, blocks the platelet $P2Y_{12}$ receptor in a reversible manner and displays pharmacological properties that translate into a faster onset of action in comparison to clopidogrel.[5] Several clinical studies have supported the safety, tolerability, and efficacy of ticagrelor in the setting of stable coronary disease and acute myocardial infarction (MI), and a greater absolute benefit over clopidogrel, as well.[6,7] In the PLATO trial (Platelet Inhibition and Patient Outcomes),[3] ticagrelor demonstrated superiority in comparison with clopidogrel in patients with ACS, reducing cardiovascular morbidity[8] and mortality[9] with a similar incidence of coronary artery bypass grafting–related major bleeding, although the rate of non–coronary artery bypass grafting–related major bleeding was higher with ticagrelor. In addition, a post hoc analysis of the PLATO trial has suggested that ticagrelor reduces sudden death in comparison with clopidogrel[9] and has also been shown to lower the risk of recurrent ischemic events, including cardiovascular and coronary heart disease death, in diabetic patients with a history of MI.[10] Faster onset of action and more potent and reversible receptor binding are possible explanations for the increased superiority of ticagrelor over clopidogrel. Yet, ticagrelor has been shown, beyond its antiplatelet activity, to exert off-target effects likely contributing to the clinical benefit. Specifically, ticagrelor inhibits the equilibrative nucleoside transporter-1 (ENT-1) that is implicated in the cellular uptake of adenosine. Adenosine is locally formed at sites of ischemic tissue damage and exerts a wide range of cardiovascular benefits (eg, vasodilation, inhibition of platelet aggregation, and leukocyte adherence to the vessel wall).[11,12] Hence, ticagrelor inhibition of ENT-1 and consequent local adenosine increase may lead to additional protective effects.[13] So far, several antiplatelet agents have been shown to exert cardioprotective effects. As such, in patients who have ST-segment–elevation myocardial infarction (STEMI), intracoronary administration of abciximab has been shown to reduce infarct size assessed by cardiac MRI (CMR)[14] and clopidogrel has been shown to reduce cardiac enzyme release.[15] Ticagrelor, has been demonstrated in a rat model of transient coronary occlusion to diminish the size of infarction, an effect not observed on clopidogrel treatment.[16] In the present study, we aimed to compare the efficacy of ticagrelor and clopidogrel in protecting the heart against tissue injury and dysfunction in a swine model of closed-chest MI by using 3T-CMR.

METHODS

Expanded Materials and Methods are available in the online-only Data Supplement.

The study protocol was approved by the institutional ethics committee (CSIC-ICCC) and all animal procedures were performed in strict accordance with the guidelines from Directive 2010/63/EU or the National Institutes of Health (NIH) guidelines (NIH Publication No. 85-23, revised 1996).

Experimental Design

Online-only Data Supplement Figure I includes a diagram depicting the experimental protocol. In brief, pigs were allocated to randomly and blindly receive the following before MI induction: (1) placebo-control (n=9); (2) loading dose of clopidogrel (600 mg; n=8); (3) a loading dose of ticagrelor (180 mg; n=8); and (4) a loading dose of ticagrelor followed by an intravenous infusion of an adenosine A1/A2-receptor antagonist {8-(*p*-sulfophenyl)theophylline [8SPT]; 4 mg/kg; n=7}.[17] These starting dosages of clopidogrel and ticagrelor were selected because they achieved similar inhibition of ADP-induced aggregation. The timing and dose of administration, 2 hours for ticagrelor and 4 hours for clopidogrel before

MI induction, was based on a crossover dose-finding pilot study, the details of which are provided in the online-only Data Supplement Methods. Throughout the 24 hours post-MI, the animals received the corresponding maintenance doses of the antiplatelet agents. As such, ticagrelor was administered twice daily (90 mg every 12 hours) and clopidogrel-treated animals received a single 75-mg maintenance dose. Then animals were anesthetized and brought to the CMR facility. Thereafter, animals were euthanized, and histopathologic analysis of infarct size was assessed and myocardial samples were collected for molecular analysis.

Study End Points

The cardiovascular end points of the following study included the assessment of the beneficial effects of ticagrelor over clopidogrel on the infarcted cardiac tissue (infarct size and edema formation) and cardiac performance (contractility) acutely post-MI.

Experimental Induction of MI

MI was experimentally induced by a 1-hour total balloon occlusion of the mid–left anterior descending coronary artery as previously described.[18] Left ventricular ejection fraction (LVEF) was assessed by echocardiography before inducing ischemia (prior-MI) and on reperfusion (post-MI) to monitor the degree of MI induction.

3T-CMR Acquisition and Data Analysis

The studies were performed on a 3.0T-CMR system (Achieva, Philips) by operators blinded to the study medication as previously reported.[18] Details of the technical parameters for all CMR sequences are provided in online-only Data Supplement Table I, and the details of the protocol analysis have been detailed elsewhere.[18]

Platelet Function Assay and Coagulation Function and Dynamics

The present study aimed to evaluate whether ticagrelor in comparison with clopidogrel provides cardioprotective effects when dosed to equal inhibition of ADP-induced platelet aggregation. To define the doses to use, we conducted a crossover dose-finding pilot study in 4 naïve animals. The methodology and results of this pilot study are detailed in the online-only Data Supplement Methods. Based on these results (online-only Data Supplement Figure II), 180 mg of ticagrelor and 600 mg of clopidogrel were selected, which, in turn, concur with the dosages used in the PLATO trial.[3] Ticagrelor was administered 2 hours and clopidogrel 4 hours before experimental MI. Conventional coagulation function parameters and thromboelastometric coagulation analysis were also evaluated in this pilot study to discard any potential effect on blood coagulation (online-only Data Supplement Table II). In the main study, platelet function was additionally monitored before treatment (baseline), prior-MI induction, post-MI induction, and at 24 hours by light transmittance aggregometry and results were reported as inhibition of platelet aggregation. Blood count (System 9000) and bleeding time were also determined by using the Simplate device in the ear skin and recorded as time until bleeding cessation.

Analyses of Markers of Edema in the Myocardium

We performed molecular analysis of 2 proteins reported to be involved in edema formation. As such, we assessed aquaporin-4,[19] a protein with a key role in intracellular edema formation,[20] and AMP-activated protein kinase (AMPK),[21] which, besides sensing cardiac energy metabolism,[22] has been shown to protect against edema formation by preserving vascular permeability.[21] To that end, tissue samples, obtained from the ischemic and remote myocardium, were processed for the analysis of: (1) mRNA and protein levels of aquaporin-4; and (2) AMPK, AMPK phosphorylated at Thr^{172} (P-AMPK), and downstream effectors endothelial nitric oxide synthase (eNOS) phosphorylated at Ser^{1177} (P-eNOS) and CD36 protein levels. In addition, aquaporin-4 expression was analyzed in different cardiac cell types in vitro (porcine aortic endothelial cells, porcine fibroblasts, and HL-1 cardiomyocytes).

Myocardial Enzymes and Cyclooxygenase-2 Activity

Cardiac troponin-I was evaluated in all swine 24 hours post-MI using a high-sensitivity Pig Elisa kit.

Previous work in rodents[16] has reported the ability of ticagrelor to induce cardiac cyclooxygenase-2 (Cox2) activation. We further assessed this hypothesis in a more complex preclinical model (swine) and determined whether it was an adenosine-mediated effect. Accordingly, we evaluated myocardial Cox2 activity and prostaglandin F1α release as previously described.[16]

Statistical Analysis

After assessment for normal distribution of the data (ie, Shapiro-Wilk normality test), the pilot study was evaluated by repeated-measures analysis of variance and results reported as mean±standard error of the mean, whereas the main study by nonparametric statistical analyses and results are reported as medians and interquartile range. Comparisons between groups at end points were performed by Kruskal-Wallis and Mann-Whitney followed by post hoc Bonferroni correction. A linear mixed model was applied for analysis of repeated measurements across time. All statistical tests conducted were 2-sided. Statistical significance was considered $P<0.05$ and we used the Statview and R packages

RESULTS

Myocardial Impact of MI Induction

Two pigs in the placebo-control arm died of nonresolved coronary thrombotic occlusion observed on balloon deflation and removal. Final numbers of animals and data set analysis are detailed in online-only Data Supplement Figure I.

Echocardiography assessment of global LVEF demonstrated that all animals displayed a comparable contractile function before MI induction (≈65% LVEF; online-only Data Supplement Figure IIIA). Sixty minutes of complete coronary balloon occlusion resulted in a

significant and similar impairment in cardiac contractility among all 4 groups (online-only Data Supplement Figure IIIB and IIIC). Evans blue dye (stains all myocardial tissue blue except the area perfused by the occluded coronary artery) revealed that a similar amount of tissue was jeopardized by left anterior descending coronary artery occlusion in the 4 animal groups (placebo: 47.5% left ventricle [LV] [42.3%–51.6%]; clopidogrel: 46.9%LV [38.8%–53.0%]; ticagrelor: 48.2%LV [37.9%–52.4%]; and ticagrelor+8SPT: 51.2%LV [48.1%–53.4%]; online-only Data Supplement Figure IIID).

Effect of Clopidogrel and Ticagrelor on Platelet Function

Platelet activation at baseline was comparable among all animals (ADP 10 μmol/L: 54.8%, 52.1%, 50.4%, 49.7% maximal aggregation; ADP 15 μmol/L: 57.5%, 56.2%, 55.0%, 56.0% maximal aggregation; ADP 20 μmol/L: 63.3%, 66.2%, 64.0%, 64.2% maximal aggregation, for placebo-control-, clopidogrel-, ticagrelor-, and ticagrelor+8SPT–administered animals, respectively). The degree of platelet inhibition induced by clopidogrel and ticagrelor (±8SPT) throughout the experimental period on challenge with ADP is shown in Figure 1. In comparison with placebo, clopidogrel and ticagrelor exerted a high and consistent inhibitory effect on platelet function at MI (degree of inhibition of platelet aggregation of control: 5.6%, 0.0%, 8.4%; clopidogrel: 60.0%, 71.9%, 68.2%; ticagrelor: 56.2%, 60.5%, 62.2%; ticagrelor+8SPT: 53.5%, 54.5%, 52.0%, for 10, 15, and 20 μmol/L ADP, respectively). This inhibitory effect persisted up to 24 hours post-MI (time of CMR analysis). 8SPT administration did not influence the high platelet inhibitory effect already exerted by a loading dose of ticagrelor. Both clopidogrel and ticagrelor treatment resulted in an increased bleeding time in comparison with placebo. Yet, despite a similar degree of platelet inhibition, clopidogrel resulted in a bleeding time almost twice as long as that seen with ticagrelor±8SPT ($P<0.001$; Figure 1D), an effect already detected prior-MI. Neither clopidogrel nor ticagrelor altered any hematologic parameter (online-only Data Supplement Table III).

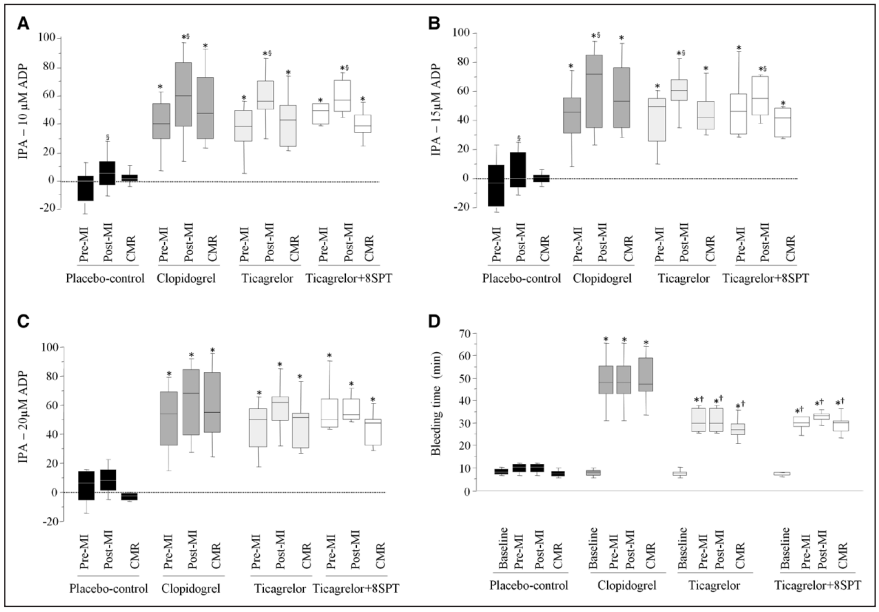

Figure 1. Platelet function and bleeding time.
Inhibition of platelet aggregation (IPA) triggered by ADP 10 μmol/L (**A**), 15 μmol/L (**B**), and 20 μmol/L (**C**) assessed by light transmittance aggregometry. **D,** Bleeding time. *$P<0.05$ versus placebo-control animals; †$P<0.05$ versus clopidogrel-treated animals; §$P<0.05$ versus prior-MI. Placebo-control n=7 animals/group; clopidogrel n=8 animals/group; ticagrelor n=8 animals/group; ticagrelor+8SPT n=7 animals/group. CMR indicates cardiac MRI; MI, myocardial infarction; and 8SPT, 8-(*p*-sulfophenyl)theophylline.

Ticagrelor Reduces Cardiac Damage to a Larger Extent Than Clopidogrel: CMR Analysis

The CMR data 24 hours post-MI is presented in Table. Compared with placebo-control, clopidogrel and ticagrelor treatment resulted in a significantly smaller infarct size in terms of both absolute infarct mass and percentage of affected LV myocardium (22.8 g LV, 15.7 g LV, and 12.0 g LV, respectively; Table). However, ticagrelor administration reduced infarct size to a significantly greater extent than clopidogrel (further 3.7 g LV; 23.5% relative reduction; P=0.0026). The same pattern was observed in serum troponin-I levels at 24 hours post-MI (Table; P=0.0021 versus clopidogrel). In fact, a direct correlation was observed between infarct mass (assessed by CMR) and troponin-I levels (lineal correlation coefficient r=0.98; P<0.001). The extent of edema was increased 24 hours post-MI and showed a transmural distribution in all animals. Although edema volume did not differ between placebo and clopidogrel (23.4 g versus 21.6 g LV; P=0.33), it was found to be significantly reduced in ticagrelor-treated animals by 5.3 g LV (24.5% relative reduction; P=0.004 versus placebo-control and clopidogrel; Table). It is interesting to note that administration of 8SPT reversed the reduction in infarct size and antiedema effects observed in ticagrelor-treated animals. As such, the amount of edema in 7 of 8 ticagrelor-treated animals was below the median of the clopidogrel- and ticagrelor+8SPT–treated arms (Figure 2A). Linear regression analysis demonstrated that the amount of edema was highly correlated (r=0.74; P<0.001) with infarct mass (Figure 2B). We also detected a good correlation (r=0.831; P<0.001) between infarct volume as assessed by histology (2,3,5-triphenyltetrazolium chloride staining) and the CMR-assessed infarct mass, although some shrinkage always occurs in the postmortem tissues (Figure 3).

In comparison with placebo, both clopidogrel- and ticagrelor-treated animals showed similar 10% relative improvement of LVEF (4% absolute improvement; P=0.01) and similar 21% relative reduction of left ventricular end-diastolic volume and left ventricular end-systolic volume (P=0.04) 24 hours post-MI (Table).

Ticagrelor Reduces Aquaporin-4 Expression and Activates AMPK Signaling in the Ischemic Myocardium

Ticagrelor treatment was associated with a significant reduction in aquaporin-4 gene (Figure 4A) and protein (Figure 4B) expression in the ischemic myocardium 24 hours post-MI in comparison with placebo-control and clopidogrel reaching levels similar to those observed in the remote myocardium. Administration of 8SPT abolished ticagrelor effects on aquaporin-4 expression. To identify cells expressing aquaporin-4, isolated cell cultures were established. Aquaporin-4 was not expressed in isolated fibroblasts nor in endothelial cells (porcine aortic endothelial cells), but it was highly expressed in HL-1 cardiomyocytes (online-only Data Supplement Figure IV).

As per AMPK, we observed that ticagrelor treatment induced activation of the AMPK-signaling pathway in the ischemic myocardium (Figure 5A) with the consequent activation and enhanced protein expression of its downstream effectors eNOS (Figure 5B) and CD36 (Figure 5C), in comparison with the placebo-control and clopidogrel-treated groups (P<0.05). Again, administration of the adenosine receptor antagonist, 8SPT, abolished ticagrelor activation of the AMPK signaling (Figure 5A through 5C). No differences were observed among the 4 groups in the remote myocardium, and no changes were observed

Table. Analyses of Cardiac Damage and Function: 3T-CMR Analyses and Serum Troponin-I Levels 24 Hours After Myocardial Infarction Induction

		Placebo-Control	Clopidogrel	Ticagrelor	Ticagrelor+8SPT
CMR analyses of cardiac anatomic parameters	LV mass, g	70.0 (64.1–73.7)	72.2 (69.3–74.7)	70.6 (67.9–74.4)	66.5 (65.0–70.2)
	Edema, g LV	23.4 (20.9–31.1)	21.6 (19.5–25.2)	16.3 (14.2–19.9)*‡	24.6 (22.8–25.3)
	Edema, % LV	36.2 (33.9–43.2)	30.1 (26.6–34.5)	23.1 (20.2–24.4)†§	36.8 (33.6–39.4)
	Infarct mass, g LV	22.8 (17.3–25.8)	15.7 (14.2–16.2)*	12.0 (10.6–12.9)†§	14.9 (14.6–16.1)*
	Necrosis, % LV	31.1 (25.9–39.1)	20.9 (19.3–22.8)†	16.4 (15.5–17.9)†§	22.4 (21.8–23.9)*
	No reflow, g LV	4.6 (2.1–6.0)	2.0 (1.5–2.8)*	2.1 (1.8–3.0)*	2.2 (2.0–2.6)*
CMR analyses of cardiac functional parameters	LVEF, %	43.0 (42.0–43.6)	47.2 (45.4–48.2)†	47.2 (45.4–51.0)*	48.7 (46.6–51.0)*
	LVEDV, mL	93.0 (87.6–98.1)	73.7 (68.9–81.3)†	77.4 (71.8–89.2)*	84.4 (76.9–86.8)*
	LVESV, mL	54.0 (49.2–55.6)	39.5 (36.3–41.9)†	39.2 (37.3–46.0)*	44.2 (40.4–45.7)*
Serum troponin levels	Troponin, ng/mL	19 (16.5–21.7)	13.4 (13.0–14.0)†	10.9 (9.3–11.4)†§	14.2 (12.2–16.1)*

Placebo-control animals n=7; clopidogrel-treated animals n=8; ticagrelor-treated animals n=8; ticagrelor+8SPT administered animals n=7. CMR indicates cardiac MRI; LV, left ventricle; LVEF, left ventricular ejection fraction; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; and 8SPT, 8-(*p*-sulfophenyl)theophylline. *P<0.05 versus placebo-control animals; †P<0.005 versus placebo-control animals; ‡ P<0.05 versus clopidogrel-treated animals; § P<0.005 versus clopidogrel-treated animals.

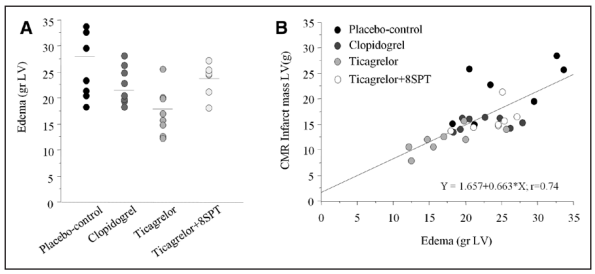

Figure 2. Edema analysis by 3T-CMR.

A, Individual data and median of edema in the different animal groups. **B,** Correlation between infarct mass (assessed by CMR) and the degree of edema (gr LV). Placebo-control n=7 animals/group; clopidogrel n=8 animals/group; ticagrelor n=8 animals/group; ticagrelor+8SPT n=7 animals/group. CMR indicates cardiac MRI; LV, left ventricle; MI, myocardial infarction; and 8SPT, 8-(*p*-sulfophenyl)theophylline.

as for AMPK expression in the different animal groups and cardiac regions (Figure 5A).

