Universitat Autònoma de Barcelona

GENÈTICA APLICADA A LA CLÍNICA NEUROVASCULAR

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Tesis doctoral

Departament de Medicina

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Aquesta tesi doctoral s'ha realitzat al Laboratori de Recerca Neurovascular de l'Institut de Recerca de l'Hospital Universitari Vall d'Hebron, grup de recerca consolidat per l'Agència de Gestió d'Ajuts Universitaris i de Recerca de la Generalitat de Catalunya 2009-2013 (Nº expedient SGR 432; Grup de Recerca en Malalties Neurovasculars).

El Laboratori de Recerca Neurovascular és membre de les xarxes RENEVAS (RD06/0026/0010), European Stroke Network (EUSTROKE 7FP Health F2-08-202213), International Genetics Stroke Consortium i del Consorci Espanyol de Genètica (Genestroke). El projecte Geno-tPA ha rebut el suport de les beques FIS (PJ060586) i Ramón Areces (2006).

M''agradaria donar les gràcies, en primer lloc, als meus directors de tesi: Israel Fernández, Joan Montaner i José Álvarez Sabín per la seva confiança i consell durant aquests anys.

No puc oblidar-me tampoc dels meus companys de laboratori, en particular tots els becaris que, com jo, s'han engrescat a l'aventura d'una tesi (la Tere, la Morancho, les Mires i la Jessy). ¡Ànims perquè, tard o d'hora, tot arriba! El Manolo i la Dolors, amb el SPSS i els programilles del "R", o la Cris Nafria que m'ha demanat un munt d'històries; la Maite i la Pilar, que sempre m'aporten el punt de vista clínic tant necessari. La Miri pels seus consells a cultius i la Sophie per les seves correccions d'anglès i francès ¡Merci beaucoup! I tots els neuròlegs de la unitat d'ictus i els auxiliars (Sandra i Borja) i tècnics (Anita i Cris) per la seva ajuda a la recollida de mostres, la base d'aquest treball. També vull agrair als postdocs (l'Anna Rosell, la Maria Borrell, la Mar i la Lidia) els seus valuosos consells als lab-meetings.

Als membres del laboratori de patologia Mitocondrial (el Marc, el Javi, la MªCarmen, la Gise, el Ramiro, el Lluis, l'Emiliano, el Tomàs, la Yolanda i la MªJesús) i els seus caps (Ramón, Elena i Toni) perquè el laboratori 104 ha estat una segona llar per mi.

A la Dorita del grup de Cardiologia, per introduir-me al món de la coagulació i als residents i adjunts de Bioquímica (Dra. Chacón, Roser, Marieta, Paula, Clara, Albert,...) per fer-me un cop de mà amb el nefelòmetre i el Triturus ¡I iniciar-me a la cuina japonesa!

A les Roses, el Ricardo, la Marichu, la Marta Valeri, l'Àlex, la Irene i la Fàtima de la UCTS, perquè no podria comptar els picoxips o les seqüències que m'han "punxat" i sempre s'han implicat a la recerca que fem. I a les noies de Pneumologia (Maribel, Laura, Sara, Lola i Chus) perquè baixar a fer extraccions de DNA amb elles sempre és un plaer.

A tothom qui va colaborar al projecte CADASIL, perquè la seva ajuda des del primer dia no té preu: els Drs. García-Patos, Bassas, Ferrer, Fernández, Pozo i en especial l'Esther Solé, amb qui hem fet mà a mà la posada a punt del diagnòstic genètic. I també a tots els qui m'han donat consell sobre la via Notch: el Valentí, la Lide, el Pep, el Jordi, la Sònia, el Ramón Gimeno,... I a tota la Unité 740 de l'INSERM (Charles, Marie, Valérie, Barbara, Céline, Minh, Snaigune, Mélanie, Françoise, Anne Blécon, Gwéndola, Pierre, Alexandra, Anne Joutel i Elisabeth) per la seva acollida durant els mesos que vaig passar a París. Van ensenyar-me que a la recerca, les idees són molt més importants que els diners.

D'altra banda, també vull donar les gràcies a tots aquells amb qui m'ho he passat molt bé dins i fora del lab: en Borja i el David, les noies d'Anatomia Patològica o les d'Oncologia, per la mítica gresca amb el ron "Arehucas" o els viatges a Madrid i Menorca.

I fent memòria, agrair a tota la colla dels ADHs (Xavi, Sergio, Gènia, Armando, Jone, Esther, Emma, Xavier i Jaume) i la UAB (Edu, Papitu i Ton) per engrescar-me a fer recerca durant aquelles pràctiques de l'AGAUR.

No voldria acabar sense donar les gràcies també als meus amics i la família, perquè són qui t'ajuden a oblidar-te de la feina quan els resultats no t'acompanyen. I com no, a la Laura, la meva xicota, qui sempre m'ajuda a veure les coses d'una altra manera i no ser tan caparrut. I la seva familia, perquè em fan sentir un més de la colla i ens ho passem d'allò més bé viatjant amunt i avall.

Segur que m'he deixat algú, perquè heu estat molts els que m'heu ajudat perquè hagi pogut escriure aquest treball, espero que em perdoneu. **¡GRÀCIES A TOTS!**

I. ÍNDEX

I.I ÍNDEX D'ABREVIATURES

A2M: **A**lfa-**2**-**M**acroglobulina

ADN: **À**cid **D**esoxirribo**n**ucleic

AIF: ***A****poptosis* ***I****nducing* ***F****actor*

AIT: **A**tac **I**squèmic **T**ransitori

AMPA: **À**cid **a**mino-3-hidroxi-5-**m**etil-4-isoazol **p**ropiònic

Apaf-1: ***A****poptosis* ***p****rotease* ***a****ctivating* ***f****actor 1*

ASCO: ***A****therosclerosis*; ***S****mall Vessel Disease*; ***C****ardiac Source*; ***O****ther cause*

ASK: ***A****ustralian* ***S****trepto****k****inase trial*

ASP: ***A****ncrod* ***S****troke* ***P****rogram*

ATLANTIS: ***A****lteplase* ***T****hrombolysis for* ***A****cute* ***N****oninterventional* ***T****herapy in* ***I****schemic* ***S****troke*

ATP: **A**denosina **T**rifostat

AUST: ***A****ustralian* ***U****rokinase* ***S****troke* ***T****rial*

BHE: **B**arrera **H**emato**e**ncefàlica

BI: ***B****arthel* ***I****ndex*

BMP: ***B****one* ***M****ineralization* ***P****roteins*

CADASIL: ***C****erebral* ***A****utosomal* ***D****ominant* ***A****rteriopathy with* ***S****ubcortical* ***I****nfarcts and* ***L****eukoencephalopathy*

CAPTORS: ***C****ollaborative* ***A****ngiographic* ***P****atency* ***T****rial* ***O****f* ***R****ecombinant* ***S****taphylokinase*

CARASIL: ***C****erebral* ***A****utosomal* ***R****ecessive* ***A****rteriopathy with* ***S****ubcortical* ***I****nfarcts and* ***L****eukoencephalopathy*