Ticagrelor Enhances Myocardial Cox2 Expression and Activity and 6-Keto-prostaglandin F1α Release

Ticagrelor was associated with higher Cox2 activity (Figure 6A) and 6-keto-prostaglandin F1α levels (the stable metabolite of prostacyclin; Figure 6B) in comparison with clopidogrel and placebo-control animals. Cox2 activity was barely detectable in the myocardium of the placebo-control group.

DISCUSSION

Although the clinical benefit of $P2Y_{12}$ inhibitors in STEMI patients undergoing coronary reperfusion therapy has always been attributed to their antithrombotic action,

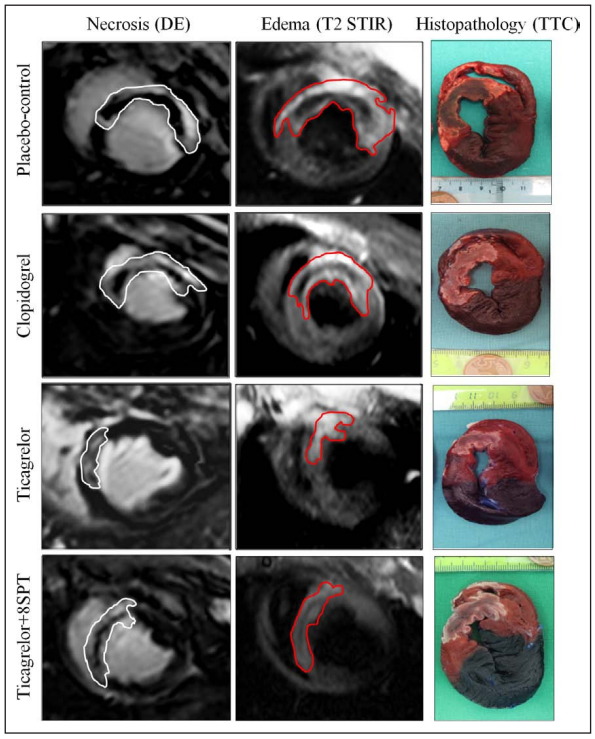

Figure 3. 3T-CMR and gross anatomic analysis of cardiac damage.

Representative short-axis CMR images showing the extent of edema (hyperintense area in T2-weighted CMR sequence), myocardial necrosis (delayed enhancement [DE] after gadolinium injection), and histological TTC staining. Images demonstrate the correspondence between the infarcted area in histology (negative TTC staining; white) and the extent of DE in the CMR. Placebo-control n=7 animals/group; clopidogrel n=8 animals/group; ticagrelor n=8 animals/group; ticagrelor+8SPT n=7 animals/group. CMR indicates cardiac MRI; 8SPT, 8-(*p*-sulfophenyl)theophylline; STIR, short tau inversion recovery; and TTC, 2,3,5-triphenyltetrazolium chloride.

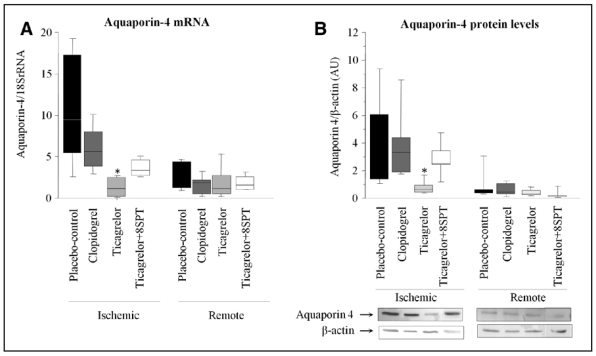

Figure 4. Myocardial aquaporin-4 expression.
mRNA (**A**) and protein (**B**) expression of aquaporin-4; **P*<0.05 versus placebo-control, clopidogrel- and ticagrelor+8SPT-treated animals. Placebo-control n=7 animals/group; clopidogrel n=8 animals/group; ticagrelor n=8 animals/group; ticagrelor+8SPT n=7 animals/group. 8SPT indicates 8-(*p*-sulfophenyl) theophylline.

experimental and clinical reports have suggested that some of these agents might reduce infarct size.[15,16]

In the present study, we found, in a preclinical animal model and by CMR, that ticagrelor and clopidogrel enhance myocardial viability in comparison with placebo-control animals 24 hours post-MI. Yet, and most interesting, we provide evidence that ticagrelor limits cardiac injury (necrosis and edema) to a greater extent than clopidogrel when dosed to a similar degree of platelet $P2Y_{12}$ inhibition. The $P2Y_{12}$-independent ticagrelor-related cardioprotective

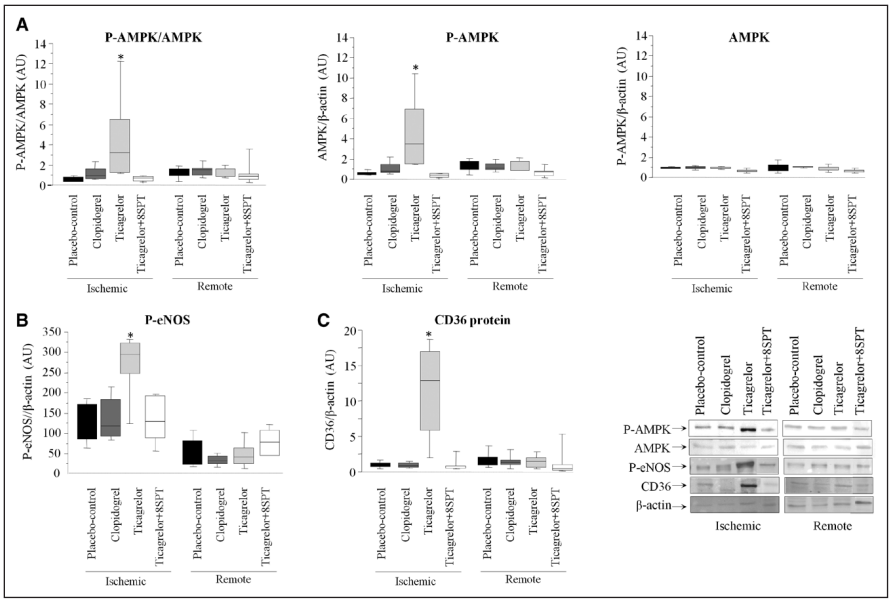

Figure 5. Analysis of myocardial AMPK signaling pathway activation.
A, P-AMPK/AMPK ratio, P-AMPK, and AMPK. **B,** eNOS activation. **C,** CD36 protein expression and their representative protein bands. **P*<0.05 versus placebo-control, clopidogrel- and ticagrelor+8SPT-treated animals. Placebo-control n=7 animals/group; clopidogrel n=8 animals/group; ticagrelor n=8 animals/group; ticagrelor+8SPT n=7 animals/group. AMPK indicates AMP-activated protein kinase; eNOS, endothelial nitric oxide synthase; P-AMPK, AMPK phosphorylated at Thr^{172}; P-eNOS, eNOS phosphorylated at Ser^{1177}; and 8SPT, 8-(*p*-sulfophenyl)theophylline.

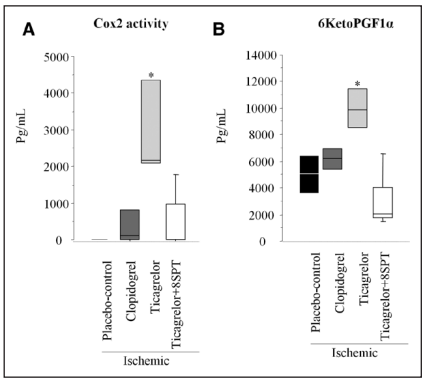

Figure 6. Effect of ticagrelor on myocardial Cox-2 activity.

Cardiac analysis of Cox2 activation (**A**) and 6-keto-PGF1α release (**B**) in the ischemic cardiac tissue. *$P<0.05$ versus placebo-control, clopidogrel- and ticagrelor+8SPT-treated animal. Placebo-control n=7 animals/group; clopidogrel n=8 animals/group; ticagrelor n=8 animals/group; ticagrelor+8SPT n=7 animals/group. Cox2 indicates cyclooxygenase-2; 6-keto-PGF1α, 6-keto-prostaglandin F1α; and 8SPT, 8-(*p*-sulfophenyl) theophylline.

effects are mediated through adenosine and are associated with a modulation of aquaporin-4 expression and AMPK-signaling activation in the ischemic cardiac tissue.

Chronic treatment with ticagrelor has been demonstrated to diminish the size of infarction in comparison with clopidogrel via an adenosine-dependent mechanism in a rat model of transient coronary occlusion.[16] The same research group using the same model has also shown that acute treatment with ticagrelor just before reperfusion reduces infarct size and that this translates into an improved heart function 4 weeks later.[23] However, in contrast to swine, translation of results in rodents is highly limited because of fundamental differences in heart anatomophysiology.[24] In fact, the ESC-Working Group Cellular Biology of the Heart Position Paper highlighted that studies in large animal models is an obligatory step before initiating human trials in cardioprotection.[25] Moreover, the ability to perform high-resolution CMR allows us to more accurately depict the protective effect of ticagrelor on cardiac damage beyond tissue necrosis, and provide data on edema, microvascular obstruction (no-reflow), and left ventricular contractibility and volumes, parameters of key importance for further clinical translatability.

Myocardial edema, a pathological consequence mainly related to ischemia and further reperfusion, has been shown to contribute to cell death,[26] yet the mechanisms and players of edema formation are only partially understood.[19] We observe that ticagrelor-administered animals modulate critical components involved in both intracellular and extracellular myocardial edema formation such as aquaporin-4 and AMPK, respectively, via adenosine-related mechanisms. First, we detected that ticagrelor-administered animals show a marked attenuation in both aquaporin-4 mRNA and protein expression, an effect reversed on 8SPT administration. Aquaporin-4, a transmembrane channel critically involved in cellular water balance, has been demonstrated to be primarily responsible for the development of cerebral edema.[20] However, although present in human cardiomyocytes, aquaporin-4 regulation and function remain largely unknown.[27] A recent study demonstrated that cardiomyocyte aquaporin-4 activity was enhanced after ischemia, and its upregulation was associated with increased myocyte swelling and size of infarction.[28] In fact, aquaporin-4 expression has been shown to coincide with myocardial edema and cardiac dysfunction in a mouse model of MI, an effect not observed by other cardiac aquaporins.[29] Most important, recent studies in mice have demonstrated that adenosine signaling regulates aquaporin-4 expression[30] and that aquaporin-4 is found significantly reduced in ENT-1 null mice versus their wild-type counterparts.[31] Altogether, these observations allow us to propose a link between ticagrelor, subsequent ENT-1 blockade, adenosine increase and signaling, and eventual aquaporine-4 diminishment in the infarcted heart.

We also report that ticagrelor administration is associated with an enhanced activation of the AMPK-signaling pathway in the ischemic cardiac region and consequent activation/increase in eNOS and CD36 downstream effectors. Besides being a key sensor of energy status, AMPK has recently been shown to preserve endothelial barrier permeability preventing ventricle edema formation on cardiac sepsis.[21] We therefore speculate that the enhanced AMPK-signaling activation observed in ticagrelor-treated animals may have attenuated vascular dysfunction because of MI induction limiting the passage of plasma into the interstitial compartment of the ischemic damaged myocardium.[32] It is worth mentioning that several recent studies have proposed that activated AMPK protects against sepsis-induced organ damage and inflammation.[33] These observations may also explain in part the post hoc analysis of the PLATO trial showing the ticagrelor-associated reduction in sepsis mortality in ACS patients.[34]

AMPK is mainly activated on extracellular increase in adenosine levels, and a recent study has shown impaired AMPK phosphorylation in adenosine A2B receptor–deficient mice.[35] A study in patients with ACS has revealed that adenosine plasma concentrations are significantly higher in ticagrelor-treated than in clopidogrel-treated patients, likely enhancing adenosine biological effects on the cardiovascular system.[36] Moreover, adenosine has been attributed to afford cardioprotection in ischemic conditioning (pre, post, and remote) by interacting with adenosine receptors,[37] which, in turn, has been shown

to reduce edema formation in humans.[38] So far, clinical studies with adenosine in STEMI patients have yielded mixed results.[24] In a post hoc subgroup analysis of the AMISTAD-II trial (Acute Myocardial Infarction Study of Adenosine) a significant reduction in infarct size was reported in the high-dose adenosine subgroup reperfused within 3 hours.[39] However, no evidence of adenosine-cardioprotective effects was observed in larger clinical trials by CMR.[40] Adenosine and adenosine analogues have been shown in animal models to mimic ischemic preconditioning and to potentially prevent lethal myocardial reperfusion injury after an ischemic episode.[37] However, systemic infusion of exogenous adenosine, with a half-life counted in seconds, may be quite different from a continuous enhancement of local endogenous adenosine release at sites of ischemia or tissue injury as provided by ticagrelor. In fact, the REDUCE-MVI trial (Reducing Micro Vascular Dysfunction in Acute Myocardial Infarction)[41] is currently testing whether ticagrelor, via ENT-1/adenosine–mediated mechanisms, improves microvascular function in revascularized STEMI patients.

Adenosine-receptor activation can also induce eNOS phosphorylation[42] which, in turn, has been shown to be essential for downstream upregulation of Cox2 activity and consequent reduction in cardiac damage. Ticagrelor increases Cox2 activity and 6-keto-prostaglandin F1α release in the damaged myocardium via adenosine receptor–related mechanisms.[16,43] It is important to notice that we did not treat pigs with aspirin to investigate the sole effect of $P2Y_{12}$ inhibition in the infarcted myocardium. Indeed, during primary percutaneous coronary intervention, ticagrelor-related Cox2/prostaglandin I2 cardioprotective effects may be acutely abolished on aspirin loading.[44] Moreover, ticagrelor has shown a reduced efficacy in North American patients where higher maintenance doses of aspirin (>300 mg) are more widely prescribed.[45] However, ticagrelor may overcome such aspirin-loading dose effects and resume its Cox2-related cardioprotecitve effects when daily low-dose aspirin therapy is given. Indeed, low doses of aspirin do not affect Cox2 activity, which, in concert with ticagrelor-derived adenosine-dependent benefits, may contribute to explaining ticagrelor benefits reported long after MI in PEGASUS patients (Prevention of Cardiovascular Events in Patients With Prior Heart Attack Using Ticagrelor Compared to Placebo on a Background of Aspirin-Thrombolysis in Myocardial Infarction 54).[46]

As to cardiac performance, LVEF was significantly improved in both antiplatelet-treated arms in comparison with the placebo-control arm, yet no differences were observed between the actively treated groups despite lower myocardial damage in the ticagrelor-treated animals. To ascertain whether the acute cardioprotective benefit exerted by ticagrelor over clopidogrel within 24 hours of infarction translate into an attenuated cardiac-remodeling process and improved cardiac output, long-term follow-up studies are required. This is particularly interesting because a reduction in edema has been shown to improve cardiac function over time.[47]

Bleeding time was also markedly shorter in ticagrelor-treated animals versus clopidogrel-treated pigs. Bleeding time, assessed by a small incision in the ear skin, mainly screens for defects of primary hemostasis and coagulation. We did not detect differences in conventional coagulation function testing or clot dynamics among all animals, allowing us to exclude the interference of ticagrelor with the intrinsic and extrinsic coagulation cascade and fibrinogen. The data in terms of separation of bleeding and antiplatelet/antithrombotic effect in this pig model nicely confirms prior published data generated in rats and dogs.[48] The reason for this is currently unclear but is likely related to irreversible (thienopyridines) versus reversible (ticagrelor) binding mode of action to the $P2Y_{12}$ receptor. Comparing the clinical data in PLATO[3] and TRITON (The Efficacy and Safety of Initial Triple Versus Initial Dual Oral Combination Therapy in Patients With Newly Diagnosed Pulmonary Arterial Hypertension),[4] one can argue that the animal data translate also into a wider separation between efficacy and bleeding in patients, because both clinical studies tested a similar increased platelet inhibition versus clopidogrel, but prasugrel appeared to induce more severe bleeding. Studies are currently underway to determine how ticagrelor modulates the formation of the plug to achieve an earlier hemostasis.

Fernández-Jiménez et al[49] recently performed a CMR study in swine to determine the dynamics of edema formation after 40 minutes of coronary artery occlusion followed by reperfusion. The authors detected a rapid and marked increase in tissue water at 2 hours of reperfusion that was largely resolved at 24 hours, but then a second increase in water content appeared. In contrast, we detect edema 24 hours after MI. This discrepancy is likely explained by the differences between the ischemic insults. Longer ischemic periods have been shown to result in larger infarcts and subsequently enhance myocyte damage and edema formation.[50]

Our study has some limitations. First, we did not perform the study in all the animals reported within the sample size calculation (n=10). Because we adhere to the 3R (replacement, reduction, and refinement) code of the European Union in animal use and euthanization, we felt that it was unethical to include more animals to corroborate the already statistically significant results reached with a lower number. Second, in the present study, we performed CMR analysis at 24 hours; therefore, we cannot draw conclusions regarding the potential benefits of ticagrelor in the longer term. However, longer-term studies are warranted. Third, our mechanistic study has not included comorbidities found in most STEMI patients. We cannot ascertain from our present studies whether reduction of edema and cardioprotection are attributable to an effect of ticagrelor on aquaporin-4 expression and AMPK signaling or if these latter are modulated because

ticagrelor reduced edema through a different mechanism. Further studies are needed to elucidate whether the association is a cause or an effect.

CONCLUSIONS

We have shown the increased capability of ticagrelor to reduce MI-induced cardiac damage (edema and necrosis) over clopidogrel by CMR analyses in a preclinical animal model. Moreover, we demonstrate that ticagrelor antiedema protective effects are mainly mediated through adenosine and are associated with a reduction in aquaporin-4 levels and the activation of AMPK signaling in the ischemic myocardium.

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DISCLOSURES

Dr Carlsson is currently employed by AstraZeneca. The other authors do not have any conflicts of interest to disclose.

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FOOTNOTES

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$P2Y_{12}$ antagonists and cardiac repair post-myocardial infarction: global and regional heart function analysis and molecular assessments in pigs

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Aims $P2Y_{12}$ antagonists are the standard in antiplatelet therapy but their potential effects on functional myocardial recovery and cardioprotection post-myocardial infarction (MI) are unknown. We investigated in a preclinical model of MI whether ticagrelor and clopidogrel differently affect cardiac repair post-MI.

Methods and results Pigs either received: (i) clopidogrel (600 mg; 75 mg/qd); (ii) ticagrelor (180 mg; 90 mg/bid); and (iii) placebo control. MI was induced by mid-left anterior descending coronary artery balloon occlusion (60 min) and animals received the maintenance doses for the following 42 days. Serial cardiac magnetic resonance was performed at Day 3 and Day 42 for the assessment of global and regional cardiac parameters. We determined cardiac AMP-activated protein kinase (AMPK), Akt/PKB, aquaporin-4, vascular density, and fibrosis. In comparison to controls, both $P2Y_{12}$ antagonists limited infarct expansion at Day 3, although ticagrelor induced a further 5% reduction ($P < 0.05$ vs. clopidogrel) whereas oedema was only reduced by ticagrelor (≈23% $P < 0.05$). Scar size decreased at Day 42 in ticagrelor-treated pigs vs. controls but not in clopidogrel-treated pigs. Left ventricular ejection fraction was higher 3 days post-MI in ticagrelor-treated pigs and persisted up to Day 42 ($P < 0.05$ vs. post-MI). Regional analysis revealed that control and clopidogrel-treated pigs had severe and extensive wall motion abnormalities in the jeopardized myocardium and a reduced myocardial viability that was not as evident in ticagrelor-treated pigs (χ^2 $P < 0.05$ vs. ticagrelor). Only ticagrelor enhanced myocardial AMPK and Akt/PKB activation and reduced aquaporin-4 levels ($P < 0.05$ vs. control and clopidogrel). No differences were observed in vessel density and fibrosis markers among groups.

Conclusions Ticagrelor is more efficient than clopidogrel in attenuating myocardial structural and functional alterations post-MI and in improving cardiac healing. These benefits are associated with persistent AMPK and Akt/PKB activation.

Keywords $P2Y_{12}$ receptor antagonists • Cardiac repair • CMR analysis • Wall motion • AMPK/Akt pathway

1. Introduction

Dual antiplatelet therapy with the cyclooxygenase inhibitor aspirin and a $P2Y_{12}$ receptor antagonist has been pivotal in the era of percutaneous coronary intervention as a mainstay treatment of ischaemic heart disease.[1] Until recently, their primary indication was to reduce the risk of thrombosis. However, it has become increasingly accepted that $P2Y_{12}$ antagonists may exert additional

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benefit via off-target effects. As such, $P2Y_{12}$ antagonists have demonstrated, at both experimental[2,3] and clinical[4,5] levels, to reduce the size of infarction. However, regardless of their antiplatelet efficacy, $P2Y_{12}$ antagonists have been shown to differ in their ability to limit infarct size.[6–9] This is of key importance since infarct size is a major determinant of post-ST-elevation myocardial infarction (STEMI) morbidity and mortality.[10] We have recently demonstrated by cardiac magnetic resonance (CMR) in a preclinical experimental model of myocardial infarction (MI) that both clopidogrel and ticagrelor administration prior-MI acutely reduce infarct size measured at 24 h, and that the effect was significantly greater in ticagrelor-treated animals.[2] Interestingly, the cardioprotective effects of ticagrelor were associated with the attenuation in oedema formation, an effect not reported in clopidogrel-treated pigs.[2] These differences suggest that the degree of cardioprotection may vary between $P2Y_{12}$ antagonist classes (clopidogrel/thienopyridine vs. ticagrelor/cyclopentyltriazolopyrimidine) partly explaining the clinical benefit found in ticagrelor-treated patients in the PLATO trial.[11,12] Although the underlying molecular mechanisms by which platelet $P2Y_{12}$ antagonists mediate cardioprotection remain to be elucidated, they have been shown to exhibit an ischaemic conditioning-like effect.[4] Particularly, ticagrelor has been shown to protect the heart against MI mainly through adenosine-related mechanisms.[2,7] In this regard, by blocking the equilibrative nucleoside transporter 1 (ENT-1) transporter, ticagrelor prevents erythrocyte re-uptake of extracellular adenosine released upon the ischaemic insult consequently leading to elevated and persistent local extracellular adenosine levels.[13] Adenosine, in turn, has shown to trigger and mediate endogenous cardioprotection.[14]

Infarct size determines the progression to left ventricular (LV) remodelling and eventual development of heart failure.[15] The LV healing process has been arbitrarily divided into an early phase (within 3 days) and a late phase (beyond 3 days). The early phase involves expansion of the infarct zone whereas the late phase involves the global LV function.[16] The effect of ticagrelor on cardiac response post-MI has previously been assessed in a rat model, where chronic ticagrelor administration resulted in smaller infarcts and improved ventricular function at 4 weeks, effects not reported for clopidogrel.[8] However, no data are yet available on the effects of both $P2Y_{12}$ antagonists on global functional and anatomical parameters, as well as on regional parameters of cardiac remodelling (16-segment model) in a large animal model by use of state-of-the art imaging modality. Hence, to understand the full range of effects elicited by the two classes of $P2Y_{12}$ antagonists, we have investigated in a pig MI model and by segmented CMR imaging at 3 days (early phase) and at a late phase (42 days) post-MI, whether administration of the drugs in the setting of MI and chronically thereafter reduces infarct size and consequently attenuates LV dysfunction. Furthermore, structural and molecular analyses have been performed.

2. Methods

The experimental procedures with animals were reviewed and approved by the Institutional Animal Care and Use Committees (CEEA-IR) and authorized by the Animal Experimental Committee of the local government (#5601) in accordance to the Spanish law (RD 53/2013) and European Directive 2010/63/EU. In addition, the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and has followed the ARRIVE guidelines and committed to the 3Rs of laboratory animal research and consequently used the minimal number of animals to reach statistical conclusion.[17]

All animals were allowed to acclimate 7 days prior to any intervention and housed in individual cages under light-controlled conditions and room temperature.

2.1 Experimental design

Regular chow-fed Landrace pigs ($n = 24$; weight ≈43 kg; Specipig SL, Spain) were randomly (Excel randomized function) distributed into three groups to blindly receive: (i) placebo control ($n = 8$); (ii) a loading dose of clopidogrel (600 mg; $n = 8$; 4 h prior-MI); and (iii) a loading dose of ticagrelor (180 mg; $n = 8$; 2 h prior-MI induction).

The study timing and doses were chosen based on our previously published data with the goal to reach comparable degree of platelet inhibition. As such, adenosine diphosphate (ADP) (20 μM)-induced platelet aggregation was inhibited by 68.2% in clopidogrel-treated animals and by 62.2% in ticagrelor-treated pigs [P = non significant (NS)].[2] In addition, we performed a sub-study in six pigs in which we assessed the platelet inhibitory effects of both treatment regimes by light transmittance aggregometry induced by ADP (5, 10, and 20 μM) over a follow-up of 8 days. As shown in Supplementary material online, *Figure S1* and in line with our previous work,[2] administration of the selected treatment regimes lead to a comparable degree of platelet inhibition in both groups that persisted over time. These results allow us to exclude any potential effect of the drugs due to different power in platelet inhibition. These dosages, in turn, concur with the dosages used in the PLATO trial.[2,11]

Pigs underwent temporary (1 h) balloon occlusion of the mid-left anterior descending coronary artery (experimental MI),[2] and thereafter, were brought back to the animal facility to recover. Throughout the following 42 days, the pigs received the daily maintenance doses of the antiplatelet agents (90 mg/bid for ticagrelor and 75 mg/qd for clopidogrel, respectively). To ensure the blind administration of the treatment, we directly and daily gave the pills to a technician of the animal facilities in charge of animal maintenance but not involved in conducting the experimental protocol. At Day 3 and Day 42 post-MI, animals were anaesthetized and brought to the 3T-CMR facility for blind assessment of global and regional parameters. Animals were euthanized after the last CMR analysis (Day 42).

2.1.1 Experimental induction of MI

On the day of MI-induction animals received buprenorphine (0.03 mg/kg) and cefazoline (25 mg/kg) as prophylaxis for pain and wound infection, respectively, and then anaesthetized by administrating an intramuscular injection of ketamine (30 mg/kg), xylazine (2.2 mg/kg), and atropine (0.05 mg/kg). Once tranquilized, animals were endotracheally intubated and anaesthesia was maintained by isoflurane inhalation (2%). Prior to starting the MI-induction procedure, an infusion of amiodarone (300 mg) and lidocaine (150 mg) in 1.000 mL of saline (250 mL/h) was initiated as prophylaxis for malignant ventricular arrhythmias through a line placed in the marginal ear vein and a bolus of heparin (100 U/kg) was administered to prevent clot formation in the catheters. MI was experimentally induced by a minimally invasive closed chest myocardial balloon occlusion of the mid-portion of the left anterior descending coronary artery as previously described.[2] Complete coronary ischaemia (verified by angiography) was maintained for 1 h. At the end of the ischaemic period, the balloon was completely deflated and animals were allowed to recover. Electrocardiographic (ECG) and haemodynamic parameters were continuously recorded throughout the entire procedure and the

echocardiographic system (Phillips iE33) equipped with a S5-1 sector array transducer was used prior- and post-MI induction to document the initial worsening of left ventricular ejection fraction (LVEF, M-mode analysis using the parasternal long-axis view).[18,19]

2.1.2 3T-CMR acquisition

CMR studies were conducted serially in all animals at Day 3 (early remodelling phase) and Day 42 (late remodelling phase) post-MI. The studies were performed on a 3.0T-CMR system (Achieva®, Philips, Amsterdam, The Netherlands) and CMR image acquisition was carried out by a CMR-specialized technician blinded in terms of treatment. For CMR studies, animals were anaesthetized with an intramuscular injection of a cocktail composed of ketamine, xylazine, and atropine and maintained by a continuous intravenous infusion of propofol to ensure mechanical ventilation. Once the animals were positioned in a head-first supine position with a flexible phased-array surface coil placed over the chest, ECG gating was used to acquire still images of the heart. The following dedicated CMR sequences were acquired in all cases: 'cine' Balanced Steady-State Free Precession (bSSFP) imaging sequence to assess wall motion (WM) and cardiac function; T2-weighted short-tau inversion recovery (T2w-STIR) sequence to assess myocardial oedema; early gadolinium enhancement to study microvascular obstruction (no-reflow phenomenon); and late gadolinium enhancement (LGE) to assess the amount and extent of myocardial necrosis. All the CMR studies followed the same scheme. First, scout images [T1-turbo field echo (TFE) sequence] were obtained to localize the true axes of the heart and define a field of view involving the whole heart. Afterwards, the bSSFP cine imaging was performed in both horizontal and vertical long axes (four-chamber and two-chamber views) and in multiple contiguous short-axis images covering the whole LV. In the short-axis cine sequence, we acquired 24 cardiac phases of every slice to guarantee a correct evaluation of the WM and heart function. Once the cine sequences were acquired, a T2w-STIR sequence was obtained to assess myocardial oedema. Thereafter, a gadolinium-based contrast agent was injected intravenously (Gd-GTPA, Magnevist®, Berlex Laboratories Inc., Wayne, NJ, USA) at a dose of 0.1 mmol/kg. The early gadolinium enhancement sequence was acquired 1 min after the administration of the contrast. The LGE sequences were obtained 10 min after the administration of contrast. Details of the technical parameters for CMR sequences have been previously published.[2,20]

2.1.3 3T-CMR data analysis

2.1.3.1 Global functional and anatomical parameters. CMR data were independently analysed using dedicated software (QMass MR v.7.6, Medis, Leiden, The Netherlands) by a CMR-trained radiologist blinded to the study medication. The protocol of analysis for global functional/anatomical parameters was performed as previously reported.[2,21] Briefly, LV epicardial and endocardial borders were traced in each image of the cardiac phases representing the end diastole and end systole in order to obtain the left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), and LVEF. LGE scar size was assessed by using manual planimetric segmentation on each slice. Same method was used for the assessment of myocardial oedema and areas of microvascular obstruction. The area of myocardial oedema was defined as the extent of the LV demonstrating high signal intensity on T2w-STIR images. The size of infarction (Day 3) or scar (Day 42) was quantified from the extent of myocardial enhancement in the LGE CMR sequence.

2.1.3.2 Cardiac remodelling parameters: WM and myocardial viability. LV regional analysis to each of the 16 American Heart Association myocardial segments were performed.[22] The 16-segments and not the 17-segments model were used for evaluation of WM abnormalities as the tip of the normal apex (Segment 17) does not move. To this end, an inferior right ventricular insertion point was identified and used as a reference point from which the LV was divided into the 16 circumferentially equal sectors. The endocardial and epicardial borders of the LV were defined in all the slices representing the end-diastolic and end-systolic phases. Thereafter, the end-systolic wall thickness (EST), the end-diastolic wall thickness (EDT), and the WM (WM = EST - EDT) were automatically calculated. In addition, we also determined the number of dysfunctional segments and myocardial viability as previously reported.[23] Accordingly, these two parameters were calculated as follows: dysfunctional segments were considered those segments with an EST <2 mm, whereas myocardial recovery was assessed as the percentage of segments with an EST at Day 3 <2 mm (dysfunctional segments) that presented an EST at day 42 ≥2 mm (recovered segments).[23] These measurements and analyses were performed in: (i) the 16 segments (the entire LV; Supplementary material online, *Figure S2A*); (ii) only including the jeopardized segments (mid- and apical-antero/septal segments; target segments 7, 8, 13, and 14; Supplementary material online, *Figure S2B*); and (iii) including those segments contra-lateral to the infracted region (basal-, mid-, and apical-inferior/lateral segments; target segments 4, 5, 10, 11, 15, and 16; Supplementary material online, *Figure S2C*).

Table 1 Serial CMR imaging analyses: oedema and no-reflow assessed at Day 3 post-MI

	Group	3 days post-MI
Oedema (g LV)	Control	23.3 (21.8–26.2)
	Clopidogrel	22.4 (19.9–24.1)
	Ticagrelor	16.8 (15.1–18.7)***
Oedema (% LV)	Control	36.6 (33.6–40.6)
	Clopidogrel	32.3 (29.7–32.9)
	Ticagrelor	25.8 (23.2–29.2)***
No-reflow (g)	Control	2.8 (2.4–3.9)
	Clopidogrel	1.8 (1.4–2.5)*
	Ticagrelor	2.0 (1.6–2.3)*

Data were analysed by the Kruskal–Wallis test followed by the Mann–Whitney *U* test with *post hoc* Bonferroni correction, and results are reported as median and interquartile range. $N = 8$ animals per group.
CMR, cardiac magnetic resonance; LV, left ventricle.
*$P < 0.05$ vs. control.
**$P < 0.05$ vs. clopidogrel.

2.1.4 Scar size analysis by TTC staining

Although the investigation of scar size by triphenyltetrazolium chloride (TTC) staining late after reperfusion is not universally accepted, we performed the analysis with the intention of gathering some additional information. At Day 42 and after CMR analysis, hearts were arrested with potassium chloride, rapidly excised, sliced, and stained with TTC in order to determine the size of the scar by planimetry using the National Institute of Health software Image J.

2.1.5 Assessment of blood adenosine concentration

We assessed blood adenosine concentrations just prior MI-induction and prior reperfusion (at 1 h ischaemia). For this purpose, 4 mL of blood were directly collected into a tube containing stop solution to avoid adenosine degradation and generation. The stop solution contained 4.2 mM Na2EDTA,

Table 2 Serial CMR imaging analyses: cardiac damage at Day 3 and Day 42 post-MI

	Group	3 days post-MI	42 days post-MI
LV mass (g)	Control	64.8 (62.9–67.3)	87.6 (80.8–97.6)*
	Clopidogrel	68.9 (62.5–74.5)	104.5 (95.5–104.9)*
	Ticagrelor	67.1 (63.3–68.4)	93.0 (89.5–98.3)*
Infact size (g LV)	Control	17.1 (14.8–17.7)	11.5 (9.5–13.2)*
	Clopidogrel	13.6 (11.5–17.0)	12.0 (10.8–13.4)*
	Ticagrelor	10.6 (9.7–11.6)**-***	8.2 (7.9–10.1)**-***
Infarct size (% of LV)	Control	25.2 (22.2–27.3)	13.0 (11.2–15.7)*
	Clopidogrel	20.7 (18.2–24.1)**	11.6 (10.2–15.1)*
	Ticagrelor	16.3 (10.1–18.5)**-***	9.1 (8.3–10.3)**-***

Data were analysed by the Kruskal–Wallis test followed by the Mann–Whitney *U* test with *post hoc* Bonferroni correction, and results are reported as median and interquartile range. $N = 8$ animals per group.
CMR, cardiac magnetic resonance; LV, left ventricle.
*$P < 0.05$ vs. 3 days post-MI.
**$P < 0.05$ vs. control.
***$P < 0.05$ vs. clopidogrel.

5 μM EHNA [Erythro-9-(2-hydroxy-3-nonyl) adenine], 79 μM AOPCP (α,β-Methylene-ADP), 1 μM Pentostatin, 100 IU of Heparin, and 0.9% NaCl. Blood samples were immediately centrifuged at 4°C and 1000 × g for 20 min and high-weigh proteins were removed by using a 10 KDa filters (Merck-Millipore). Chromatographic analysis was performed using an Agilent 1200 Series HPLC. Briefly, 50 μL of the filtrated blood samples were injected onto a 4.6 × 150 mm, 5 μm C18 columns (Agilent Technologies). The starting mobile phase consisted of 100% of methanol (MeOH) for 1 min followed by 46% of MeOH and 56% of 50 mM Na_2HPO_4 buffer (pH = 4) for 30 min and finally 100% of Na_2HPO_4 for 1 min. The full cycle was performed with a flow rate of 500 μL/min. A standard curve was generated with pure adenosine added to stop solution in buffer and processed as the blood samples. HPLC chromatograms were analysed by the Analyst Software (Sciex).

2.1.6 Assessment of myocardial AMP-activated protein kinase and Akt/PKB expression and activation and aquaporin-4 expression

Our previous findings in the acute setting of MI revealed a significant association between acute ticagrelor cardioprotective effects and activation of AMP-activated protein kinase (AMPK) and a reduction in aquaporin-4.[2] AMPK is known to regulate cardiac energy metabolism and to exert protection against oedema formation.[24,25] In addition, previous studies in rodents have supported the capability of chronic ticagrelor treatment to induce protein kinase B (Akt/PKB) activation, a pro-survival protein kinase.[26,27] In the present study, we sought to determine whether chronic administration of ticagrelor modulates cardiac aquaporin-4 expression, and AMPK and Akt/PKB expression and/or activation at 42 days post-MI. To this end, tissue samples obtained at sacrifice from the infarcted and non-infarcted myocardium of all animals were processed for protein and mRNA isolation. We performed western blot analysis to determine aquaporin-4, AMPK and Akt/PKB protein expression, and degree of activation (AMPK phosphorylated at Thr^{172} or P-AMPK and Akt phosphorylated at Ser^{473} or P-Akt). Results were normalized to β-actin (aquaporin-4).We also analysed the transcript levels of aquaporin-4 and AMPK by real time PCR-7000 Sequence Detection System of ABIPRISM (Applied Biosystems). The threshold cycle (Ct) values were determined and normalized to the housekeeping gene 18SrRNA in order to adjust to equal amounts of RNA.

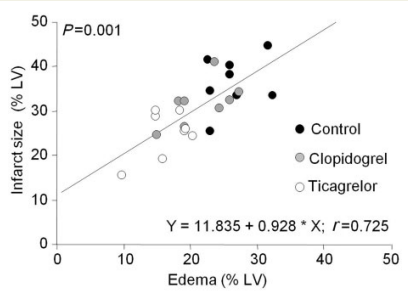

Figure 1 Linear regression analysis between oedema (% LV) and infarct size (% LV) assessed by cardiac magnetic resonance imaging at 3 days post-MI.

2.2 Statistical analysis

Data were evaluated by the non-parametric Kruskal–Wallis test followed by the Mann–Whitney *U* test with Bonferroni correction. Results are reported as medians and interquartile range (IQR), and the χ^2 of independence test was performed for the regional segmental analyses of dysfunctional segments, and linear regression analysis was performed to compare oedema and infarct size. Comparisons were carried out with the StatView (SAS Institute Inc.) and R Statistical Package. A two-sided *P* value less than 0.05 was considered statistically significant.

3. Results

3.1 Impact of MI on cardiac contractility through echocardiographic assessment

All animals showed a similar reduction of LVEF of around 30% at reperfusion as compared to baseline. In the control animals, LVEF was reduced

Table 3 Serial CMR imaging analyses: left ventricular performance at Day 3 and Day 42 post-MI

	Group	3 days post-MI	42 days post-MI
LVEDV (mL)	Control	85.1 (75.6–86.6)	113.0 (110.3–122.0)*
	Clopidogrel	84.8 (79.8–99.3)	129.1 (125.6–134.3)*
	Ticagrelor	85.2 (73.7–93.0)	124.0 (104.4–137.3)*
LVESV (mL)	Control	43.6 (38.6–48.7)	60.9 (56.8–69.2)*
	Clopidogrel	44.7 (38.5–47.7)	66.6 (64.33–73.5)*
	Ticagrelor	36.7 (33.1–43.4)	55.1 (48.6–64.0)*
LVEF (%)	Control	44.8 (43.4–47.8)	48.2 (45.4–50.2)
	Clopidogrel	50.6 (44.9–55.3)	47.2 (44.2–51.2)
	Ticagrelor	53.3 (49.3–57.3)**	54.5 (52.2–58.0)**-***

Data were analysed by the Kruskal–Wallis test followed by the Mann–Whitney *U* test with *post hoc* Bonferroni correction, and results are reported as median and interquartile range.
N = 8 animals per group.
CMR, cardiac magnetic resonance; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEF, left ventricular ejection fraction.
*$P < 0.05$ vs. 3 days post-MI.
**$P < 0.05$ vs. control.
***$P < 0.05$ vs. clopidogrel.