CASES: ***C****anadian* ***A****lteplase for* ***S****troke* ***E****ffectiveness* ***S****tudy*

CCS: ***C****ausative* ***C****lassification of* ***S****troke system*

CHARGE: ***C**ohorts for **H**eart and **A**ging **R**esearch in **G**enomic **E**pidemiology*

CLOTBUST: ***C**ombined **L**ysis **O**f **T**hrombus in **B**rain ischemia with **U**ltrasound in **ST**roke*

CNV: ***C**opy **N**umber **V**ariants*

COMA: ***CO**mbining **M**onteplase with **A**ngioplasty*

DEDAS: ***D**ose **E**scalation of **D**esmoteplase for **A**cute ischemic **S**troke*

DEFUSE: ***D**iffusion and perfusion imaging **E**valuation **F**or **U**nderstanding **S**troke **E**volution*

DFI: ***D**eterioration **F**ollowing **I**mprovement*

DHPLC: ***D**enaturing **H**igh **P**erformance **L**iquid **C**hromatography*

DIAS: ***D**esmoteplase **I**n **A**cute **S**troke*

DSPA: **D**e**s**mote**p**las**a**

DTC: **D**oppler **T**rans**c**raneal

ECA: **E**nzim **C**onversor de l'**A**ngiotensina

ECASS: ***E**uropean and **A**ustralian **C**ooperative **S**troke **S**tudy*

EGF: ***E**pidermal **G**rowth **F**actor*

ELAM-1: ***E**ndothelium **L**eukocyte **A**dhesion **M**olecule 1*

EPC: ***E**ndothelial **P**rogenitor **C**ells*

EPITHET: ***E**cho**P**lanar **I**maging **TH**rombolytic **E**valuation **T**rial*

ESTAT: ***E**uropean **S**troke **T**reatment with **A**ncrod **T**rial*

FADD: ***F**as **A**ssociated protein with **D**eath **D**omain*

FDA: ***F**ood and **D**rugs **A**dministration*

Gb3: Globotriosilceramida

GOM: ***G**ranular **O**smiophilic **M**aterial*

GOS: ***G**lasgow **O**utcome **S**cale*

GWAS: ***G**enome-**W**ide **A**ssociation **S**tudies*

HHT: ***H**ereditary **H**emorrhagic **T**elangiectasia*

HI: ***H**emorrhagic **I**nfarction*

HIC: **H**emorràgia **I**ntra**c**erebral

HTA: **H**iper**t**ensió **A**rterial

ICAM-1: ***I**nter-**C**ellular **A**dhesion **M**olecule-1*

IL-1: **I**nter**l**euquina 1

IMS: ***I**nterventional **M**anagement of **S**troke*

IPZ: **I**nhibidor depenent de **P**roteïna **Z**

ISEDIC: **I**mpacto **S**ocial de los **E**nfermos **D**ependientes por **Ic**tus

IST-3: *Third **I**nternational **S**troke **T**rial*

ISTR: ***I**nternational **S**troke **T**reatment **R**egistry*

KO: ***K**nock-**O**ut*

LACI: ***LA**cunar **C**erebral **I**nfarction*

LRP: ***L**DL **R**elated **P**rotein*

MAST-E: ***M**ulticenter **A**cute **S**troke **T**rial-**E**urope*

MAST-I: ***M**ulticenter **A**cute **S**troke **T**rial-**I**taly*

MAV: **M**alformacions **A**rterio**v**enoses

MCP-1: ***M**onocyte **C**hemotactic **P**rotein-1*

GM-CSF: ***G**ranulocyte-**M**acrophage **C**olony **S**timulating **F**actor*

MMP: ***M**atrix **M**etalo**p**roteinases*

mRS: ***R**ankin **m**odified **S**cale*

MTHFR: **M**etil **T**etra-**H**idro**f**olat **R**eductasa

NIHSS: ***N**ational **I**nstitute of **H**ealth **S**troke **S**cale*

NINDS: ***N**ational **I**nstitute of **N**eurological **D**iseases and **S**troke*

NMDA: **N**-**m**etil-**D**-**a**spartat

NO: ***N**itric **O**xide*

NOS: ***N**itric **O**xide **S**ynthetase*

nPA: Lanoteplasa

OCSP: ***O**xfordshire **C**ommunity **S**troke **P**roject*

OR: ***O**dds **R**atio*

PACI: ***P**artial **A**nterior **C**erebral **I**nfarction*

PAI-1: ***P**lasminogen **A**ctivator **I**nhibitor 1*

PARP1: ***P**oly **A**DP-**R**ibose **P**olymerase 1*

PC: **P**roteïna **C**

PDE4D: **F**osfo**die**stearasa 4D

PDGF: ***P**latelet **D**erived **G**rowth **F**actor*

PECAM-1: ***P**latelet **E**ndothelial **C**ell **A**dhesion **M**olecule-1*

PH: ***P**arenchymal **H**ematoma*

PID: ***P**eri-**I**nfarct **D**epolarization*

POCI: ***PO**sterior **C**erebral **I**nfarction*

PROACT: ***PRO**lyse in **A**cute **C**erebral **T**hromboembolism*

pro-uPA: Pro-uroquinasa

RBD: ***R**eceptor **B**inding **D**omain*

RER: **R**etícle **E**ndoplasmàtic **R**ugós

ROS: ***R**eactive **O**xygen **S**pecies*

rPA: Reteplasa

RR: **R**isc **R**elatiu

SAK: ***Sta**philo**k**inase*

SERPIN: ***SER**in **P**rotease **IN**hibitor*

SITS-MOST: ***S**afe **I**mplementation of **T**hrombolyisis in **S**troke - **MO**nitoring **ST**udy*

SK: ***S**trepto**k**inase*

SNP: ***S**ingle **N**ucleotide **P**olymorphisms*

SODD: ***S**ilencer **O**f **D**eath **D**omain*

SSS-TOAST: ***S**top **S**troke **S**tudy*-TOAST

STARS: ***S**tandard **T**reatment with **A**lteplase to **R**everse **S**troke*

STAT: ***S**troke **T**reatment with **A**ncrod **T**rial*

TACI: ***T**otal **A**nterior **C**erebral **I**nfarction*

TAFI: ***T**hrombin **A**ctivable **F**ibrinolysis **I**nhibitor*

TFPI: ***T**issue **F**actor **P**athway **I**nhibitor*

TH: **T**ransformació **H**emorràgica

TIBI: ***T**hrombolyisis **I**n **B**rain **I**schemia*

TIMI: ***T**hrombolysis **I**n **M**yocardial **I**nfarction*

TIMP: ***T**issue **I**nhibitor of **M**etallo**p**roteinases*

TNF: ***T**umor **N**ecrosis **F**actor*

TNK: Tenecteplasa

TOAST: ***T**rial of **O**RG 10172 in **A**cute **S**troke **T**reatment*

t-PA: ***T**issue **P**lasminogen **A**ctivator*

TRADD: ***T**NF**R**-**A**ssociated **D**eath **D**omain*

TRUMBI: ***TR**anscranial low-frequency **U**ltrasound-**M**ediated thrombolysis in **B**rain **I**schemia*

u-PA: ***U**rokinase **P**lasminogen **A**ctivator*

UPR: ***U**nfolded **P**rotein **R**esponse*

VCAM-1: ***V**ascular **C**ell **A**dhesion **M**olecule-1*

VSMC: ***V**ascular **S**mooth **M**uscle **C**ell*

vWF: ***V**on **W**illebrand **F**actor*

WTCCC: ***W**elcome **T**rust **C**ase-**C**ontrol **C**onsortium*

II. EXECUTIVE SUMMARY

This PhD work presents results of genetics in stroke focusing in Geno-tPA study, which involved more than 1.000 individuals, nowadays the most extensive pharmacogenetic study for t-PA treatment during the acute phase of Ischemic Stroke.

The aims of this study were to identify SNPs associated with:

1) t-PA efficacy (early recanalization, reocclusions and functional outcome)

2) t-PA security (hemorrhagic transformation and in-hospital death)

1) The most extensive study of t-PA safety **(paper 4)** included 4 different cohorts in several steps of analysis, subgroups were derived for model evaluations and functional analysis.

First, we attempted to genotype 250 **S**ingle **N**ucleotide **P**olymorphisms (SNP) in cohort A of 531 patients and cohort B of 354 individuals and we selected the most interesting variants for HT and in-hospital death prediction, as indicated by low p-values in both groups.

Then, these SNPs and all the associated clinical variables were used to generate a predictive model either by Logistic Regression or CRT classification tree analysis.

These models were evaluated in cohort C, consisting of 287 t-PA treated patients and cohort D of 491 ischemic stroke patients not receiving t-PA.

Finally, several functional studies were carried out in subgroups of samples from our

hospital to determine the mechanism by which these variants lead to clinical outcomes. As a result, we hypothesisize that rs669 in A2M gene may influence A2M activity, rs1801020 in F12 gene would influence F12 levels and rs1695 would modify substrate specificity and higher circulating levels of these enzyme.

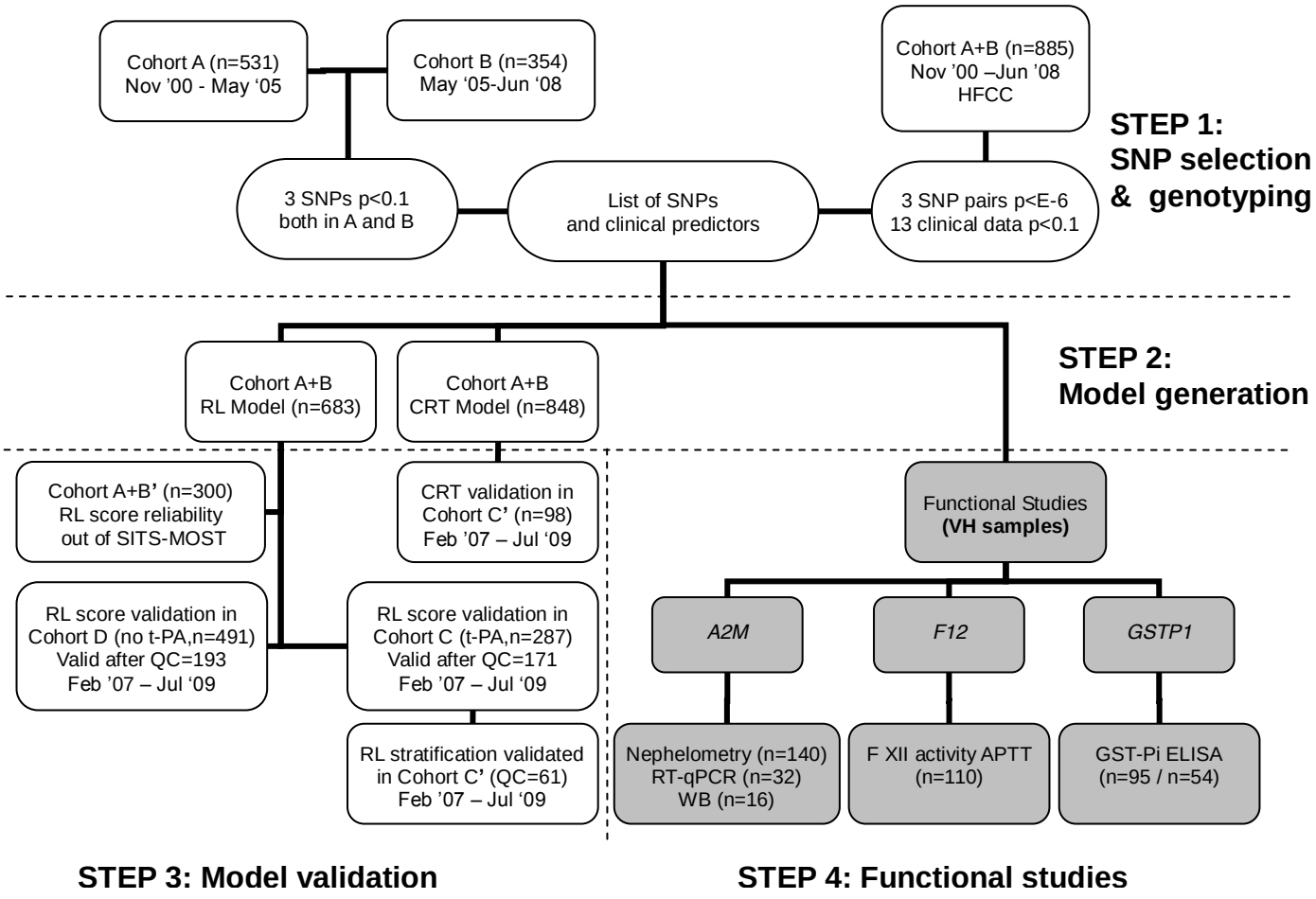


Figure I. Workflow of the t-PA safety study (article 6), the most complex study presented in this PhD work

2) Regarding t-PA efficacy, we genotyped the same 250 SNPs in cohorts A and B and the best variants were combined with clinical to generate a predictive model **(paper 3)**. However, continuous Transcranial Doppler monitorization for recanalization assesment was only available in cohorts A and B, and we were not able to validate these models in cohorts C and D. Functional studies showed how rs1063856 influenced FVIII levels on admission, but we could not find a functional effect of the other variants rs1143627 and

rs16944 in IL1B gene or rs2070584 in TIMP1 gene.

Reocclusion studies, which required longer Doppler monitorization were only available in our center (Vall d'Hebrón hospital) and we could not obtain any independent cohort for replication **(papers 1 and 2)**. Thus, we have found 3 independent variants 4G/5G (rs1799768) of *PAI-1* gene, -1C>T (rs1883832) of CD40 gene and -7A>G (rs1800801) of MGP gene that were associated with higher reocclusion risk after effective thrombolytic therapy, but SNP validation is pending. However, two of them (rs1799768 and rs1883832) influenced PAI-1 and CD40 gene expression, respectively.