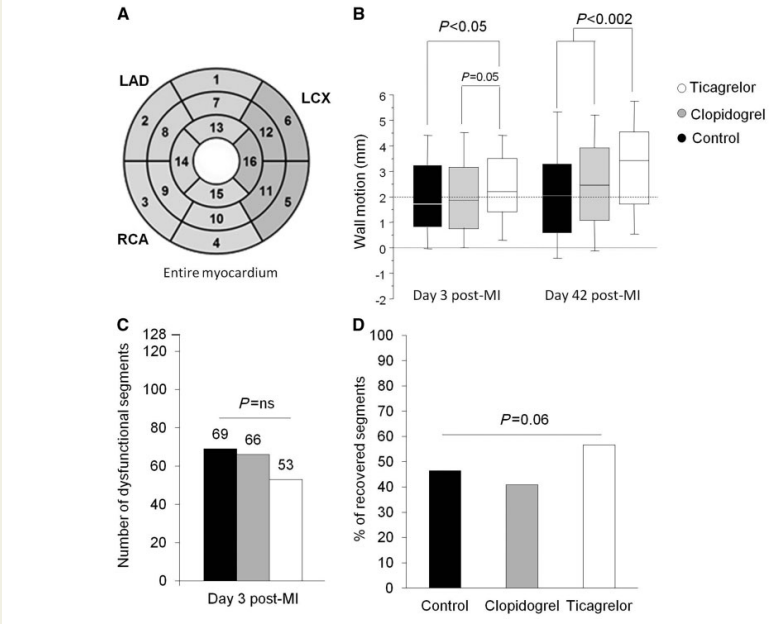

Figure 2 Analysis of myocardial regional parameters in the 16-segment model (American Heart Association). (*A*) Segments included in the analysis (128 segments/group). (*B*) WM regional analysis over time. (*C*) Number of dysfunctional segments (WM <2 mm). (*D*) Percentage of dysfunctional segments at Day 3 post-MI (WM <2 mm) that have been recovered (WM ≥2 mm) at Day 42 post-MI (i.e. myocardial viability). WM was evaluated by the non-parametric Kruskal–Wallis test followed by the Mann–Whitney *U* test with Bonferroni correction. Results are reported as medians and IQR. The χ^2 test was performed for the regional segmental analyses of the number of dysfunctional/recovered segments. Wall motion = (end-systolic wall thickness) - (end-diastolic wall thickness); *N* = 8 animals per group.

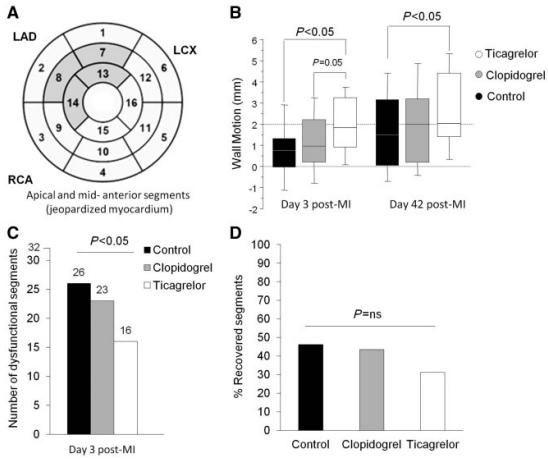

Figure 3 Regional analysis of functional parameters in the jeopardized segments according to the 16-segment model (American Heart Association). (*A*) Segments included in the analysis are highlighted in grey (32 segments/group). (*B*) Wall motion analysis over time. (*C*) Number of dysfunctional segments (WM <2 mm). (*D*) Percentage of dysfunctional segments at Day 3 post-MI (WM <2 mm) that have been recovered (WM ≥2 mm) at Day 42 post-MI (i.e. myocardial viability). Wall motion was evaluated by the non-parametric Kruskal–Wallis test followed by the Mann–Whitney *U* test with Bonferroni correction. Results are reported as medians and IQR. The χ^2 test was performed for the regional segmental analyses of the number of dysfunctional/recovered segments. Wall motion = (end-systolic wall thickness) - (end-diastolic wall thickness); *N* = 8 animals per group.

from 73.2 (72.3–74.8)% to 43.3 (39.4–47.4)%, in the clopidogrel-treated animals from 72.0 (70.9–76.2)% to 45.5 (40.8–47.8)% and in the ticagrelor-administered animals from 75.2 (69.7–75.9)% to 43.3 (42.3–44.6)%.

3.2 Blood adenosine levels

Blood adenosine levels increased post-MI (i.e. prior reperfusion) in ticagrelor-administered animals by 1.29 ± 0.54 μM ($P < 0.05$ vs. baseline). In contrast, adenosine plasma levels in clopidogrel-treated pigs did not significantly increase vs. baseline (1.09 ± 1.05 μM; *P* = NS).

There was a trend towards higher adenosine levels in the ticagrelor-administered animals as compared with clopidogrel-treated pigs ($P = 0.09$).

3.3 Effects of ticagrelor, clopidogrel, and placebo in global anatomical and functional parameters

3.3.1 Cardiac damage

The extent of oedema at Day 3 post-MI did not differ between placebo and clopidogrel-treated pigs ($P = 0.33$; *Table 1*). However, in the ticagrelor-treated pigs, oedema was reduced by 7 g (27%) LV vs. control and by 6 g (20%) LV vs. clopidogrel ($P < 0.05$ vs. control and clopidogrel-treated pigs, respectively).

Compared to control animals, both P2Y$_{12}$ antagonists significantly and to a similar extent attenuated no-reflow (*Table 1*).

Both clopidogrel and ticagrelor significantly ($P < 0.05$) limited infarct size expansion as compared to controls at Day 3 (*Table 2*). Ticagrelor resulted in a stronger reduction as compared with clopidogrel ($P < 0.05$). A direct correlation was observed between infarct size and oedema formation (linear correlation coefficient $r = 0.725$; $P = 0.001$; *Figure 1*).

As expected, the serial CMR assessment at 42 days post-MI verified the reduction on the fibrotic scar in all animals. Ticagrelor pigs, however, consistently showed a more pronounced reduction in the size of the scar as compared to both clopidogrel-treated pigs and controls ($P < 0.05$; *Table 2*). The attenuation in scar formation by ticagrelor was highly significant and the size was below the median in clopidogrel-treated pigs. Post-mortem TTC-staining supported this observation [control: 9.5 (9.0–10.25)% LV; clopidogrel: 7.8 (7.3–8.7)% LV; and ticagrelor: 6.0 (5.5–7)% LV; $P < 0.05$ ticagrelor vs. control and clopidogrel].

3.3.2 Cardiac function

CMR functional analyses showed a higher LVEF at 3 days post-MI in ticagrelor-treated pigs that was maintained at Day 42 post-MI ($P < 0.05$ vs. control and clopidogrel groups; *Table 3*). No changes in LVEF were detected in control and clopidogrel-treated pigs over time. LV volumes (LVEDV and LVESV) significantly increased over time ($P < 0.05$ vs. Day 3 post-MI). Ticagrelor displayed a positive trend ($P = 0.06$) towards LVESV improvement vs. both control and clopidogrel-treated pigs (*Table 3*).

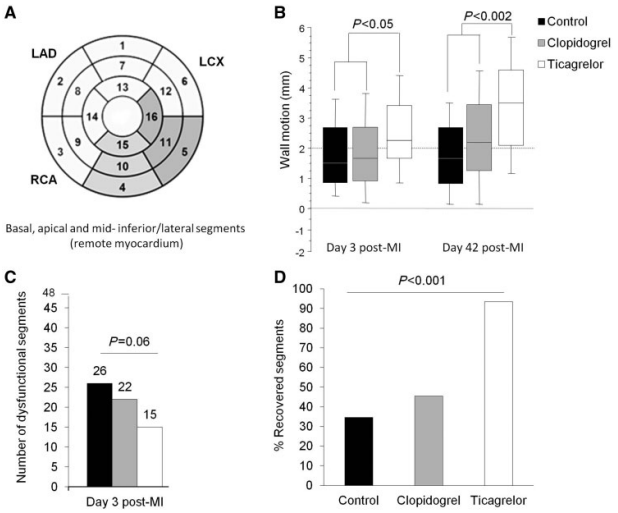

Figure 4 Regional analysis of the remote myocardium (contra-lateral to the infarcted heart) according to the 16-segment model (American Heart Association). (*A*) Segments included in the analysis are highlighted in grey (48 segments/group). (*B*) Wall motion analysis over time. (*C*) Number of dysfunctional segments (WM <2 mm). (*D*) Percentage of dysfunctional segments at Day 3 post-MI (WM <2 mm) that have been recovered (WM ≥2 mm) at Day 42 post-MI (i.e. myocardial viability). Wall motion was evaluated by the non-parametric Kruskal–Wallis test followed by the Mann–Whitney *U* test with Bonferroni correction. Results are reported as medians and IQR. The χ^2 test was performed for the regional segmental analyses of the number of dysfunctional/recovered segments. Wall motion = (end-systolic wall thickness) - (end-diastolic wall thickness); *N* = 8 animals per group.

3.4 Effects of ticagrelor, clopidogrel, and placebo on regional parameters associated with LV remodelling

Firstly, we evaluated WM abnormalities in the 16 segments (128 segments/group in total; *Figure 2A*). As shown in *Figure 2B*, the mean overall segmental WM at Day 3 post-MI was 1.7 mm in control and 1.9 mm in clopidogrel-treated pigs, whereas it was 20% higher in ticagrelor-treated pigs (2.2 mm; $P < 0.05$ vs. control and $P = 0.05$ vs. clopidogrel-treated animals). Regarding the number of dysfunctional segments, 41% of the segments ($n = 53$) were found to be dysfunctional (WM <2 mm) in the ticagrelor-treated pigs 3 days post-MI as compared with 54% ($n = 69$) and 52% ($n = 66$) in control and in clopidogrel pigs, respectively (*Figure 2C*). At Day 42 post-MI, both control and clopidogrel pigs increased their WM to 2.1 mm and 2.4 mm, respectively, whereas it increased up to 3.4 mm in the ticagrelor group ($P < 0.002$; *Figure 2B*). Overall myocardial viability was superior in the ticagrelor-treated pigs as compared with the clopidogrel and control pigs (57% vs. 41% and 46%, respectively, χ^2 $P = 0.06$; *Figure 2D*).

The target segments (i.e. jeopardized myocardium; $n = 32$ segments/group, *Figure 3A*) were severely dysfunctional (akinetic and/or dyskinetic) in control and in clopidogrel pigs (0.75 mm and 0.9 mm, respectively), whereas they were mildly dysfunctional (hypokinetic) in the ticagrelor pigs (1.85 mm; $P \leq 0.05$ vs. control and clopidogrel; *Figure 3B*). In line with these observations, the proportion of dysfunctional segments at Day 3 post-MI was 80% ($n = 26$ segments) and 72% ($n = 23$ segments) in the control and clopidogrel pigs, whereas it was 50% ($n = 16$) in the ticagrelor group (χ^2 $P < 0.05$; *Figure 3C*). All groups showed an improvement at 42 days post-MI (control: 1.4 mm; clopidogrel: 2.1 mm; ticagrelor: 2.2 mm; *Figure 3B*) and the rate of functional recovery showed no statistical differences between the three groups (*Figure 3D*).

Finally, we assessed the WM in the inferior-lateral myocardium (i.e. remote myocardium; $n = 48$ segments/group; *Figure 4A*). Although mild, WM abnormalities were detected in a higher number of segments in control (dysfunctional segments $n = 26$; WM: 1.5 mm) and clopidogrel-treated pigs (dysfunctional segments $n = 22$; WM: 1.6 mm) as compared to ticagrelor-treated pigs (dysfunctional segments $n = 15$; WM: 2.3 mm; $P < 0.05$ vs. control and clopidogrel; χ^2 $P = 0.06$; *Figure 4B* and *C*). Moreover, at 42 days post-MI, WM in the ticagrelor pigs was completely restored and even tended to be hyperkinetic ($P < 0.002$ vs. control and clopidogrel; *Figure 4B*). Myocardial viability increased up to 93% in the ticagrelor group whether such functional improvements were not detected in the remote myocardium in control and clopidogrel pigs (χ^2 $P < 0.0001$; *Figure 4B* and *D*).

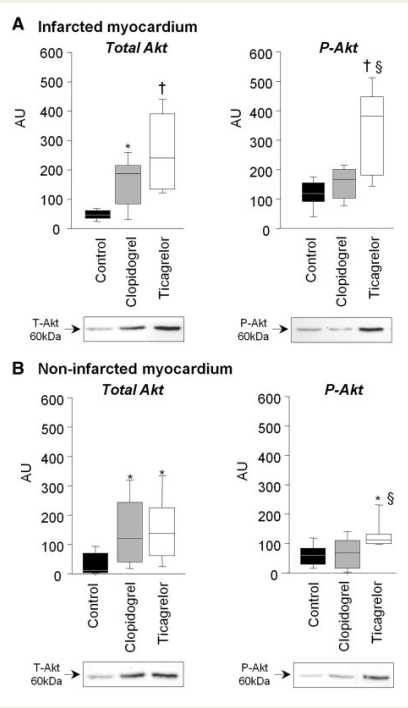

Figure 5 Expression and activation of the cardioprotective kinase Akt/PKB. (*A*) Infarcted myocardium; (*B*) Non-infarcted myocardium. *$P < 0.05$ vs. control pigs; †$P < 0.001$ vs. control pigs; §$P < 0.05$ vs. clopidogrel-treated pigs. Data were evaluated by the non-parametric Kruskal–Wallis test followed by the Mann–Whitney *U* test with Bonferroni correction. Results are reported as medians and IQR.

3.5 Molecular effects of P2Y$_{12}$ inhibition on aquaporin-4 expression and AMPK and Akt/PKB activation

Both P2Y$_{12}$ antagonists enhanced Akt/PKB protein levels in the entire myocardium (*Figure 5A* and *B*). Yet, ticagrelor-treated animals showed higher levels of activated Akt as compared to both clopidogrel and controls.

Ticagrelor-treated animals showed a reduced aquaporin-4 protein expression in the infarcted myocardium with regards to clopidogrel and placebo-treated animals (*Figure 6A* and *B*). There was an enhanced expression of activated AMPK in the entire myocardium of ticagrelor-treated pigs as compared with the control- and clopidogrel-treated groups (*Figure 6A* and *B*; $P < 0.05$).

No changes in aquaporin-4 or AMPK transcript levels were observed among the three different groups in the entire heart. As such, aquaporin-4 gene expression levels (aquaporin-4/18SrRNA) were 0.3 (0.2–0.4) in controls-, 0.3 (0.2–0.3) in clopidogrel-, and 0.2 (0.1–0.3) in ticagrelor-treated animals in the infarcted myocardium while in the non-infarcted myocardium the levels were 0.1 (0.1–0.2) in controls-, 0.3 (0.1–0.2) in clopidogrel-, and 0.3 (0.1–0.3) in ticagrelor-treated animals. As per AMPK, mRNA levels were control 1.3 (0.3–2.5), clopidogrel 1.4 (0.5–2.2), and ticagrelor 1.2 (0.6–2.3) in the infarcted region, and control 0.2 (0.2–0.3), clopidogrel 0.2 (0.2–0.3), and ticagrelor 0.3 (0.2–0.3) AMPK/18SrRNA in the non-infarcted tissue.

3.6 Molecular effects of P2Y$_{12}$ antagonists on vascular density and fibrosis

Chronic P2Y$_{12}$ inhibition had no impact on capillary density or new vessel formation in the infarcted cardiac region. No differences were observed in either lectin staining (Supplementary material online, *Figure S3A*) or angiogenic markers (Supplementary material online, *Figure S3B*).

Regarding fibrosis, all groups showed a comparable fibrotic response at a protein expression level in the infarcted cardiac region ($P < 0.05$ vs. non-infarcted; Supplementary material online, *Figure S4*).

4. Discussion

We have recently demonstrated in pigs (the best-suited animal model for the assessment of cardioprotective therapies)[28,29] and by CMR that despite both clopidogrel and ticagrelor protect against ischaemia/reperfusion (I/R) injury, ticagrelor does so to a greater extent than clopidogrel most likely as a consequence of its off-target properties.[2] In the present study, we expand this findings to the chronic setting and evidence, by the same experimental approach that administration of ticagrelor in the setting of MI and chronically thereafter, for the following 42 days improves cardiac contractility, limits scarring, and restores WM in the jeopardized myocardial segments. These beneficial effects are not seen in clopidogrel-treated pigs (Supplementary material online, *Figure S5*). Interestingly, the cardiac benefits of ticagrelor are found associated with molecular read-outs. As such, ticagrelor-treated animals have higher activated AMPK and Akt/PKB than clopidogrel-treated pigs. This association does not imply a causative effect of ticagrelor on AMPK/Akt but indicates overall cardiac healing. In the last decades, the improved management of acute MI patients has reduced morbidity and mortality in the acute phase; however, this success has paradoxically resulted in an increase in the incidence of heart failure due to adverse LV remodelling post-MI.[15] As a consequence, treatment efforts are currently focus on limiting the damage in the setting of acute ischaemic heart disease as well as on favouring LV repair post-infarction. A previous study in rats reported ticagrelor's ability to reduce scar size at 4 weeks post-MI.[8] Herein, we provide the first evidence that ticagrelor attenuates the deleterious effects of MI and favours the repair process in a clinically relevant pig model of MI. We demonstrate that the previously reported anti-oedema effects afforded by ticagrelor acutely 24 h post-MI[2] remain, and are even more evident at 3 days post-MI. Indeed, whereas ticagrelor diminished myocardial oedema formation by 24.5% at 24 h,[2] it was further reduced by 32% at 72 h supporting the anti-oedema effects of ticagrelor.

We also show that, despite both clopidogrel and ticagrelor lowered the percentage of necrotic tissue at 3 days post-MI, the effect mediated by ticagrelor was significantly greater to that of clopidogrel, effects that correlated with the amount of oedema. Although the pathophysiological

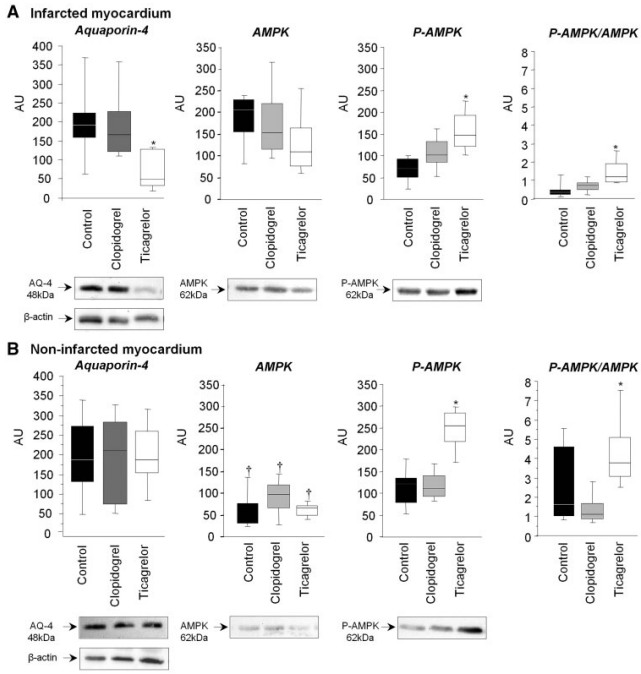

Figure 6 Expression levels of aquaporin-4, AMPK, and P-AMPK. (*A*) Infarcted myocardium. (*B*) Non-infarcted myocardium. *$P < 0.05$ vs. control and clopidogrel-treated pigs; †$P < 0.05$ vs. infarcted myocardium. Data were evaluated by the non-parametric Kruskal–Wallis test followed by the Mann–Whitney *U* test with Bonferroni correction. Results are reported as medians and IQR.

mechanisms that drive oedema formation remain to be elucidated, an oedematous reaction is known to burst upon ischaemia/reperfusion and persists for at least 1 week post-MI thereby contributing to cell injury.[30] Hence, the ability of ticagrelor to diminish oedema formation may have contributed to limit myocyte cell death, an essential feature for successful myocardial healing in the remodelling process. We must consider, however, that assessment of intracellular and/or extracellular myocardial water content would have allowed us to better determine the impact of ticagrelor over the detrimental effects of oedema on cardiac structural and functional parameters. Nevertheless, we have previously demonstrated that ticagrelor anti-oedema effects are associated with the activation of AMPK and a reduction in aquaporin-4 expression.[2] AMPK plays a critical role as a sensor to maintain cardiac energy metabolism. Yet, it also displays a pleiotropy of other cardioprotective actions including the ability to limit infarct size in the setting of ischaemia/reperfusion[31,32] and stimulate wound healing, thereby preventing structural and functional alterations post-MI.[32,33] Importantly, this kinase is activated by increase in adenosine nucleotide levels. In the present study, we evidence that animals chronically treated with ticagrelor show a persistent activation of AMPK above the levels observed in clopidogrel- or control-animals likely in response to increased adenosine availability. Chronic treatment with ticagrelor also reduces aquaporin-4 expression beyond the acute oedematous phase indicating a continuous preventive effect of ticagrelor on cardiac permeability and excess fluid reabsorption.[34] We also demonstrate in pigs the ability of ticagrelor to induce endogenous cardioprotection via Akt/PKB activation.[7,8] As such, despite both $P2Y_{12}$ antagonists enhance myocardial Akt expression only ticagrelor-treated animals show a significant increase in Akt/PKB activation. Whether it is a downstream effect related to the activation of the adenosine-receptor or AMPK signalling remains to be determined.[35] Nevertheless, all together our molecular data indicate that chronic administration of ticagrelor provides a sustained activation of protective cardiac signalling pathways that extends well-beyond the ischaemia/reperfusion period and may have contributed to restore the damaged heart. In this regard, we also report that scarring remains significantly smaller in ticagrelor-treated animals as compared to clopidogrel-treated and control pigs at 42 days post-MI.

The marked attenuation in cardiac damage detected in ticagrelor pigs may largely explain the improvement in LV function at 3 days post-MI and maintained at 42 days and the clear trend towards LVESV recovery. Importantly, ESV has been shown to be the most important predictor of mortality after acute MI[36] and LVEF, besides reflecting the size of infarction, is a strong clinical prognostic indicator for survival post-infarction.[37] No effects on cardiac function were previously reported for ticagrelor-treated animals at 24 h post-MI.[2] One can argue that chronic administration of ticagrelor may have eventually resolved cardiac dysfunction due to ischaemia (i.e. hibernating and/or stunned myocardium).[38] In line with this hypothesis, Braunwald and Kloner[38] described that the presence of oedema may prolong myocardial stunning for hours and even for few days post-MI. Importantly, beyond LVEF and volumes, regional abnormalities have also been associated with death and/or major cardiovascular events post-acute MI.[39] Here, the results presented on the 16-segment regional WM analysis supports the ability of ticagrelor to limit overall regional contractile dysfunction as compared to clopidogrel. Most interestingly, target segment analyses of the jeopardized myocardium reveals that, despite the expected ischaemia-related contractile impairment, chronic ticagrelor administration attenuates contractile dysfunction during the early phase post-MI and further restores WM and enhances myocardial viability at later stages. Interestingly, AMPK-deficient mouse hearts have shown compromised scar contractility at 4 weeks post-MI.[33]

In addition to myocardial stunning and the size of the infarct, the patency of the infarct-related artery irrigated zone (no-reflow phenomenon) also determines the cardiac repair process.[40] In this regard, the degree of microvascular obstruction was similarly diminished at 3 days post-MI by both $P2Y_{12}$ antagonists likely because their inherent antiplatelet/anti-inflammatory properties were similar by design in our experimental setting.[41] Supporting these data, $P2Y_{12}$ antagonists have shown to reduce platelet-mediated myocardial damage in the setting of ischaemia/reperfusion by attenuating platelet activation.[42]

Finally, no differential effect was observed in animals administered $P2Y_{12}$ antagonists as compared to controls with regards to vascular density, angiogenesis, and on the fibrotic response within the infarcted tissue.

There are limitations for the clinical translatability of our findings that deserve to be acknowledged. First, although we did not carry out cardiac CMR assessment before MI-induction we did evaluate each animal over time. Second, we did not treat pigs with aspirin as occurs in patients suffering from MI in order to investigate the sole effect of $P2Y_{12}$ inhibition in the infarcted myocardium. Third, both $P2Y_{12}$ antagonists were given orally to pigs prior coronary occlusion (pre-conditioning approach) instead of being administered during coronary occlusion as occurs in MI-patients. The fact that pigs need to be fully anaesthetized before experimental MI-induction does not allow us to perform this optimal therapeutic approach.

5. Conclusion

In this pig MI model, chronic administration of ticagrelor but not clopidogrel improves cardiac healing. We demonstrate that treatment with ticagrelor is associated with acute and long-term benefit on structural and functional ventricular parameters as assessed by serial CMR imaging, and is associated with persistent activation of the cardioprotective and adenosine-dependent AMPK/Akt pathway. These findings may help to gain a better understanding of the mechanisms that may have contributed to the lower morbidity/mortality at 12 months observed in ticagrelor-treated patients as compared to clopidogrel in the PLATO trial.[11]

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: L.C. is currently employed by AstraZeneca. All other authors have nothing to disclose.

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Resultados

4

Resultados

En los artículos presentados hemos estudiado y comparado, en el contexto de un IAM, los potenciales efectos cardioprotectores de dos inhibidores del receptor plaquetario $P2Y_{12}$:

- Clopidogrel: tienopiridina de segunda generación, inhibidor competitivo e irreversible del receptor $P2Y_{12}$.
- Ticagrelor: antiagregante no tienopiridínico (triazolopirimidina), inhibidor no competitivo y reversible del receptor $P2Y_{12}$.

Para ello, hemos diseñado dos estudios experimentales basados en un modelo porcino de alta traslacionabilidad clínica. En ambos estudios hemos trabajado con dosis antiplaquetarias equivalentes (igual grado de inhibición plaquetaria) para evitar que potenciales beneficios fueran atribuidos a un mayor efecto antiplaquetario de alguno de los dos fármacos.

Los resultados se han evaluado *in vivo* mediante resonancia magnética cardíaca 3T y *post mortem* a través de estudios histológicos y moleculares del tejido miocárdico. El primero de los dos estudios se centraba en los efectos inmediatos (primeras 24 h) postinfarto, mientras que el segundo estudiaba las fases precoz y tardía (3 d y 42 d postinfarto) de remodelado ventricular izquierdo adverso.

4.1
Resultados
Efectos inmediatos

4.1.1 Resonancia magnética

Los estudios de RMC permitieron determinar cómo, en comparación con placebo, tanto clopidogrel como ticagrelor redujeron el tamaño del infarto. Sin embargo, ticagrelor lo hizo en mayor grado que clopidogrel obteniendo una reducción relativa de la necrosis postinfarto de un 23,5 % (diferencia absoluta de 3,7 g de masa necrótica). El tamaño de tejido necrótico presentó una relación directa con los niveles plasmáticos de troponina I (coeficiente de correlación lineal $R = 0{,}98$). En consecuencia, el tratamiento con ticagrelor se asoció a menor elevación de troponina I plasmática.

	Control (placebo)	**Clopidogrel**	**Ticagrelor**
Necrosis (g)	22,8 (17,3–25,8)	15,7 (14,2–16,2)	12,0 (10,6–12,9)
% necrosis (masa VI)	31,1 (25,9–39,1)	20,9 (19,3–22,8)	16,4 (15,5–17,9)
Troponina I	19 (16,5–21,7)	13,4 (13,0–14,0)	10,9 (11,3–9,4)

Tabla 4. Tamaño de infarto medido por RM y de forma subrogada a través de la determinación de troponina I.

	Control (placebo)	Clopidogrel	Ticagrelor
Edema (g)	23,4 (20,9–31,1)	21,6 (19,5–25,2)	16,3 (14,2–19,9)
% necrosis (masa VI)	36,2 (33,9–43,2)	30,1 (26,6–34,5)	23,1 (20,2–24,4)

Tabla 5. Tamaño de edema medido por RM.

El volumen de edema miocárdico también fue menor en los animales tratados con ticagrelor respecto a los tratados con placebo o clopidogrel (disminución relativa frente a placebo de un 24,5 %). Es interesante destacar que el tratamiento con clopidogrel no condicionó una disminución del edema miocárdico. Es decir, sólo ticagrelor se asoció a una disminución estadísticamente significativa en el grado de edema postinfarto.

Además, los efectos positivos observados tras el tratamiento con ticagrelor fueron bloqueados por la administración de un antagonista de los receptores de adenosina (8-p-sulfofenil-teofilina, 8SPT), hallazgo que sugiere que los efectos cardioprotectores de ticagrelor están mediados por adenosina.

	Ticagrelor	Ticagrelor + 8SPT
Necrosis (g)	12,0 (10,6–12,9)	14,9 (14,6–16,1)
% necrosis (masa VI)	16,4 (15,5–17,9)	22,4 (21,8–23,9)
Edema (g)	16,3 (14,2–19,9)	24,6 (22,8–25,3)
% edema (masa VI)	23,1 (20,2–24,4)	36,8 (33,6–39,4)

Tabla 6. Valores de infarto (necrosis) y edema en los animales tratados con ticagrelor sin y con administración de 8SPT.

4.1.2 Análisis moleculares

Los análisis moleculares del tejido miocárdico *post mortem* reforzaron los resultados obtenidos en los estudios de RMC (*in vivo*).

El menor volumen de edema detectado por RMC, se tradujo a nivel molecular con una menor expresión del gen y proteína acuaporina 4 en los animales tratados con ticagrelor.

En este grupo también se objetivó una mayor activación de la proteína AMPK (forma activa = AMPKp) con mayores *ratios* AMPKp/AMPK. De igual forma, se detectó mayor expresión de eNOS y CD36, dos moléculas implicadas en la cascada metabólica desencadenada por AMPKp.

Finalmente, ticagrelor también se asoció con mayor actividad de la enzima cicloxigenasa 2 (Cox2) y 6-ceto-prostanglandina F1a (metabolito

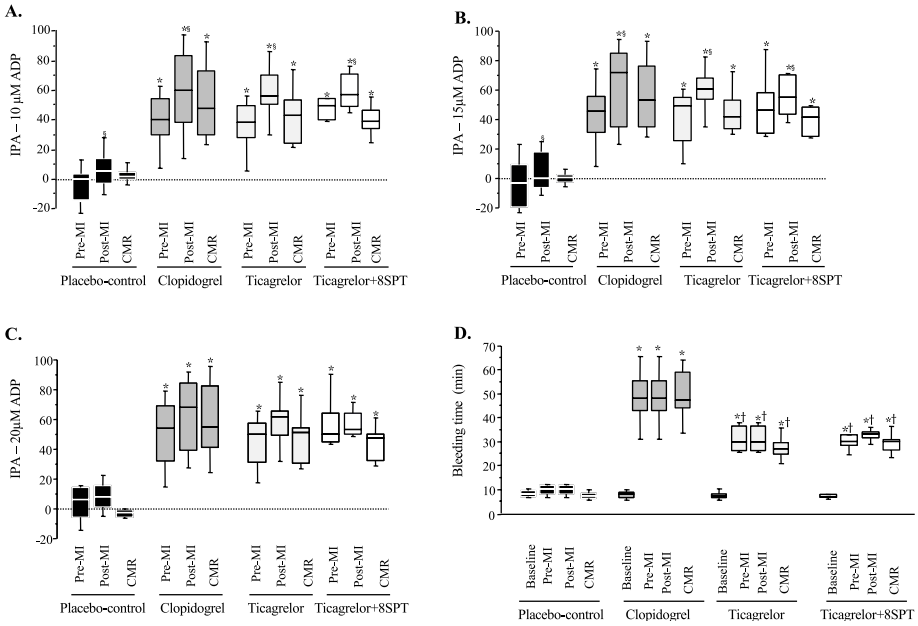


Figura 7. Los gráficos A, B y C ilustran el grado de inhibición de agregación plaquetaria (IPA) con concentraciones crecientes de ADP (10, 15 y 20 µmol/L respectivamente). Tanto clopidogrel como ticagrelor ejercieron una inhibición alta y constante de la función plaquetaria (IPA en grupo control: 5,6 %, 0,0 %, 8,4 %; clopidogrel: 60,0 %, 71,9 %, 68,2 %; ticagrelor: 56,2 %, 60,5 %, 62,2 %; ticagrelor + 8SPT: 53,5 %, 54,5 %, 52,0 %, para 10, 15 y 20 µmol/L ADP, respectivamente). Este efecto inhibitorio persistió hasta el momento de realizar la CMR (24 post IM). La administración de 8SPT no influyó en el alto efecto inhibidor de plaquetas ejercido por ticagrelor. El gráfico D ilustra el tiempo de sangrado. Tanto clopidogrel como ticagrelor aumentaron el tiempo de sangrado en comparación con el placebo. Sin embargo, a pesar de un grado similar de inhibición plaquetaria, clopidogrel lo aumentó casi el doble que ticagrelor ± 8SPT.

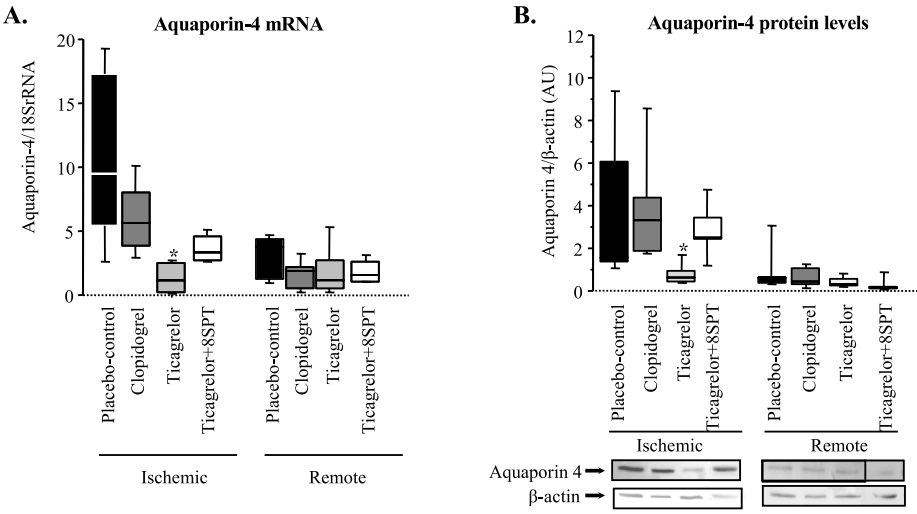


Figura 8. *Box plot* representando los niveles de RNA mensajero (A) y expresión de la proteína acuaporina 4 (B) en los diferentes grupos. Los animales tratados con ticagrelor mostraron una reducción significativa tanto en la expresión del gen como de la proteína.

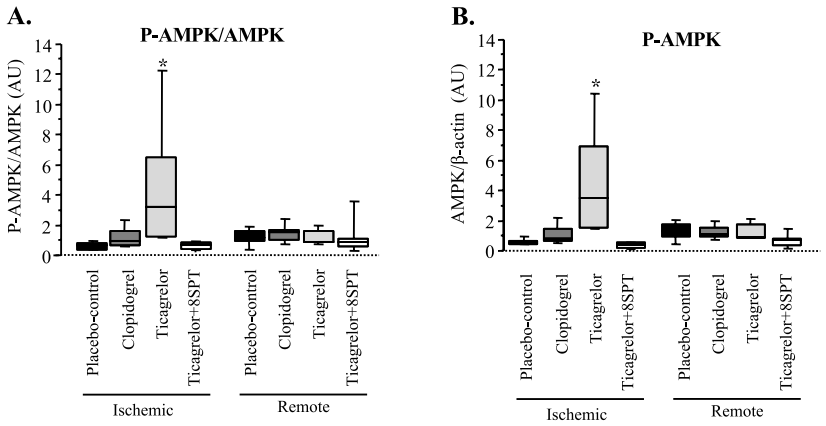
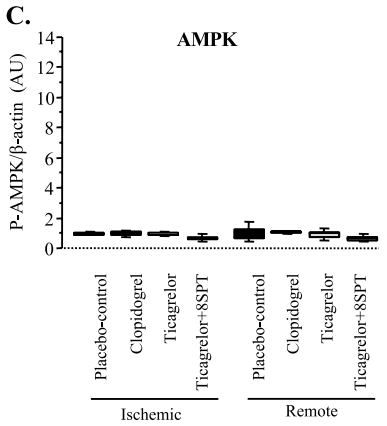


Figura 9. Tres gráficos *box plot* que ilustran la relación entre p-AMPK/AMPK (activa/inactiva), y los niveles de p-AMPK (activa) y AMPK (inactiva) en los diferentes grupos. Únicamente el tratamiento con ticagrelor indujo una activación significativa de la vía de señalización de AMPK en el miocardio isquémico.



estable de la prostaciclina), molécula con efectos antiagregantes y vasodilatadores.

Al igual que en los estudios de RMC, todos los resultados favorables a ticagrelor fueron revertidos por el inhibidor del receptor de adenosina (8SPT).

Las conclusiones en las primeras 24 h postinfarto son las siguientes:

- Ticagrelor redujo el daño miocárdico evaluado por RMC en mayor grado que clopidogrel.
  - Redujo el edema postinfarto en comparación con clopidogrel.
  - Redujo la necrosis postinfarto en mayor medida que clopidogrel.
- Ticagrelor (pero no clopidogrel) se asoció a vías moleculares mediadas por adenosina que reducen la formación de edema miocárdico.
  - Redujo la expresión de acuaporina 4, proteína con un papel clave en la formación de edema intramiocárdico (regula el paso del agua a través de la membrana celular).
  - Aumentó la activación de proteína cinasa activada por AMP (AMPK), proteína relacionada con una menor permeabilidad vascular y menor edema extracelular.

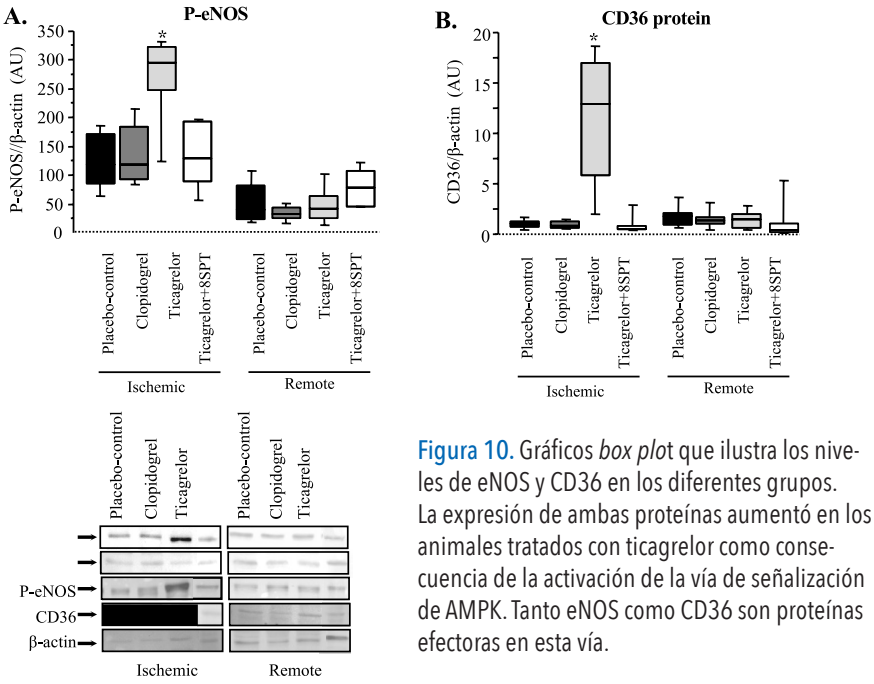


Figura 10. Gráficos *box plot* que ilustra los niveles de eNOS y CD36 en los diferentes grupos. La expresión de ambas proteínas aumentó en los animales tratados con ticagrelor como consecuencia de la activación de la vía de señalización de AMPK. Tanto eNOS como CD36 son proteínas efectoras en esta vía.