Finally, we also studied CADASIL, the most important monogenic disease that causes stroke and we found two new mutations causative of CADASIL artheriopathy **(papers 5 and 6)**. Besides, we have diagnosed the first case of the more aggresive CARASIL disease in caucasic population **(paper 7)**, another monogenic disease that had been described only in Asia. The inclusion of these relevant cases is the reason we have choose the title "Genetics applied to neurovascular clinic", to englobe all the work presented here.

In this report, we start defining stroke and its subtypes (ischemic or hemorrhagic) and the different classifications we can do based on diagnostic procedures: ethiologic classification (e.g. TOAST) or clinical classification (eg. OCSP).

Thereafter, we present the biological phenomena that occur after vessel occlusion: membrane depolarization, neurotransmitters release, edema formation, generation of ROS, inflammation and the mechanisms of death which cause tissue damage. Following with biological background, the coagulation cascade and the fibrinolytic pathway are

presented, as most of the following results are related to hemostasis.

In the fourth point, we present epidemiologic risk factors that lead to stroke and we develop the genetic influence in stroke development in the fifth point. The next following points would explain in detail the 2 syndromes for which new mutations are presented: CADASIL and CARASIL diseases.

In the eight point, we discuss different treatments assayed for Ischemic Stroke: all the fibrinolytic treatments tested in clinical trials and their current status (8.1) and all the alternative therapies developed in the following years (sonotrombolysis, mechanical devices, neuroprotective and neurorreparative agents). As the only thrombolytic drug approved for Ischemic Stroke treatment is t-PA, which focus our pharmacogenetic study, we further explain in depth the treatment protocol and exclusion criteria for this drug, its end-points (early recanalization and functional outcome) and possible complications (reocclusion and hemorrhagic transformation). In each category, we describe the current clinical and biochemical predictors for each outcome. Finally, we introduce the concept of pharmacogenetics and the previous results which made us believe that genetic background influences response to t-PA therapy.

The objectives of this PhD. work are specifically: on the one side, build predictive models for t-PA efficacy (either reocclusion **(papers 1 and 2)** or early recanalization **(paper 3)**) and t-PA safety (hemorrhagic transformation and in-hospital mortality **(paper 4)**). On the other side, describe new pathological mutations in genetic small vessel diseases such as CADASIL **(papers 5 and 6)** and CARASIL **(paper 7)** diseases.

The models generated could be the first step for individualized therapy for ischemic stroke.

However, the current predictive capacity should be improved by adding new clinical, biochemical or genetic items until an AUC over 0.9 would be reached and it could guide medical decisions. Nevertheless, they could uncover new therapeutic targets for t-PA coadjuvant treatments.

Most importantly, the genetic diagnosis of the first CARASIL case in caucasic population should introduce it in differential diagnosis of small vessel diseases whenever the patient met clinical criteria.

A. INTRODUCCIÓ

1. L' ictus (definició)

El terme ictus prové del llatí ictus-us i significa “cop” en relació al carácter sobtat de la patologia vascular cerebral. L’ictus o infart cerebral es defineix com una alteració neurològica focal, transitòria o permanent, causada per un transtorn del flux sanguini cerebral. S'utilitza per referir-se de forma genèrica a la isquèmia cerebral (obstrucció d'un vas, 80-85% dels casos) com a l'hemorràgia cerebral (ruptura i extravassació de sang, 15-20%) **(figura 1)**.

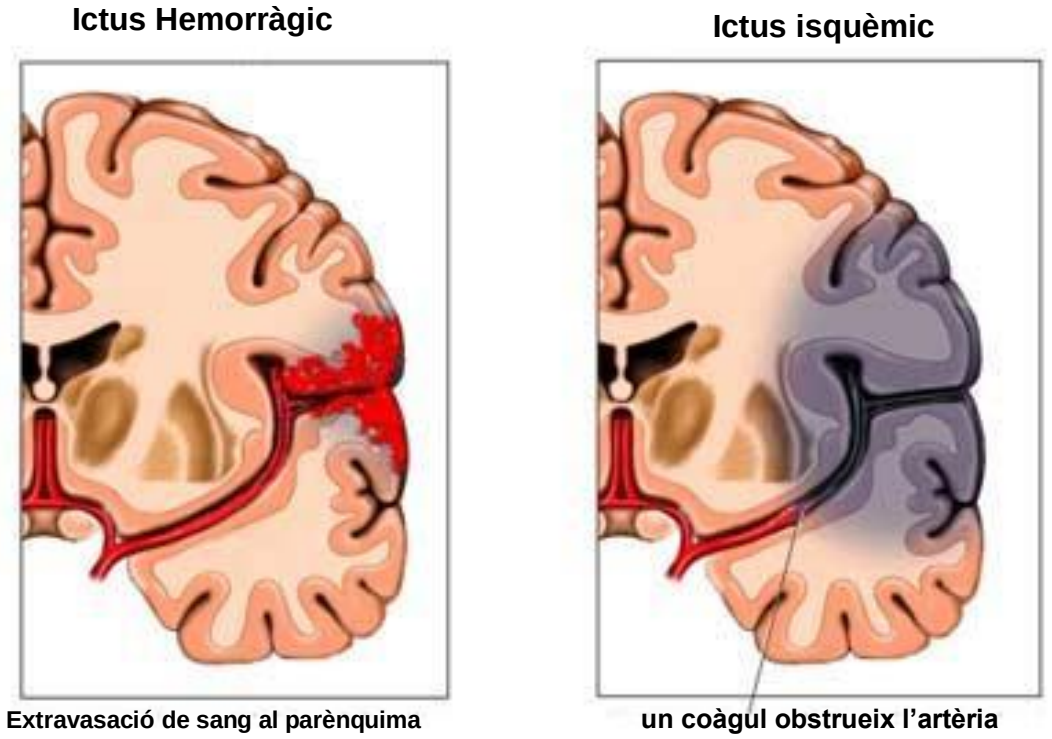


Figura 1. Representació de la isquèmia, modificat de la web: http:// royalbucks.co.uk

Les conseqüències de l'ictus depenen de la zona de l'encèfal afectada i poden ser: afàsia, disàrtria, hemiparèsia, pèrdua de consciència, vertigen, problemes de visió o cefalea, entre d'altres. La seva reversibilitat dependrà de la regió afectada, el temps d'oclusió i l'evolució clínica del pacient.

Malgrat els avenços a la prevenció, diagnòstic i tractament, és la tercera causa de mort i la primera de discapacitat de l'adult als països industrialitzats (Lloyd-Jones et al, 2010), tot i que una nova classificació recent la situa en quart lloc, darrera les malalties cardiovasculars, el càncer i les malalties cròniques respiratòries (Roger et al, 2011).

A Espanya, l'ictus (8,21% del total) és la segona causa de mort després de la cardiopatia isquèmica (9,29% del total), la primera causa en dones (9,81% del total) (INE, 2008). L'any 2006, la prevalença de la malaltia era del 2,5% (Lloyd-Jones et al, 2010), arribant al 7,5% a la població més gran de 65 anys (Medrano et al, 2006). A Barcelona, cada dia es produexien un promig de 10-12 nous casos d'ictus, comptabilitzant-se 4.000 al cap de l'any; arribant als 10.000 casos anuals a Catalunya i més de 80.000 a l'Estat Espanyol.

L'estudi ISEDIC *(***I**mpacto **S**ocial de los **E**nfermos **D**ependientes por **IC**tus*)* va demostrar que el 45% dels individus que sobreviuen a un ictus pateixen seqüel·les físiques i mentals importants (ISEDIC, 2004) i el cost promig per pacient s'ha estimat en 15.597-25.782$ anuals pel període 2010-2050 (Lloyd-Jones et al, 2010) o 13.383€ per any (Truelsen et al, 2005). Si no es realitza una prevenció eficaç, és previsible que l'impacte de l'ictus augmenti amb l'envelliment progressiu de la població, ja que la malaltia es presenta principalment a partir dels 65 anys, el que s'estima representarà un 30% de la població l'any 2050.

1.1 L'Ictus Hemorràgic

L'ictus hemorràgic es produeix com a conseqüència de la ruptura d'un vas intracraneal. En funció de la localització de la sang extravasada, podem definir 5 categories: **H**emorràgia **I**ntra**c**erebral (HIC), hemorràgia subaracnoidea, hemorràgia intraventricular i hematomes subdural i extradural.

Podem diferenciar 2 grups etiològics dins les HICs: HIC traumàtiques i les no traumàtiques o espontànies. Posteriorment, les classificarem en primàries (per efecte de la **H**iper**t**ensió **A**rterial (HTA) o acumulació de dipòsits beta-amiloide a l'angiopatia amiloide) o secundàries per presència de vasos congènitament anormals (aneurismes saculars, fístules arteriovenoses, telangiectàssies, angiomes cavernosos), vasos neoformats (hemorràgia intratumoral), processos inflamatoris (vasculitis, aneurismes micòtics) o tractaments amb fàrmacs (ex. trombolítics, antiagregants, anticoagulants).

Taula 1. Diferents etiologies de les hemorràgies intracerebrals.

- Hipertensió arterial crònica
- Angiopatia Amiloide Cerebral
- Malformacions vasculars
    - Malformacions Arteriovenoses (MAVs)
    - Telangectàsies
    - Angiomes cavernosos o cavernomes
    - Angiomes venosos
- Aneurismes (saculars, infecciosos, traumàtics o neoplàsics)
- Diàtesi hemorràgiques
    - Coagulopaties primàries: hemofilia A (dèficit FVIII), hemofilia B (dèficit FIX), malaltia de Von Willebrand (dèficit vWF), afibrinogenèmia (manca de FI o fibrinògen).
    - Coagulopaties secundàries: púrpura trombocitopènica idiopàtica i púrpura trombòtica trombocitopènica, coagulació intravascular disseminada, trombocitopènia i síndromes mieloproliferatius, mieloma múltiple.
    - Ús de fàrmacs antitrombòtics: antiagregants (ex. aspirina), anticoagulants (ex. acenocoumarol) i trombolítics (ex. activador tissular del plasminògen, t-PA)
- Tumors cerebrals primaris o metastàsics
- Vasculopaties
    - Vasculitis sistèmiques
    - Vasculitis aïllades del Sistema Nerviós Central
    - Vasculitis infeccioses

- Altres vasculopaties: sarcoïdosi, Moya-Moya, Behçet o disecció arterial

- Causes relacionades amb un pic hipertensiu
  - Fàrmacs (efedrina, inhibidors de la monoamina oxidasa) o drogues (amfetamines, cocaïna) amb efectes simpaticomimètics
  - Estimulació del nervi trigeminal
  - Hipotermia
  - Secundari a cateterisme cardíac
  - Shock per picadura d'escorpió o vespa
- Causes relacionades amb canvis del fluxe cerebral
  - Després d'endarterectomia o angioplàstia carotídea
  - Després d'intervenir una cardiopatia congènita
  - Després de transplantament de cor
- Altres causes
  - Consum d'alcohol o metanol
  - Migranya
  - Trombosi venosa cerebral

El tractament de les HIC depèn de la causa de l'hemorràgia: si és iatrogènica per tractament anticoagulant, s'atura el tractament i s'administra vitamina K o protamina per aturar el sagnat. Si és d'origen hipertensiu, s'inicia tractament hipotensor. Quan la causa és la ruptura d'un aneurisma, es realitza l'evacuació quirúrgica de l'hematoma o s'embolitza amb diversos materials (*coils*, microfilaments,...) que promouen la formació d'un coàgul i el segellament de l'aneurisma. El tractament quirúrgic està contraindicat en pacients majors de 75 anys o que arriben molt deteriorats a l'hospital (Towfighi et al, 2005). Existeixen altres tractaments experimentals com l'administració de FVII recombinant, que redueix l'hemorràgia, però no millora l'evolució clínica del pacient (Mayer et al, 2009).

1.2 L'Ictus Isquèmic

A l'ictus isquèmic, la circulació d'una part de l'encèfal es veu interrompuda per la obstrucció d'un vas sanguini, que condueix a una disminució de l'arribada d'oxígen i glucosa al cervell i la conseqüent interrupció de l'activitat metabòlica al territori afectat.

1.2.1 Classificació etiològica

La classificació etiològica més utilitzada és la TOAST (***T****rial of* ***O****RG 10172 in* ***A****cute* ***S****troke* ***T****reatment*), estratificant els següents grups (Adams et al, 1993):

A) Aterosclerosi de gran vas o aterotrombòtic

Isquèmia de tamany mig / gran, de topografia cortical o subcortical i localització carotídea o vertebrobasilar, al que es compleixen algun d'aquests supòsits:

A1- Aterosclerosi amb estenosi ≥50 % de l'artèria extracraneal ipsilateral a la oclusió o de l'arteria intracraneal de gran calibre (cerebral mitja, cerebral posterior o basilar), en absència d'altres patologies.

A2- Aterosclerosi amb estenosi <50 % a les mateixes artèries en absència de cap altra etiologia i en presència de dos o més dels factors de risc vascular següents: edat > 50 anys, hipertensió arterial, hipercolesterolèmia, tabaquisme o diabetes mellitus.

B) Cardioembolisme

Isquèmia generalment de tamany mig / gran, de topografia habitualment cortical, on s'observa alguna de las següents cardiopaties embolígenes:

Presència d'un coàgul o un tumor intracardíac, estenosi mitral reumàtica, prótesi aòrtica o mitral, endocarditis, fibrilació auricular, malaltia del nodus sinusal, aneurisma ventricular esquerre, hipocinèsia cardíaca global, acinesia causada per un infart agut de miocardi, infart agut de miocardi als 3 mesos previs.