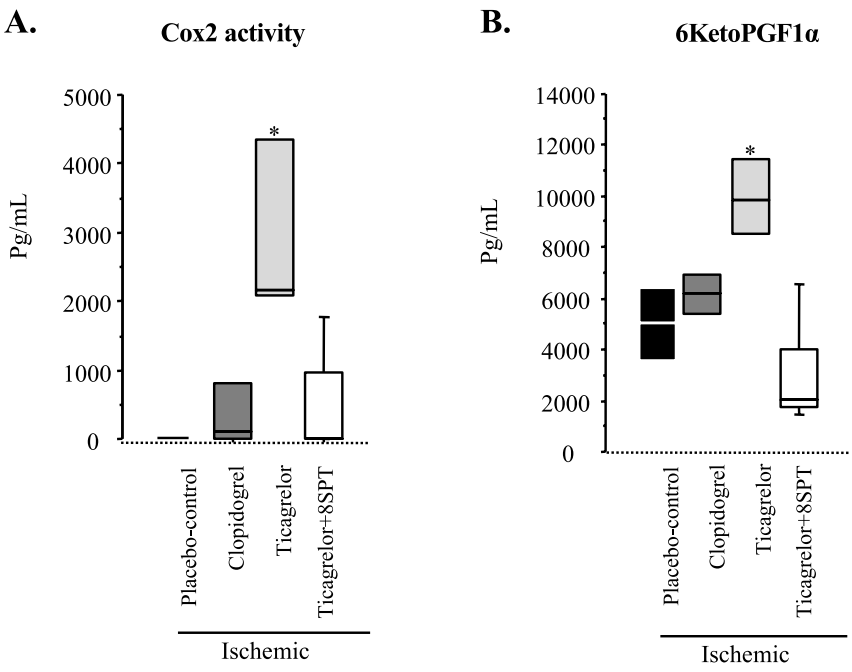


Figura 11. Gráficos *box plot* representando la concentración de enzima Cox2 y el metabolito activo de la prostaglandina (6KetoPGF1α). La activación de la enzima eNOS es esencial para la activación de Cox2 y la subsiguiente producción de prostaglandina, molécula con propiedades antiagregantes y vasodilatadoras.

4.2
Resultados
Fases precoz y tardía (3 y 42 días postinfarto)

4.2.1 Daño miocárdico

Los estudios de RM en fase precoz se alinearon con los estudios realizados en las primeras 24 h postinfarto. Tanto el edema como la necrosis fueron menores en los animales tratados con ticagrelor en comparación con clopidogrel y los animales placebo-control. Del mismo modo, clopidogrel consiguió disminuir el tamaño de infarto respecto al grupo control, pero en menor medida que ticagrelor, y no modificó la formación de edema postinfarto.

4.2.2 Función cardíaca global

En comparación con los grupos control y clopidogrel, la FEVI de los animales tratados con ticagrelor fue mejor a los días 3 y 42 d.

	Control (placebo)	Clopidogrel	Ticagrelor
FEVI 3 d (%)	44,8 (43,4–47,8)	50,6 (44,9–55,3)	53.3 (49,3–57,3)*
FEVI 42 d (%)	48,2 (45,4–50,2)	47,2 44,2–51,2)	54,5 (52,2–58,0)**

Tabla 7. FEVI a los tres y cuarenta y dos días después del infarto.

* Un 15 % y 5 % superior respecto a placebo y clopidogrel.
** Un 12 % y 13 % superior respecto a placebo y clopidogrel.

4.2.3 Función cardíaca segmentaria

Para evaluar la función cardíaca segmentaria se calculó, utilizando un software dedicado, la motilidad parietal. Ésta se definió como la diferencia en mm entre el grosor miocárdico en sístole y el grosor miocárdico en diástole (grosor sist. – grosor diast.). Además, se definieron los conceptos de segmentos disfuncionales y segmentos viables.

- Segmentos disfuncionales: aquellos con una motilidad parietal < 2mm.
- Segmentos viables: segmentos disfuncionales a día 3 que dejan de serlo a día 42.

El grupo tratado con ticagrelor evidenció una mayor motilidad parietal media que los otros dos grupos, menor número de segmentos disfuncionales en la fase precoz y mayor número de segmentos viables en la fase tardía.

Al considerar únicamente el área en riesgo, el grado de disfunción regional fue mucho menor en el grupo tratado con ticagrelor que en los otros dos.

Motilidad parietal media	Control (placebo)	Clopidogrel	Ticagrelor
Precoz (3 d)	1,7 mm	1,9 mm	2,2 mm*
Tardía (42 d)	2,1 mm	2,4 mm	3,4 mm
Segmentos disfuncionales 3 d	54 %	52 %	41 %
Segmentos viables 42 d	41 %	46 %	57 %

Tabla 8. Valoración de la motilidad segmentaria medida a través de: motilidad parietal media de todos los segmentos, % de segmentos disfuncionales a los 3 d (engrosamiento parietal <2 mm) y % de segmentos viables a los 42 d (eran disfuncionales, pero se han recuperado).
* Aumento relativo de un 23 % y 14 % respecto a placebo y clopidogrel respectivamente).

	Control (placebo)	Clopidogrel	Ticagrelor
Motilidad área en riesgo (3 d)	0,75 mm	0,9 mm	1,85 mm
Segmentos disfuncionales	80 %	72 %	50 %

Tabla 9. Motilidad segmentaria media del área en riesgo y % de segmentos disfuncionales.

Aunque de forma leve, también se identificaron alteraciones de la motilidad segmentaria el miocardio remoto (pared inferolateral). Al igual que en el resto de regiones, éstas fueron menores en el grupo tratado con ticagrelor.

	Control (placebo)	Clopidogrel	Ticagrelor
Motilidad miocardio remoto	1,5 mm	1,6 mm	2,3 mm
Segmentos disfuncionales	26 %	22 %	15 %

Tabla 10. Motilidad segmentaria media del miocardio remoto y % de segmentos disfuncionales.

4.2.4 Dilatación ventricular

Aunque los resultados no fueron estadísticamente significativos (p = 0,06), ticagrelor mostró una clara tendencia positiva en referencia al volumen telesistólico (menor dilatación en comparación con placebo y clopidogrel).

	Volumen telesistólico (ml)	
	3 d	42 d
Control (placebo)	43,6	60,9
Clopidogrel	44,7	66,6
Ticagrelor	36,7	55,1

Tabla 11. VTSVI a los 3 d y 42 d en los diferentes grupos.

4.2.5 Adenosina plasmática

Se determinaron los niveles de adenosina plasmática antes de instaurar la isquemia miocárdica y durante la isquemia, inmediatamente antes de la reperfusión. Solo los animales tratados con ticagrelor mostraron mayores niveles de adenosina plasmática durante la isquemia ($p < 0,05$).

4.2.6 Proteínas implicadas en el edema miocárdico

En línea con las observaciones a 24 h postinfarto (objetivo 1), en los cerdos tratados con ticagrelor los niveles de acuaporina 4 fueron menores y los niveles de AMPK y AMPKp mayores en comparación con clopidogrel y control.

La expresión de la enzima AKT/PKB, implicada en la supervivencia celular, aumentó en los grupos tratados con clopidogrel y ticagrelor. Sin embargo, únicamente ticagrelor se asoció con un aumento de la forma activa de la enzima (p-AKT).

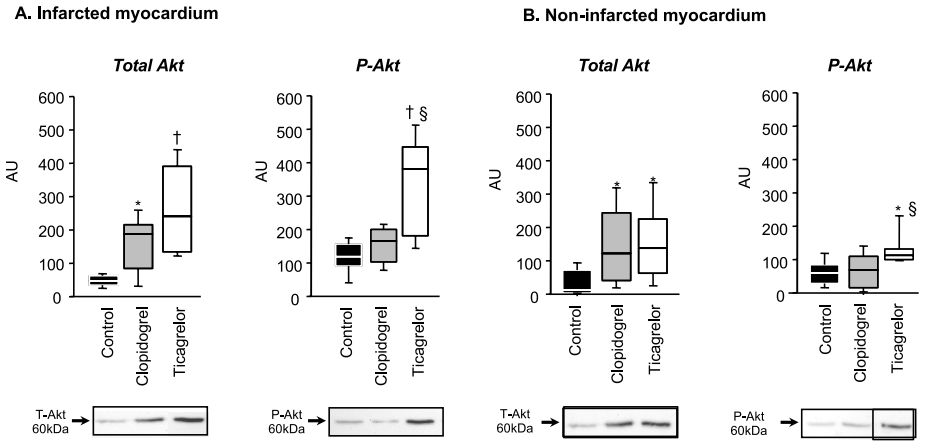


Figura 11. Gráficos *box plot* que ilustran los niveles de proteína Akt y Akt fosforilada (activa) en el miocardio infartado y miocardio remoto. Aunque tanto clopidogrel como ticagrelor mejoraron los niveles de proteína Akt/PKB en todo el miocardio (infartado y remoto), los animales tratados con ticagrelor mostraron niveles más altos de Akt-activada.

En conclusión, los beneficios inmediatos detectados sobre el daño y edema miocárdicos también se tradujeron en beneficios en parámetros de

remodelado ventricular en las fases precoz (3 d postinfarto) y tardía (42 d postinfarto):

- Menor daño miocárdico (necrosis, edema).
- Mejor función cardíaca global.
  - Mayor FEVI en fases precoz y tardía.
- Mejor función cardíaca segmentaria:
  - Menor alteración de la motilidad segmentaria en el área en riesgo.
- Mayor viabilidad de los segmentos infartados.
  - Definida como segmentos disfuncionales en fase precoz que se recuperan en la fase tardía.

A nivel molecular, estos efectos se relacionaron con:

- Mayores niveles de adenosina plasmática.
- Menor expresión de acuaporina 4.
- Mayor expresión de AMPK y AKT/ PKB (proteína-quinasa con múltiples implicaciones intracelulares relacionadas con la supervivencia celular).

4.3
Resultados
Resonancia magnética cardíaca en el modelo porcino

Si bien el protocolo de estudio de resonancia magnética cardíaca en humanos está estandarizado y es fácil de reproducir, la implementación en animales puede ser problemática y requiere de una serie de adaptaciones necesarias para la obtención de imágenes de calidad suficiente.

En el caso concreto de nuestro estudio nos encontramos con tres dificultades fundamentales.

La primera era debida a la diferencia en el campo magnético de la máquina de resonancia utilizada en los estudios animales (3 T) en relación con las máquinas utilizadas para el estudio en humanos en nuestro centro (1,5 T). Una mayor intensidad del campo magnético permite obtener imágenes con una mejor relación señal/ruido (mejor resolución espacial) pero, como contrapartida, es mucho más susceptible a artefactos que degradan la calidad final de las imágenes obtenidas (70).

En nuestro caso particular, los artefactos con mayor impacto sobre las imágenes y más difíciles de eliminar han sido los artefactos de flujo en las secuencias b-SSFP. Estos se caracterizan por la aparición de bandas heterogéneas que degradan la imagen y son debidos a la introducción de inhomogeneidades en el campo magnético por el flujo circulante de sangre (71). Siempre intentamos reducirlos garantizando la máxima homogeneidad de campo en la región a estudiar («*shimming*» antes de las secuencias de cine) y acortando el tiempo T1 lo máximo posible. Sin embargo, en algunos casos fue necesario cambiar la secuencia por otra «*spoiled Gra-*

dient Echo» con una resolución ligeramente menor, pero menos sensible a estos artefactos.

Otro aspecto problemático fue la dificultad para obtener un registro electrocardiográfico fiable en los estudios preliminares. Este hecho era especialmente evidente en los animales que acudían a la instalación de RM con frecuencias cardíacas más elevadas (> 120 lpm) y dificultaba en gran medida la adquisición de las imágenes. Para solventar este apartado fue necesario revisar el régimen anestésico inicialmente planeado (para asegurar un menor ascenso de la frecuencia cardíaca) y adquirir un sistema de sensores nuevo que garantizara una correcta adhesión a la piel de los animales.

Los posibles artefactos de movimiento fueron fáciles de evitar dado que los animales eran traídos sedados e intubados, por lo que podíamos controlar de forma precisa los periodos de apnea.

Finalmente, se modificó el protocolo de adquisición de las secuencias STIR, realce precoz y realce tardío para garantizar una adquisición que permitiera calcular el volumen de edema, MVO y necrosis de la forma más precisa posible.

Discusión y conclusiones

5

Discusión

Desde su introducción, hace ya casi dos décadas, el doble tratamiento antiplaquetario con ácido acetilsalicílico (inhibidor de la cicloxigenasa) y clopidogrel (inhibidor del receptor plaquetario $P2Y_{12}$) ha sido el pilar fundamental del tratamiento médico de los pacientes que han sufrido un SCA y van a ser sometidos a intervencionismo coronario (76). A pesar de su efectividad para reducir los eventos coronarios adversos, en este tiempo se han objetivado una serie de inconvenientes clínicamente relevantes de clopidogrel, siendo los dos más importantes: 1) Inicio de acción lento en el contexto agudo. 2) Gran variabilidad individual, existiendo un porcentaje no despreciable de pacientes con escasa respuesta a clopidogrel.

En este contexto diversos estudios han demostrado que ticagrelor superaba gran parte de las limitaciones de clopidogrel, produciendo un efecto antiplaquetario mayor y más rápido (53). Este mejor perfil antiplaquetario se traduce en una menor mortalidad y menor tasa de eventos cardiovasculares en pacientes que habían sufrido un SCA (54). Además, el efecto antiplaquetario desaparece más rápido tras la discontinuación del tratamiento con ticagrelor que con clopidogrel, hecho de gran relevancia en caso de necesitar interrumpir el tratamiento (por ejemplo, requerimiento de cirugía urgente).

La superioridad de ticagrelor frente a clopidogrel se fundamentaría en tres pilares (77): 1) ticagrelor no necesita ser metabolizado para ser activo (a diferencia de clopidogrel). 2) el principal metabolito de ticagrelor también es activo (AR-C124910XX). 3) se trata de un inhibidor reversible del

receptor $P2Y_{12}$ (justifica una desaparición de acción más rápida y también la necesidad de administrar una dosis cada 12 h en lugar de cada 24 h).

Sin embargo, una segunda hipótesis defiende que el factor diferencial de ticagrelor frente a las tienopiridinas es su capacidad para aumentar la biodisponibilidad de adenosina, principalmente vía inhibición del receptor ENT-1 e inducción de liberación de ATP por los eritrocitos.

En los estudios presentados en esta tesis doctoral hemos demostrado, en un modelo preclínico porcino de IAM, un mayor efecto cardioprotector de ticagrelor frente a clopidogrel. Si bien ambos fármacos incrementan la viabilidad miocárdica, ticagrelor limita en mayor medida el daño miocárdico (tamaño de infarto y edema) con dosis antiplaquetarias equivalentes. Estos efectos, que se objetiva en las primeras 24 h postinfarto, se traducen en la fase crónica en una mejor motilidad cardíaca, mejores parámetros funcionales y menores índices de remodelado ventricular adverso. Además, hemos demostrado que estos efectos cardioprotectores son independientes de la función antiplaquetaria y que están mediados por adenosina.

Nuestro estudio inicial pone de manifiesto que ticagrelor se asocia a menor edema y necrosis miocárdicas *in vivo* (evaluados mediante RMC) y que estos resultados se asocian a nivel molecular con menor expresión de acuaporina-4 y mayor expresión y activación de AMPK, proteínas clave en el desarrollo del edema miocárdico.

Acuaporina 4 es un canal transmembrana implicado en el transporte de agua que demostró ser crítico en el desarrollo de edema cerebral (78). Estudios animales posteriores confirmaron su implicación en el edema miocárdico post isquemia y su asociación con una mayor necrosis y disfunción miocárdicas (79, 80). Estudios más recientes han sugerido dos asociaciones clave: 1) Adenosina es capaz de regular la expresión de acuaporina 4 (81). 2) En ausencia del gen ENT-1 hay escasa expresión de acuaporina (82). Estos dos hechos nos sugieren que adenosina regula la expresión de acuaporina 4 inhibiendo el receptor ENT-1.

Por otro lado, adenosina es una de las moléculas capaces de inducir la activación de AMPK (83). AMPK, recientemente ha demostrado estimular la formación de uniones estrechas entre células endoteliales, lo que evita el aumento de la permeabilidad vascular durante el evento isquémico (84). Este hecho nos permite hipotetizar que disminuye la formación de edema miocárdico al disminuir el paso de agua y leucocitos al intersticio cardía-

co. Es importante recordar que, aparte de su papel «anti edema», la enzima AMPK juega un papel fundamental en la regulación del metabolismo energético y la supervivencia celular en condiciones de isquemia (85). Al cesar el aporte de oxígeno disminuye la producción de ATP y aumenta el AMP. La enzima AMPK se activa (fosforila), lo que a su vez estimula una serie de mecanismos dirigidos a mantener la viabilidad/supervivencia celular: 1) aumento de la captación de glucosa, 2) aumento de la glicólisis (metabolismo anaerobio de la glucosa), 3) captación y oxidación de ácidos grasos; y 4) autofagia celular (reabsorción de proteínas y orgánulos dañados).

En concordancia con la activación de AMPK, hemos objetivado mayor expresión de eNOS y CD36 (transporta ácidos grasos de cadena larga al interior de la célula), proteínas implicadas en las vías efectoras de AMPK. La enzima eNOS y promueve la formación de NO. El NO producido en el endotelio vascular ejerce un efecto vasodilatador y antiplaquetario en la microcirculación coronaria (83). A su vez, eNOS es esencial para la activación de la enzima COX2 y consiguiente formación de prostaciclina. La prostaciclina es una molécula con efecto vasodilatador, antiplaquetario y antiinflamatorio. En nuestro estudio demostramos mayores niveles de su metabolito activo (6-ceto-prostanglandina F1a) en los animales tratados con ticagrelor.

Es importante destacar que los efectos de ticagrelor quedaron anulados tras la administración del antagonista de adenosina 8SPT. Hecho que nos ha permitido inferir que los mecanismos cardioprotectores activados por ticagrelor están mediados por adenosina. En este sentido, un estudio reciente sobre SCA demostró que las concentraciones plasmáticas de adenosina eran significativamente mayores en los pacientes tratados con ticagrelor que los tratados con clopidogrel (86).

Los resultados de nuestro segundo estudio, dirigido a comprobar si los efectos beneficiosos de tricagrelor observados en la fase aguda postinfarto persistían o eran transitorios, confirman y refuerzan que el efecto cardioprotector de ticagrelor es mayor que el de clopidogrel.

En el ámbito molecular, se mantienen el incremento en la expresión y activación de AMPK así como la reducción en acuaporina 4 y se detecta una mayor activación de la proteína AKT/PKB. Esta enzima representa un nexo común de abundantes vías intracelulares con importantes implicaciones en la supervivencia, proliferación y metabolismo celular (87).

Una mayor activación de AKT/PKB pone de manifiesto una mayor activación de vías de supervivencia celular. En conjunto, los datos moleculares/celulares indican una mayor resistencia miocárdica durante el insulto isquémico en los animales tratados con ticagrelor en comparación con los tratados con clopidogrel.

Los análisis anatómicos confirman la disminución del tamaño de la cicatriz a largo plazo (42 d) en los animales tratados con inhibidores del receptor $P2Y_{12}$ respecto al grupo control. Al igual que en las primeras 24 h postinfarto, esta disminución es mayor en los animales tratados con ticagrelor que con clopidogrel. Así mismo, el edema detectado por RMC a los 3 d postinfarto mantiene el mismo patrón que en el primer día: notable disminución del grado de edema en los animales tratados con ticagrelor en comparación con los tratados con clopidogrel o placebo.

En cuanto a la función miocárdica, los animales tratados con ticagrelor presentaron mejores parámetros de contractilidad global (FEVI) y segmentaria (menos segmentos disfuncionales y más segmentos viables). Además, se observó una tendencia positiva en el volumen ventricular telesistólico que sugería menor dilatación en fase crónica. Estos hallazgos concuerdan con el menor daño miocárdico observado tanto en los estudios por imagen como en los análisis moleculares. Además de la menor necrosis, es importante reseñar el efecto beneficioso de un menor edema miocárdico, el cual ha demostrado influir negativamente en la función cardíaca global y segmentaria postinfarto (88, 89).