C) Malaltia de petit vas o infart lacunar

Isquèmia de tamany petit (<1,5 cm de diàmetre) al territori d'una artèria perforant cerebral, que habitualment ocasiona clínicament un síndrome lacunar (hemiparèsia motora pura, síndrome sensitiu pur, síndrome sensitivomotor, hemiparèsia atáxica i disartria mà-maldestre) en un pacient afectat d'hipertensió arterial o altres factors de risc vascular (diabetes mellitus, dislipèmia, tabaquisme) en absència d'altra etiologia.

D) Causa inhabitual o criptogènic

Isquèmia de tamany i localització variable en un pacient on s'han descartat l'origen aterotrombòtic, cardioembòlic o lacunar i s'identifica una altra patologia. Habitualment són malalties sistèmiques (conectivopatia, infecció, neoplàsia, síndrome mieloproliferatiu o alteracions de la coagulació) o per arteriopatia no ateroscleròtica (disecció arterial, displàsia fibromuscular, ectasis vasculars, Moya-Moya o, síndrome de Sneddon).

E) Etiología indeterminada

Isquèmia de tamany mig / gran de localització variable al qual no s'ha determinat la causa embolígena. Poden distinguir-se diverses situacions:

E1- Estudi incomplet: caldrien més proves diagnòstiques per evaluar l'etiologia

E2- Coexistència de més d'una etiología potencial.

E3- D'origen desconegut. Després d'un estudi diagnòstic exhaustiu, s'han descartat els subtipus aterotrombòtic, cardioembòlic, lacunar i criptogènic.

L'etiologia indeterminada representa el 30 - 40 % dels pacients (Mohr, 1988; Sacco et al, 1989), i aquest percentatge arriba fins el 64 % en malalts menors de 55 anys (Cabanes et al, 1993). Per aquest motiu, s'han incorporat dades d'epidemiologia i de neuroimatge a la nova classificació SSS-TOAST (***S**top **S**troke **S**tudy*) amb l'objectiu de reduir la proporció d'ictus d'etiología indeterminada (Ay et al, 2007).

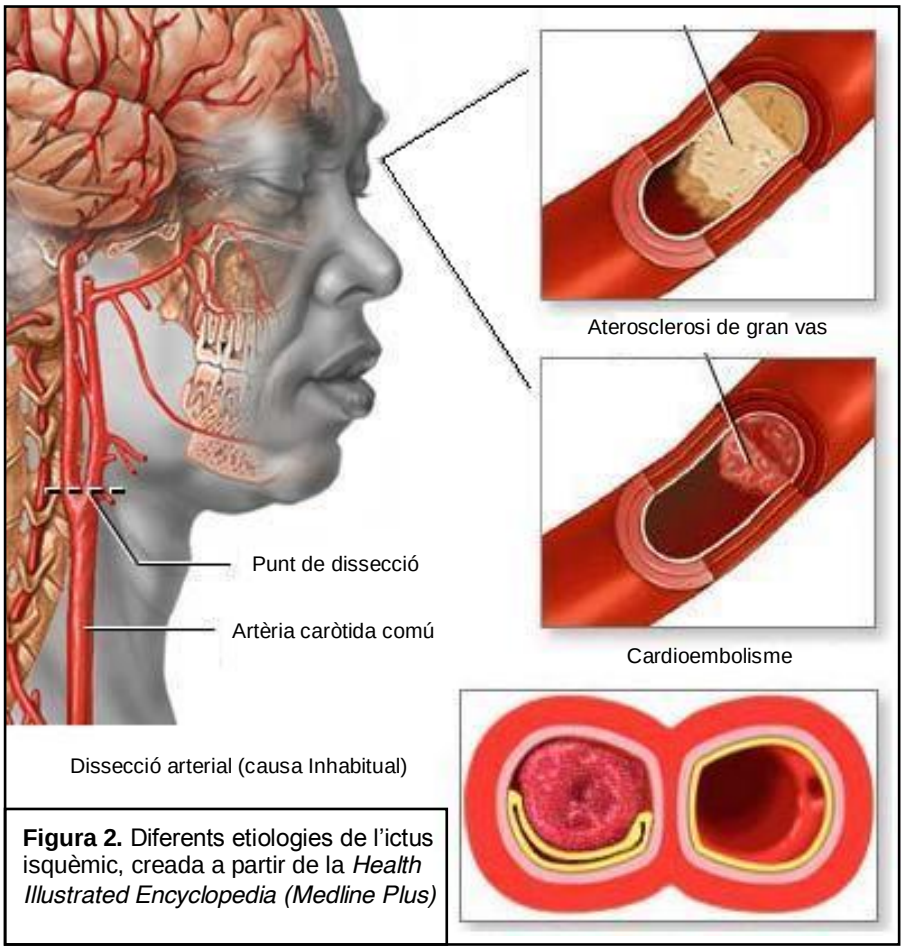


Figura 2. Diferents etiologies de l'ictus isquèmic, creada a partir de la *Health Illustrated Encyclopedia (Medline Plus)*

Per últim, l'escala ASCO (A=***A**therosclerosis*; S=***S**mall Vessel Disease*; C=***C**ardiac Source*; O=***O**ther cause*) cada pacient rep una puntuació per a cadascun dels components de l'escala, basant-se en els nivells d'evidència de les proves diagnòstiques (Amarenco et al, 2009).

Finalment, s'ha creat un paquet informàtic per introduir, de forma semiautomatitzada, les dades de la classificació etiològica, anomenat ***C**ausative **C**lassification of **S**troke system* (CCS) (Arsava et al, 2010). Al *"causative subtype"*, el programa classifica cada etiologia del TOAST en 3 categories (evident, probable, possible) i subdivideix el grup d'indeterminats en 4 categories (embolisme criptogènic, altres criptogènics, avaluació incomplerta i no classificats).

El “*phenotypic subtype*”, semblant a la classificació ASCO d'Amarenco, puntua cada subgrup en 3 categories (major, minor or absent + incomplete evaluation) i el resultat s'indica en forma gràfica.

El CCS ha estat validat internacionalment per 15 observadors, obtenint-se un coefficient d'acord Kappa de 0,8 entre ells, molt superior als obtinguts anteriorment amb la classificació TOAST (0,42 a 0,68), el TOAST automatitzat (0,44 a 0,91) o les escales mRS (0,25 a 0,64) i Barthel (0,27 a 0,68). Aquesta millora de la classificació fenotípica redueix la variabilitat de la mostra obtinguda i pot permetre la reducció del tamany mostral necessari per obtenir una mateixa potència estadística.

1.2.2 Classificació clínica OCSP

Una classificació alternativa de l'ictus és pels criteris de la OCSP *(**O**xfordshire **C**ommunity **S**troke **P**roject)* (Lindley et al, 1993), que defineix 4 grups:

- ***T**otal **A**nterior **C**erebral **I**nfarction* (TACI)
- ***P**artial **A**nterior **C**erebral **I**nfarction* (PACI)
- ***LA**cunar **C**erebral **I**nfarction* (LACI)
- ***PO**sterior **C**erebral **I**nfarction* (POCI).

Aquesta classificació, de caire més clínic, es basa en el territori vascular afectat i la gravetat de la oclusió. Al territori anterior tenim els TACI, relacionats amb les oclusions proximals i el PACI, relacionats amb les distals. El LACI no presenta simptomatologia cortical i en molts casos és produït per l'etiologia lacunar del TOAST, mentre el POCI inclou els infarts al territori vertebrobasilar.

2. Història natural de l'ictus isquèmic

El teixit nerviós és extremadament sensible a la isquèmia, això provoca que una isquèmia cerebral global (pròpia d'una parada cardio-respiratòria) de només 10 segons comporta la pèrdua de consciència, en 20 segons es frena l'activitat elèctrica i uns minuts ja provoquen déficit neurològic posteriors (Torregrosa et al, 2007).

L'ictus isquèmic és causat per una isquèmia cerebral focal i les lesions neurològiques es limiten al territori irrigat per l'artèria afectada. La reducció del flux sanguini no es dóna de forma homogènia en tot el territori afectat; existeix un nucli central que és la zona irrigada únicament per l'artèria ocluida (flux < 10 ml/100g*min) on es produeix una lesió tisular en un període breu i una regió d'hipoperfusió circundant (20 ml/100g*min > flux > 10 ml/100g*min) denominada periinfart (histològicament) o penumbra (radiològicament) que tot i estar metabòlicament compromesa, és viable temporalment degut a la irrigació de la circulació colateral i potencialment salvable si es produeix una recanalització ràpida de l'artèria ocluida (Torregrosa et al, 2007). La reversibilitat del dany a la zona de penombra depèn del grau de circulació arterial colateral, el temps d'hipoperfusió i l'estat previ del teixit.

Al voltant de l'àrea de penombra es troba una zona d'oligohèmia benigna (flux> 20 ml/100g*min) que, tot i estar funcionalment compromesa, no veu afectada la viabilitat cel·lular. Només en condicions excepcionals, com la presència d'un edema massiu, provocaria l'elevació de la pressió intracraneal i el descens de la perfusió a nivells de penombra isquèmica (Hacke et al, 1996).

2.1 Canvis hemodinàmics durant la isquèmia

Donada l'extrema sensibilitat del cervell a la isquèmia, existeixen diversos mecanismes anatòmics i regulatoris destinats a mantenir i regular el flux sanguini al parènquima cerebral. Des del punt de vista anatòmic, poden destacar-se les nombroses anastomosis arterio-arterials classificades en dos sistemes: el polígon de Willis, que inclou les artèries cerebrals anteriors, mitges i posteriors, i les anastomosis leptomeníngees de Heubner, que interconecten les branques corticals distals (Torregrosa et al, 2007) **(Figura 3)**.

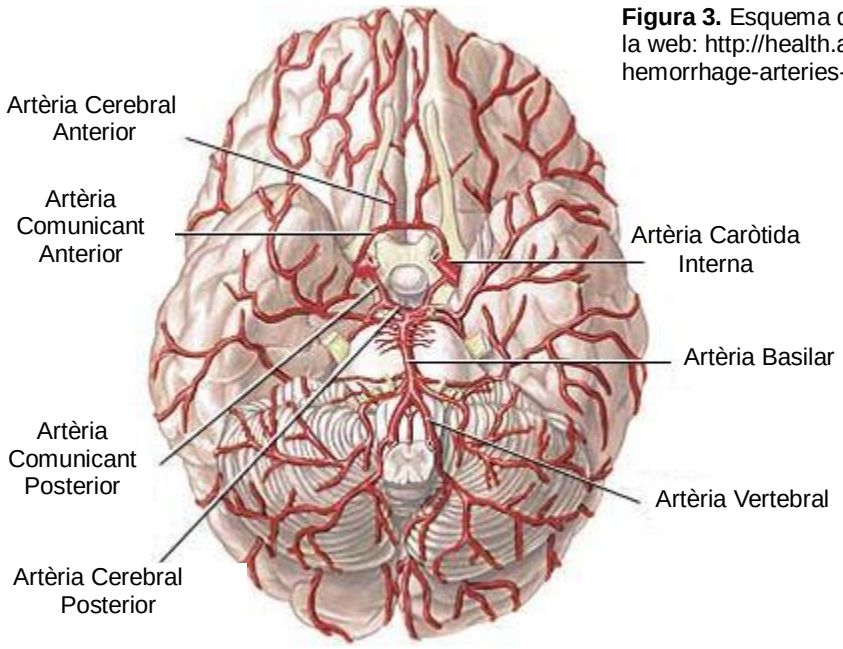


Figura 3. Esquema de la vasculatura cerebral, modificat de la web: http://health.allrefer.com/health/lobar-intracerebral-hemorrhage-arteries-of-the-brain.html.

Quan la irrigació colateral no és suficient, el fluxe sanguini es compensa mitjançant la relaxació dels vasos de resistència, també causada per la lactoacidosi del metabolisme anaeròbic. Quan els vasos arriben al seu màxim de dilatació, tant l'autorregulació com la resposta al CO_2 queden suprimides, i el flux sanguini depèn de la pressió arterial sistèmica.

2.2 La cascada isquèmica

La manca de nutrients i d'oxigen provoca una disminució dràstica dels nivells de

fosfocreatina i ATP. En cas d'isquemia perllongada, la depleció energètica altera greument les funcions cel·lulars degut a la interrupció dels processos depenents d'ATP **(figura 4)**.