Si bien ticagrelor ya había demostrado disminuir el tamaño de infarto, medido por histopatología, y mejorar la función cardíaca (vía adenosina) en modelos animales con ratas (90, 91), la anatomía y fisiología cardíacas en roedores es muy diferente a la humana. Como consecuencia los resultados eran escasamente extrapolables. Nuestros estudios confirman, por primera vez, las ventajas de ticagrelor en un modelo anatómico y fisiológico mucho más próximo al humano, usando de técnicas de imagen *gold estándar* para el análisis de la estructura/función cardíaca.

Sin embargo, a pesar de lo prometedor de nuestros resultados es necesario discutir una serie de condicionantes que podrían interferir en la práctica clínica.

Primero, para asegurar que únicamente se evaluaban los efectos de los inhibidores del receptor $P2Y_{12}$ ninguno de nuestros animales ha sido tra-

tado con AAS. En este sentido cabe comentar la interacción contraproducente que altas dosis de AAS ejercen sobre los beneficios de ticagrelor. En el estudio PLATO los resultados del tratamiento con ticagrelor fueron peores en Norte América que en otras regiones (54). Un análisis *post hoc* del estudio concluyó que esta pérdida de eficacia podía ser secundaria al hecho que en Norteamérica había más pacientes tratados con dosis altas de AAS que en otras regiones (92). La hipótesis para justificar esta discrepancia se encuentra en la inhibición, dosis dependiente, que el AAS ejerce sobre la enzima COX2. Dosis altas inhiben la enzima y por consiguiente la producción de prostanglandina. Sin embargo, dosis menores no inhiben la COX2. Dado que los efectos protectores de ticagrelor se encuentran mediados, al menos parcialmente, por el aumento de prostanglandina, estos efectos no se producen si se inhibe su producción.

Así mismo, dada la importancia de adenosina en la protección otorgada por ticagrelor, cabría esperar que los estudios clínicos estudiando la infusión directa de adenosina fueran igualmente prometedores. Sin embargo, los resultados son dispares. Si bien dosis altas de adenosina intravenosa se han asociado a menor tamaño de infarto en algunos estudios (93), en otros con más pacientes no han reportado beneficios (94). A pesar de estos resultados algo decepcionantes es acertado comentar que probablemente no es lo mismo la infusión directa de adenosina, molécula con una media de muy corta duración (segundos) al ser rápidamente captada por los eritrocitos vía receptor ENT-1, que un aumento local persistente de adenosina en el corazón infartado debido al bloqueo de este receptor.

Por último, destacar la dificultad de los modelos preclínicos para reproducir el modelo fisiopatológico humano en el que influyen múltiples factores como una edad avanzada, la presencia de comorbilidades, interacciones farmacológicas e incluso episodios previos de angina que ya pueden haber desencadenado fenómenos de condicionamiento (12). Si bien esta condición seguro que limita la traslacionabilidad de nuestros estudios, este es un hecho compartido por todos los diseños experimentales con animales.

6

Conclusión

Las conclusiones de esta tesis se resumen en los siguientes puntos:

1. Ticagrelor, independientemente de su acción antiplaquetar, presenta beneficios cardioprotectores únicos no obtenidos con el tratamiento con clopidogrel.
2. Esta protección se pone de manifiesto en las fases aguda (primeras 24h) y crónica (3d y 42d) postinfarto y se traduce en menor edema, menor necrosis y mejor función venticular en los estudios de RMC.
3. Los estudios moleculares sustentan los hallazgos de los estudios de imagen (menor expresión de acuaporina 4 y mayor expresión/activación de AMPK, akt/PKB y prostaciclina).
4. La cardioprotección asociada a ticagrelor está mediada por adenosina.

7

Líneas de futuro

Las técnicas de reperfusión coronaria han condicionado una disminución considerable en la mortalidad en la fase aguda después de un IAM, con la contrapartida de un aumento en la morbilidad a largo plazo de los supervivientes (principalmente en forma de insuficiencia cardíaca crónica). Además, es importante remarcar que a pesar de los importantes avances que se han producido en el tratamiento del SCACEST se ha alcanzado una meseta en los niveles de mortalidad aguda post infarto.

La explicación a estos dos aspectos (evolución a insuficiencia cardíaca crónica y estabilización en los niveles de mortalidad aguda) la encontramos en el fenómeno de remodelado ventricular. Éste se produce como consecuencia del daño conjunto de la isquemia y la reperfusión posterior del área infartada y es determinante en la pérdida de función ventricular y evolución hacia insuficiencia cardíaca. Si bien el daño por isquemia ha disminuido considerablemente gracias a la excelencia alcanzada en las técnicas de revascularización miocárdica, el reto actual consiste en disminuir el daño por reperfusión.

Desde el descubrimiento de los fenómenos de condicionamiento isquémico se sabe que existen mecanismos endógenos que son capaces de mejorar la supervivencia de los miocardiocitos al estrés oxidativo desencadenado por la reperfusión. Por lo tanto, en el futuro el desarrollo de nuevas terapias farmacológicas que sean capaces de replicar el fenómeno de condicionamiento isquémico van a ser esenciales para disminuir, aún más, la mortalidad por IAM y la evolución a insuficiencia cardíaca.

En este sentido, uno de los principales retos es conseguir un nivel suficiente de traslacionabilidad clínica desde los estudios preclínicos (celulares, animales) al modelo humano. A pesar de los resultados prometedores de múltiples fármacos en investigación básica, con frecuencia los éxitos de estas intervenciones no se han reproducido en los estudios clínicos en humanos. Las razones de éste fracaso son múltiples. Por un lado, mientras los estudios pre-clínicos se realizan en animales jóvenes y sanos, los humanos con cardiopatía isquémica con frecuencia son de edad avanzada y presentan otras patologías, factores de riesgo y potenciales interacciones farmacológicas debido a la existencia de co-medicaciones. Además, en los pacientes que han sufrido episodios previos de angina, este hecho podría haber desencadenado fenómenos de condicionamiento isquémico por lo que el efecto de las terapias farmacológicas sería menor.

Por lo tanto, el gran reto para los próximos años es garantizar el mayor grado de traslacionalidad clínica y, así, minimizar el fracaso de las expectativas generadas en estudios preclínicos. Para ello es recomendable seguir una serie de recomendaciones:

- Iniciar los estudios con animales de pequeño tamaño para luego confirmar los resultados en animales de mayor tamaño, con una anatomía y fisiología más similares a los humanos
- Iniciar estudios en humanos sólo después de haber obtenido unos beneficios claros y robustos en los estudios con animales.
- Definir objetivos que se acepten como marcadores surrogados de objetivos duros en la práctica clínica (ej. Tamaño del infarto como marcador de riesgo de mortalidad y desarrollo de insuficiencia cardíaca). Cuanta mayor sea la asociación entre el objetivo surrogado y el duro mejor será la reproducibilidad clínica.

Es decir, para garantizar el éxito futuro de estudios clínicos fase III es fundamental: 1) ser rigurosos con el diseño de los estudios animales, utilizando objetivos clínicamente relevantes y 2) escalar únicamente los que presentan resultados inequívocamente beneficiosos.

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Anexos

Anexo 1
Supplementary Material
Protective effects of ticagrelor on myocardial injury after infarction

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Supplemental Methods

The study protocol was approved by the institutional ethics committee (CSIC-ICCC) and all animal procedures were carried out in strict accordance to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the National Institute of Health (NIH) guidelines" (NIH Publication No.85-23, revised 1996). In addition, we have followed the ARRIVE guidelines and committed to the 3Rs of laboratory animal research.(1) Supplemental Figure 1 includes a diagram depicting the entire experimental protocol.

Materials

Ticagrelor was provided by AstraZeneca and clopidogrel was purchased from Bristol-Myers Squibb/Sanofi Pharmaceuticals. 8-(p-sulphophenyl)-theophylline (8SPT), considered a nonselective adenosine receptor antagonist, was purchased from Sigma. ELISA kit for high-sensitivity pig cardiac troponin I was from Life Diagnostics and for 6-keto-PGF1α, arachidonic acid, SC560 and SC58125 was from Cayman Chemicals. Anti-aquaporin 4, anti-Cox2, and anti-CD36 were purchased to Abcam. Anti-Ser^{1177} phospho-eNOS, anti-AMPK and anti-Thr172 phos-

phoAMPK were from Cell signaling. All the reagents for conducting light transmittance aggregometry (LTA) analysis were purchased from Menarini Diagnostica.

Experimental design

Regular chow-fed Landrace pigs (weight ≈ 45 kg; Specipig SL, Spain) were allocated to randomly and blindly receive before MI-induction: I) placebo-control (n = 9); II) loading dose of clopidogrel (600 mg; n = 8); III) a loading dose of ticagrelor (180 mg; n = 8); and IV) a loading dose of ticagrelor followed by an intravenous infusion of an A1/A2-receptor antagonist (8-p-sulfophenyl-theophylline; 8SPT; 4 mg/kg over 1 minute). These starting dosages of clopidogrel and ticagrelor were selected as they achieved similar inhibition of ADP-induced aggregation. The timing of administration (2 hours for ticagrelor and 4 hours for clopidogrel prior MI induction) was based on their pharmacodynamic profile in 4 additional animals (see below). 8SPT was delivered as a bolus and allowed to circulate for approximately 2 minutes prior inducing ischemia. The dose of 8SPT was selected according to previous work in rabbits and corrected for the BSA in pigs.(2, 3) Once the experimental MI procedure was completed, the pig was brought back to the animal facility to recover. Throughout the following 24 hours, the animals received the corresponding maintenance doses of the antiplatelet agents (90 mg *bid* for ticagrelor and 75 mg/day for clopidogrel). Twenty-four hours following the MI induction and consequently, 4 hours after the second clopidogrel dose and 2 hours after the third dose of ticagrelor, the animals were anesthetized and brought to the 3T-CMR facility for assessment of cardiac anatomy and function. Thereafter, the coronary artery was catheterized up to the same coronary segment where ischemia was induced based on anatomical markers, blood perfusion interrupted by balloon inflation, and Evans blue dye perfused through the left atrium to outline the ischemic cardiac region. The animal's heart was then arrested with potassium chloride, rapidly excised and sliced. Alternative slices were collected to: 1) delineate by Evans blue dye the boundary of the area perfused by the occluded LAD coronary artery (*i.e.*, jeopardized myocardium), and calculate infarct size by TTC staining (both measurements were performed by planimetry using the National Institute of Health software Image J); and, 2) collect myocardial samples (ischemic and remote myocardium) for molecular analysis.

Experimental induction of ischemia/ reperfusion

Ischemia/reperfusion was experimentally induced by a 1hour total balloon occlusion of the mid LAD coronary artery as previously described. (4) Briefly, on the day of infarction animals received buprenorphine (0.03 mg/kg) and cefazoline (25 mg/kg) as prophylaxis for pain and wound infection, respectively. Subsequently, animals were anesthetized for the coronary intervention by giving an intramuscular injection of ketamine (30 mg/kg), xylazine (2.2 mg/kg), and atropine (0.05 mg/kg). Animals were endotracheally intubated, and anesthesia was continued by isoflurane inhalation (2%). Anesthetic depth was clinically monitored by cessation of movement, eye position, loss of muscular tone, and absence of palpebral and pedal reflexes. Just before starting the procedure, a perfusion of amiodarone (300 mg) and lidocaine (150 mg) in 1.000 mL of saline (250 mL/h) was initiated as prophylaxis for malignant ventricular arrhythmias through a line placed in the marginal ear vein. Ischemia/ reperfusion was performed by percutaneous approach and complete coronary balloon occlusion just proximal to the second diagonal branch of the LAD. One hour after the coronary occlusion, reperfusion was instituted by balloon deflation, coronary perfusion was verified by contrast angiography, and animals were allowed to recover. Animals had continuous electrocardiographic (ECG) and hemodynamic monitoring throughout the entire procedure.

Left ventricle ejection fraction (LVEF) was assessed by transthoracic echocardiography before inducing ischemia (prior-MI) and upon reperfusion (post-MI) by using an echocardiographic system (Phillips iE33) equipped with a S5-1 sector array transducer. Left ventricle ejection fraction (LVEF) was measured in the short-axis M-mode right parasternal projection in a plane below the mitral valves and perpendicular to the LV as previously described.(5)

3T-CMR acquisition

Anesthesia was achieved by applying an intramuscular injection of a cocktail composed of ketamine, xylazine, and atropine and maintained by continuous intravenous propofol infusion. Animals were kept under me-

chanical ventilation during the procedure. The studies were performed on a 3.0T-CMR system (Achieva®, Philips, Amsterdam, The Netherlands) by operators blinded to the study medication. Animals were positioned in a head-first supine position with a flexible phased-array surface coil placed over the chest. ECG gating was used to acquire still images of the heart. The following dedicated CMR sequences were acquired in all cases: "cine" (b-SSFP) imaging sequence to assess wall motion and cardiac function, T2-STIR sequence to assess the existence of myocardial edema, early gadolinium enhancement to study the existence of microvascular obstruction (no-reflow phenomenon) and late gadolinium enhancement to assess the amount and extent of myocardial necrosis. All the CRM studies followed the same scheme. First, scout images (T1-TFE sequence) were obtained to localize the true axes of the heart and define a field of view involving the whole heart. Afterward, the bSSFP cine imaging was performed in both horizontal and vertical long axes (4-chamber and 2-chamber views) and in multiple contiguous short axis images covering the whole LV. In the short axis cine sequence 24 cardiac phases of every slice were acquired to guarantee a correct evaluation of the wall motion and heart function. Once the cine sequences were acquired a T2-STIR sequence was obtained to assess myocardial edema. Thereafter, a gadolinium-based contrast agent was injected intravenously (Gd-GTPA, Magnevist®, Berlex Laboratories Inc., Wayne, New Jersey, USA) at a dose of 0.1 mmol/kg. The early gadolinium enhancement sequence was acquired 1 minute after the administration of the contrast. The late gadolinium enhancement (LGE) sequences were obtained 10 min after the administration of contrast. Details of the technical parameters for all CMR sequences are provided in Supplemental Table 1.

3T-CMR data analysis

All CMR images were analyzed using dedicated software (QMass MR v.7.6, Medis, Leiden, The Netherlands) by a CMR-trained radiologist blinded to the study medication. The protocol of analysis has been detailed elsewhere.(6) In brief, LV cardiac borders were traced in each image of the cardiac phases representing the end diastole and end systole to obtain the left ventricle end-diastolic- and end-systolic volumes (LVEDV and LVESV, respectively) and LVEF. The area of myocardial edema was

defined as the extent of the LV demonstrating high signal intensity on T2W-STIR images.

Microvascular obstruction was obtained by delineating the areas of intramyocardial low signal intensity in early gadolinium enhancement sequences. Infarct size (necrosis) was quantified from the extent of myocardial enhancement in the LGE CMR sequence. Edema and necrosis were identified as hyperintense regions, defined as 50% of the peak myocardial signal intensity (full width half maximum) with manual adjustment when needed. If present, a central hypointense core within the area of increased signal was included in the T2W-STIR or LGE analysis.

Platelet function assay and coagulation function and dynamics

The present study aimed to evaluate whether ticagrelor as compared with clopidogrel provides cardioprotective effects when dosed to equal inhibition of ADP-induced platelet aggregation. In order to define the doses to use we conducted a cross-over pilot dose-finding study in 4 naïve animals (Supplemental Figure 2). Based on the results from this pilot study, 180 mg of ticagrelor and 600 mg of clopidogrel were selected which, in turn, concur with the dosages used in the PLATO trial.(7) These dosages were administered 2hours and 4hours prior-experimental MI, respectively, to exclude any potential delay in the pharmacodynamic profile of clopidogrel taking into consideration that in contrast to ticagrelor, clopidogrel requires metabolic activation. In all cases, antiplatelet human dosages were converted to the pig dose according to body surface area.(2) Conventional coagulation function parameters [prothrombin time, activated partial tromboplastin time (aPTT) and fibrinogen (ST4 Diagnostica Stago)] and thromboelastometric coagulation analysis by using extrinsically- and intrinsicallyactivated, and fibrin-based thromboelastometric assays (EXTEM, INTEM, and FIBTEM, respectively) were also evaluated in this pilot study to discard any effect on blood coagulation (Supplemental Table 2). In the main study, platelet function was additionally monitored before treatment (baseline), prior-MI induction, post-MI induction and at 24 hours. Platelet function was evaluated by light transmittance aggregometry (LTA; Aggrecorder II PA 3220 Menarini Diagnostic,) triggered by ADP (5, 10 and 20 μM; Menarini Diagnostic). The

maximal platelet aggregation (MPA) response was recorded and used for data analysis of the degree of inhibition of platelet aggregation (IPA). IPA was calculated using the following formula:

$$\%\,\mathrm{IPA}=[(\mathrm{MPA}_{\mathrm{baseline}}-\mathrm{MPA}_{\mathrm{post\text{-}MI\ or\ 24\,h}})/\mathrm{MPA}_{\mathrm{baseline}}]\times 100$$

as previously described.(8) Blood count (System 9000) and bleeding time was also determined by using the Simplate cutting device in the ear skin and recorded as time until bleeding cessation.

Troponin-I assessment

Serological troponin-I levels were assessed in all animals 24 hours after MI with the corresponding cardiac Pig Elisa kit (Life Diagnostics).

Analyses of aquaporin-4 expression and adenosine monophosphate activated protein kinase (AMPK) signaling pathway activation

Tissue samples were obtained from the ischemic and remote myocardium 24 hours after MI and were immediately frozen until processed. Samples were pulverized and processed for mRNA and protein isolation. We assessed: 1) mRNA and protein levels of aquaporin-4; and 2) AMPK, AMP-kinase phosphorylated at Thr^{172} (P-AMPK), and downstream effectors eNOS phosphorylated at Ser^{1177} (P-eNOS) and CD36 protein expression. Results were normalized to a housekeeping gene or β-actin, respectively. In addition, activation of AMPK was evaluated by ratio P-AMPK to total AMPK.

Myocardial Cox2 activity

Myocardial Cox2 activity and PGF1α release was assessed as previously described in all animals.(9) Briefly, myocardial samples were sectioned into three segments (20 mg each), homogenized in cold PBS (pH 7.4), and centrifuged. The supernatants were collected and stored on ice. The segments were placed into test vials with 500 µL Hanks'HEPES solution. Fifty µM AA were added to the first tube (for overcoming the po-

tential rate-limiting effects of cPLA2 that generates AA); AA + 200 µM of SC58125 (a specific Cox2 inhibitor) to the second tube; and AA + 100 µM of SC560 (a specific Cox1 inhibitor) to the third tube. After 15minute incubation at room temperature, the supernatant in each vial was aspirated and stored at −70 °C. The samples (25 µL each) were analyzed for 6-keto-PGF1α by an ELISA kit according the manufacturer instruction. The first tube represents 6-keto-PGF1α generated by both Cox1 and Cox2. Cox2 activity was calculated as 6-Keto-PGF1α levels in the first minus the second tube and Cox1 activity as 6-Keto-PGF1α levels in the first minus the third tube.

Aquaporin-4 expression in cardiac-related cells

It remains controversial which resident cardiac cell express aquaporin-4. We assessed aquaporin-4 protein expression in porcine aortic endothelial cells (PAECs), porcine fibroblasts and HL-1 cardiomyocytes. To that end, PAECs and pig Fibroblasts (0.5×10^6 cells) were cultured in 1% gelatin pretreated flasks with supplemented M-199 Earle's medium (10%FBS; 1% Penicillin/Streptomicyn, 1% L-Glutamine). HL-1 cells cultured in 1mg/cm^2 of bovine fibronectin in 0.02% Gelatin pretreated flasks with in supplemented Claycomb medium (20%FBS; 1% Penicillin/Streptomicyn, 1% L-Glutamine, 2% Norepirephrine). When cells reached confluence, they were tripsinized and plated in a 100 × 20 mm pre-treated petri-plaque with fresh medium. Upon reaching confluence they were washed twice with PBS and processed with Tripure for protein extraction.

Statistical analyses

Normal distribution of the data was assessed by applying the Shapiro-Wilk test. Analysis of the non-Normally distributed data was carried out by a non-parametric statistical analysis and results are reported as medians and interquartile range [IQR]. For independent factors (comparisons between groups) we performed Kruskal-Wallis and Mann-Whitney analysis applying post-hoc Bonferroni correction; for repeated measurements Wilcoxon and Friedman analysis. Regression slopes were compared by Student's *t*-test based on the standard error of regression models (Real

Statistics Excel Resource Pack).(10) The remaining statistical analysis was performed using the Statview package. All statistical tests conducted were two-sided and statistical significance was considered $p < 0.05$.

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Supplemental Table 1. Parameters for cardiac magnetic resonance (CMR) data acquisition and analysis. T2-STIR: short tau inversion recovery; LGE: late gadolinium enhancement.

	Voxel size (mm)	Flip angle (º)	TR (mseg)	TE (mseg)	TI (mseg)	Bandwith (Hz/pixel)
B-SSFP	1.67x1.67x8	60	4.7	2.4	-	1035
T2_STIR	1.57x1.91x10	90	2 R-R interval	80	150	544.7
LGE	1.81x1.83x5	15	3.8	1.22	270-290	344.8

In the T2-STIR sequence the repetition time is variable depending on the heart rate.
TR: Repetition time
TE: Echo time

Supplemental Table 2. **A**.Coagulation parameters assessment. **B.** Thrombus dynamics analyzed in a thromboelastography system using extrinsically- and intrinsically-activated, and fibrin-based thromboelastometric assays (EXTEM, INTEM, and FIBTEM,respectively).

MCV; Mean Corpuscular Volume, CMR; Cardiac Magnetic Resonance, PT; Prothrombin Time, aPTT; activated Partial Tromboplastin: CT: clotting time ; CFR: clot formation time; A10: clot firmness; MCF: Maximum clot firmness; MCE: Maximum clot elasticity; AUC: area under the curve.

A.

	PT ratio	aPTT ratio	Fibrinogen mg/dl
Placebo-control	1.0 [1.0-1.0]	0.8 [0.7-0.9]	209.6 [176.2-250.8]
Clopidogrel	1.0 [1.0-1.2]	0.9 [0.8-1.0]	320.1 [192.0-349.4]
Ticagrelor	1.0 [1.0-1.1]	0.9 [0.8-0.9]	217.5 [182.7-389.3]

B.

	EXTEM					
	CT (s)	**CFT (s)**	**A10 (mm)**	**MCF (mm)**	**MCE**	**AUC**
Placebo-control	57 [49-55]	40 [37-41]	71 [70-74]	71 [71-74]	253 [250-288]	7080 [7058-7326]
Clopidogrel	55 [55-57]	37 [35-40]	74 [74-74]	75 [75-76]	303 [302-317]	7434 [7423-7527]
Ticagrelor	60 [59-63]	34 [34-51]	72 [68-75]	73 [71-76]	269 [240-313]	7212 [6995-7466]

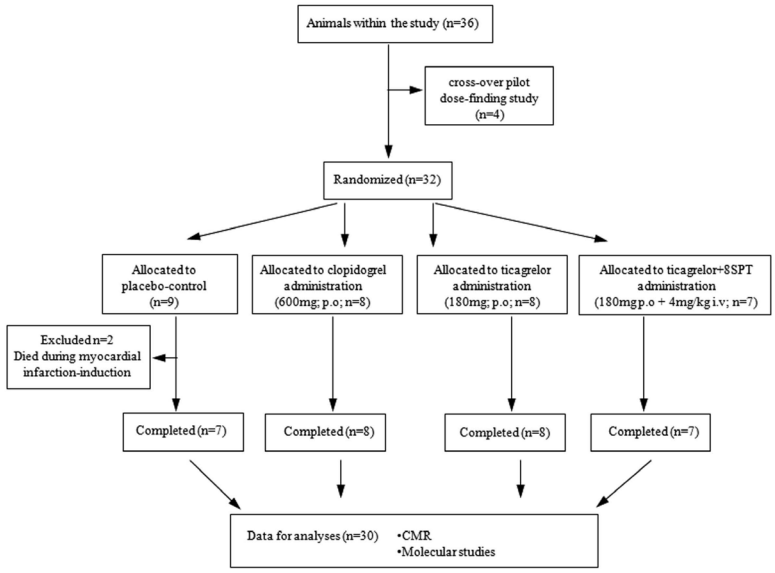
	INTEM					
	CT (s)	**CFT (s)**	**A10 (mm)**	**MCF (mm)**	**MCE**	**AUC**
Placebo-control	108 [99-145]	27 [25-34]	73 [71-75]	74 [73-76]	278 [259-312]	7212 [7109-7428]
Clopidogrel	160 [149-181]	40 [39-42]	73 [73-74]	74 [74-76]	280 [277-283]	7328 [7302-7330]
Ticagrelor	194 [184-198]	42 [41-45]	73 [71-74]	73 [72-75]	274 [258-292]	7196 [7112-7362]

	FIBTEM			
	A10 (mm)	**MCF (mm)**	**MCE**	**Plt component**
Placebo-control	47 [42-50]	48 [43-51]	94 [76-103]	230 [214-239]
Clopidogrel	41 [37-51]	41 [37-45]	70 [60-82]	236 [233-245]
Ticagrelor	38 [37-39]	39 [38-40]	63 [61-65]	211 [180-251]

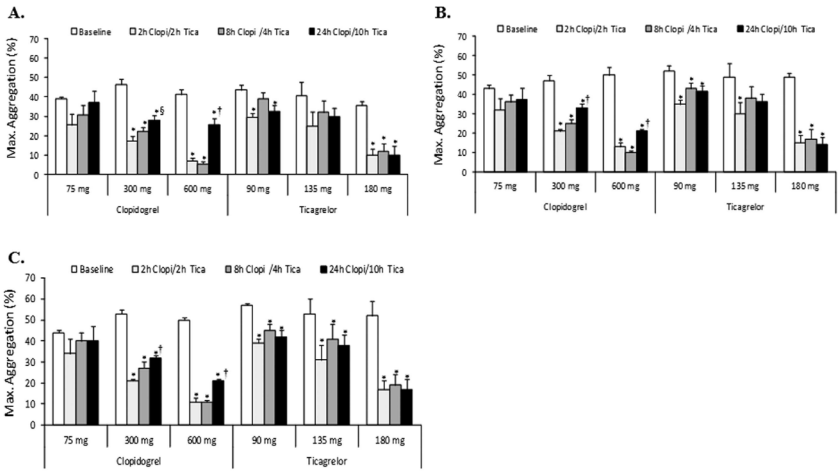
RBC: Red Blood Cells; MCV: Mean Corpuscular Volume

		RBC	Hematocrit	Platelet count	MCV
Control	Baseline	5.4 [5.3-5.9]	25.1 [23.1-26.0]	284.0 [262.5-301.5]	44.5 [41.9-45.6]
	Prior-MI	5.1 [4.9-5.5]	21.8 [21.6-23.8]	277.0 [269.5-293.0]	44.4 [42.2-45.7]
	Post-MI	5.3 [4.7-5.5]	22.5 [21.1-23.4]	261.0 [239.5-274.0]	43.7 [41.7-44.8]
	CMR	5.4 [4.8-5.6]	23.8 [20.9-24.4]	280.0 [243.5-294.0]	43.8 [42.5-45.5]
Clopidogrel	Baseline	4.9 [4.7-5.1]	21.6 [19.6-23.5]	308.0 [286.0-352.5]	44.4 [40.9-46.0]
	Prior-MI	4.8 [4.6-5.1]	21.2 [20.2-21.5]	279.5 [245.5-338.3]	43.8 [38.7-45.1]
	Post-MI	4.5 [4.4-4.9]	19.6 [18.9-20.0]	256.0 [241.0-285.0]	43.6 [38.1-44.8]
	CMR	5.1 [4.7-5.2]	22.1 [20.9-22.8]	276.5 [227.8-304.3]	44.3 [40.4-46.3]
Ticagrelor	Baseline	5.1 [4.5-5.2]	22.7 [21.6-23.0]	275.5 [244.5-299.5]	44.7 [42.3-47.2]
	Prior-MI	5.2 [4.8-5.4]	22.8 [22.7-23.6]	326.0 [288.5-368.3]	44.3 [42.7-48.1]
	Post-MI	5.1 [4.8-5.2]	22.3 [21.6-22.8]	277.5 [251.8-324.3]	43.6 [42.1-46.8]
	CMR	4.6 [4.5-5.2]	22.1 [20.8-23.2]	272.5 [245.5-302.0]	44.5 [44.1-46.9]
Ticagrelor+8SPT	Baseline	4.8 [4.5-4.9]	21.0 [19.7-21.5]	278.1 [275.3-279.1]	44.7 [41.7-46.5]
	Prior-MI	4.8 [4.5-4.9]	21.0 [19.7-21.5]	278.1 [275.3-279.1]	44.7 [41.7-46.5]
	Post-MI	4.8 [4.4-5.0]	20.3 [19.2-21.6]	261.5 [259.5-270.4]	43.7 [41.7-45.4]
	CMR	5.2 [5.2-5.6]	23.9 [22.2-24.7]	330.2 [229.1-347.8]	45.0 [43.1-47.0]

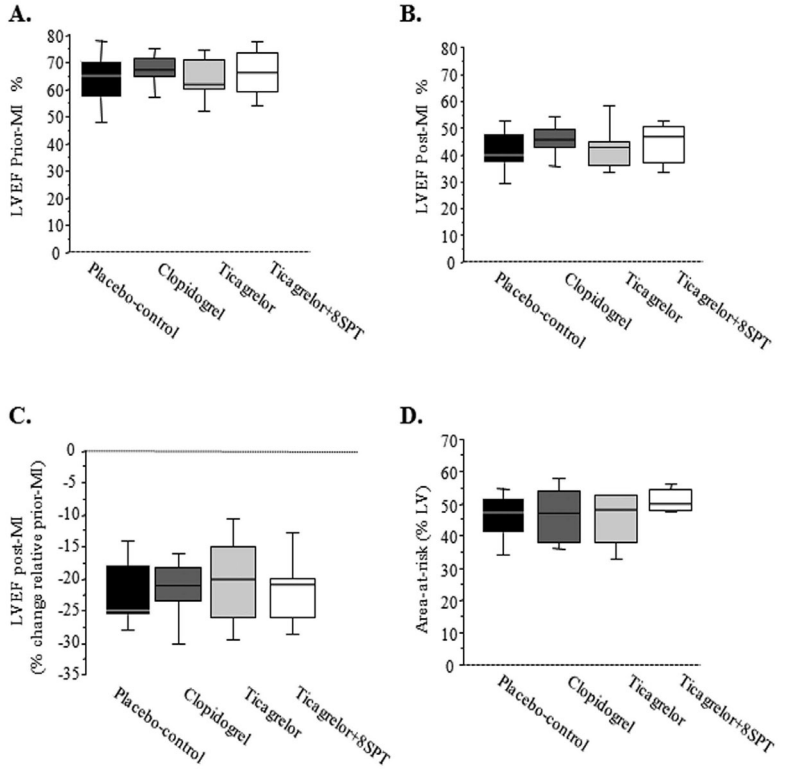
Supplemental Figure 1. Diagram depicting the experimental protocol of the study.



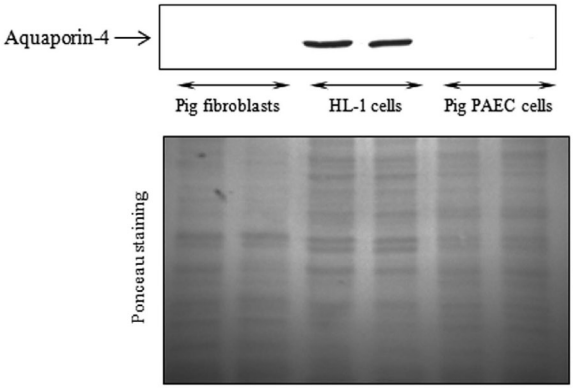
Supp Figure 2. Ligth transmittance aggregation (LTA) analysis. Platelet aggregation challenged by ADP 5µM (A), 10 µM (B) and 20 µM (C) was assessed at different time points after clopidogrel and ticagrelor administration at different oral doses, n=4 animals/group. *p<0.05 vs baseline; † p<0.05 vs previous 2h and 8h; § p<0.05 vs 2h



Supplemental Figure 3. Effect of myocardial infarction (MI) on cardiac performance assessed by echocardiography. Left ventricle ejection fraction (LVEF) prior (**A**) and post (**B**) to MI induction (pre-MI). **C.** Worsening in cardiac performance post-MI expressed as change vs baseline. **D.** Percentage of left ventricle (LV) subjected to ischemia (jeopardized myocardium). N=7-8 in each group

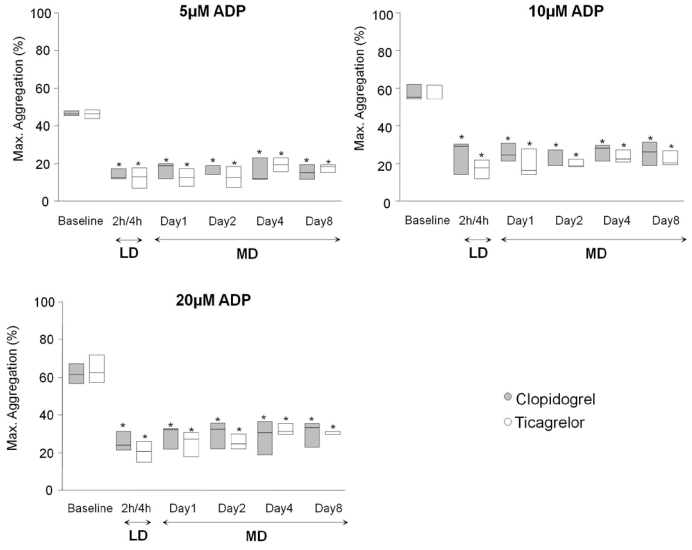


Supplemental Figure 4. Aquaporin-4 protein expression in cultured pig fibroblasts, cardiomyocytes (HL-1 cells) and pig pulmonary aortic endothelial cells (PAECs) .



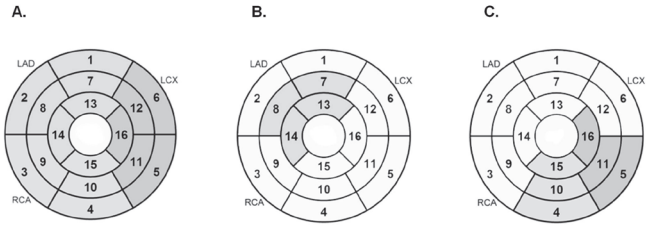
Anexo 2
Supplementary Material
$P2Y_{12}$ antagonists and cardiac repair post-myocardial infarction

Supplemental Figure 1. Light transmittance aggregometry (LTA) analysis. Platelet aggregation challenged by ADP (5, 10 and 20 μM) was assessed before treatment (baseline), and at different time points after clopidogrel and ticagrelor treatment regimen of their corresponding loading dose (LD; clopidogrel 600mg and ticagrelor 180mg) followed by their maintenance doses (MD; clopidogrel 75mg/day and ticagrelor 90mg/bid). LTA was assessed in clopidogrel- and ticagrelor- administered animals at 4h and 2h post-drug ingestion, respectively. N=3 animals/group. *p<0.05 vs baseline. Data was analyzed by Kruskal Wallis followed by Mann-Whitney with post-hoc Bonferroni correction.



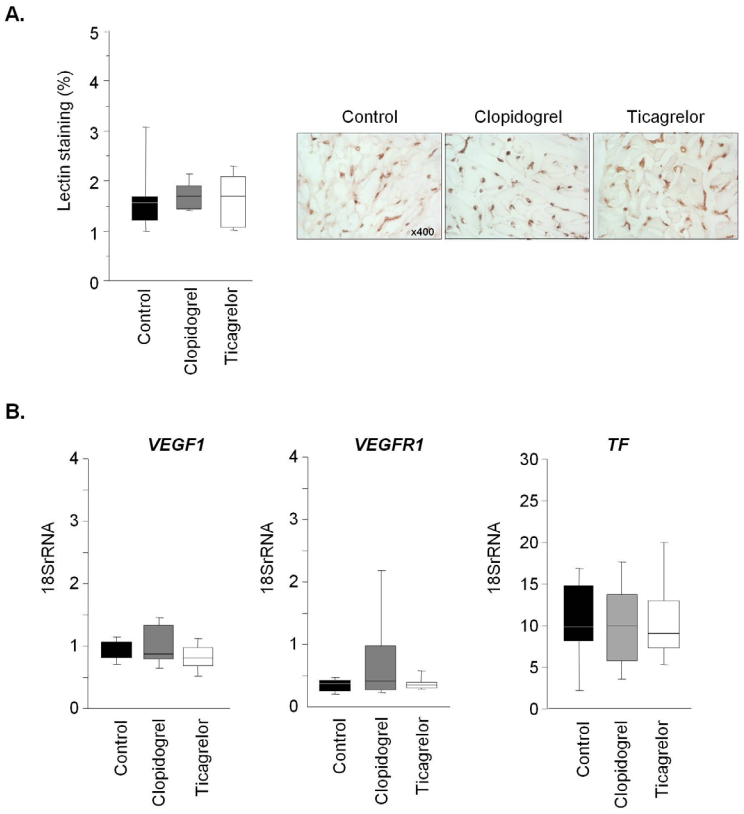
Supplemental Figure 2. Regional CMR analyses of the left ventricle (LV) following the16-segment model (American Heart Association).

A. Analysis of the entire LV; **B**. Analysis of those ischemic segments (i.e., jeopardized myocardium) **C**. Analysis of those segments contralateral to the infarcted region (i.e., remote myocardium). In grey color the target segments included within each analysis.



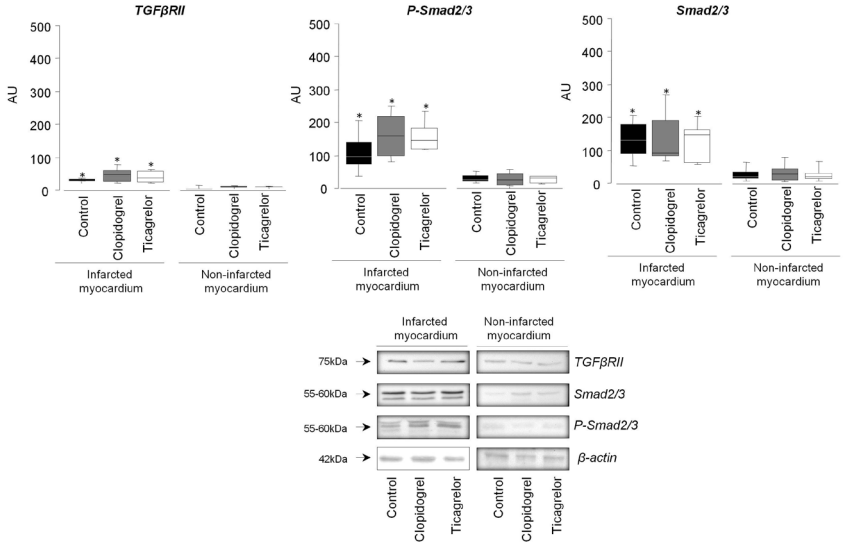
Supplemental Figure 3. Vascular density in the infarcted myocardium

A. Lectin staining. **B.** Transcript levels of several angiogenic markers. VEGF: vascular endothelial growth factor; VEGFR1: VEGF-receptor-1; TF: tissue factor. Data was analyzed by non-parametric analysis (Kruskal Wallis and Mann-Whitney) and reported as median and interquartile range.



Supplemental Figure 4. Cardiac fibrosis.

Myocardial protein expression of *TGFβRII*/Smad2/3 was significantly higher in the infarcted myocardium that in the non-infarted region (*p<0.05). In the infarcted myocardium the three groups displayed a comparable response. *TGFβRII: tissue growth factor β receptor II*. Data was analyzed by non-parametric analysis (Kruskal Wallis and Mann-Whitney) and reported as median and interquartile range.



Supplemental Figure 5. Illustration summarizing the reported findings.