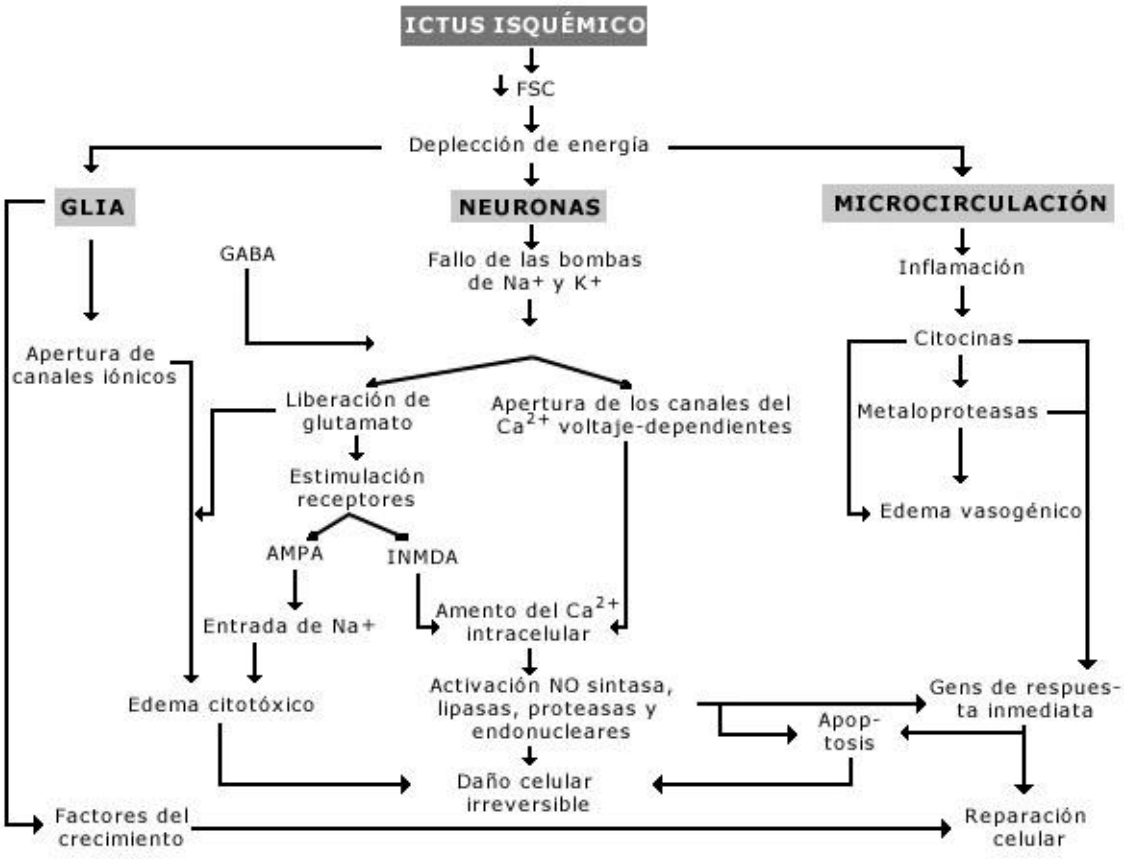


Figura 4. Esquema de les diferents vies alterades per la isquèmia cerebral. Extreta de Castillo, 2001

Es perd la capacitat de mantenir la homeòstasi iònica, produint-se la fallida de la bomba Na^+/K^+ ATPasa, i sortint K^+ de l'interior cel·lular a l'espai intersticial. En compensació, es produeix l'entrada al citosol de Na^+, Cl^- i Ca^{2+} i d'aigua per osmòsi (Siesjö, et al 1992). L'entrada d'aigua causa un edema intracel·lular i l'intercanvi iònic elimina el potencial de membrana, produint-se la despolarització de la cèl·lula ("despolarització isquèmica") que impossibilita l'activitat elèctrica neuronal. La despolarització de la membrana provoca l'obertura dels canals de Ca^{2+} depenents de voltatge, incrementant-se la concentració de calci intracel·lular i una segona despolarització ("despolarització anòxica") (Katsura et al, 1994; Lipton, 1999).

La intensa despolarització de la membrana neuronal dóna lloc a l'alliberació de vesícules pre-sinàptiques i n'impedeix la seva recaptació del botó sinàptic (Ogawa, 2007). En aquestes condicions, el glutamat i altres aminoàcids excitants (noradrenalina i adenosina) tenen efectes tòxics (Krause et al, 1988). La noradrenalina i adenosina activen l'adenilat ciclasa, incrementant els nivells de cAMP i la permeabilitat de la membrana de les cèl·lules glials, contribuint a l'edema astrocític perivascular i perineuronal (Kermer, 1999). El glutamat alliberat estimula els canals-receptors NMDA (N-metil-D-aspartat), AMPA (àcid amino-3-hidroxi-5-metil-4-isoazol propiònic), kainat i els receptors metabotròpics a la neurona postsinàptica. L'activació dels receptors AMPA augmenta l'entrada de Na^+ i controla la despolarització inicial de membrana causada pel glutamat i la movilització dels receptors NMDA; l'estimulació dels receptors NMDA provoca una nova entrada de Ca^{2+} al citosol cel·lular, agreujant l'edema citotòxic (White et al, 2000).

A diferència de les neurones, les cèl·lules glials no tenen estructures post-sinàptiques i són resistents al dany per mecanismes d'excitotoxicitat. Els astròcits pateixen un procés d'hipertròfia i proliferació, la gliosis reactiva, que es relaciona amb mecanismes de recuperació de la lesió isquèmica (Rivera et al, 2001).

El metabolisme anaeròbic de la glucosa provoca l'acumulació d'àcid làctic i fa disminuir el pH fins a nivells de 6,5 (Rehncrona et al, 1985). Aquesta acidosi desacopla el calci unit a proteïnes citoplasmàtiques, augmentant la concentració de Ca^{2+} lliure intracel·lular. Per últim, la disfunció de la bomba Mg^{2+}/Ca^{2+} ATPasa del reticle endoplasmàtic provoca l'alliberament del Ca^{2+} enmagatzemat al reticle cap al citosol.

La sobrecàrrega de Ca^{2+} incrementa la permeabilitat de la membrana i estimula

proteases, endonucleases i fosfolipases que destrueixen components estructurals de la membrana. Les fosfolipases alliberen àcids grassos com l'àcid araquidònic ó l'àcid docosahexanoic, que són metabolitzats a ecoisanoids i docosanoids i participen als processos inflamatoris. A més, el Ca^{2+} incrementa l'activitat de l'òxid nítric sintasa neuronal i endotelial (nNOS i eNOS) i la síntesi d'òxid nítric (NO), participant en el dany cel·lular a través de mecanismes d'estrès oxidatiu. La formació d'espècies reactives d'oxigen (ROS) excedeix la capacitat antioxidant de la neurona, ocasionant alteracions en els constituents proteics (ex. nitrosilació de tirosines), lipídics (ex. peroxidació no enzimàtica) i d'àcids nuclèics.

2.3 Fenòmens inflamatoris durant la isquèmia cerebral

En condicions normals, el cervell es troba aillat del sistema circulatori mitjançant la barrera hematoencefàlica (BHE), constituïda per les cèl·lules endotelials, els pericits i els peus astrocitaris.

La reducció del flux cerebral provoca una resposta inflamatòria: en primer lloc es produeix una expressió transitòria de factors de transcripció (c-fos, c-jun, zif 268, Jun-B...) (Uemura et al, 1991; Hsu et al, 1993) i les "*heat shock proteins*" que s'expressen entre la primera i segona hora després de la isquemia (Nowak et al, 1990; Welsh et al, 1992).

Una hora després de l'ictus comença l'alliberació i activació local de citocines, quimiocines, molècules d'adhesió leucocitària i enzims proteolítics, arribant al màxim al voltant de les dotze hores i mantenint-se fins 2-5 dies següents a la oclusió, provocant el creixement de l'infart (Liu et al, 1993; Wang et al, 1995).

Els astròcits, la micròglia, els leucòcits i les cèl·lules endotelials que s'activen mitjançant el procés d'isquèmia produeixen citocines inflamatòries (IL-1β, IL-6, TNF-α) i quimiocines

atractores de monòcits (ex. MCP-1) i neutròfils (ex. IL-8) (Pelidou et al, 1999; Barone & Feuerstein, 1999). L'alliberació d'aquestes citocines indueix l'expressió de molècules d'adhesió com ***V**ascular **C**ell **A**dhesion **M**olecule-1* (VCAM-1), ***I**nter-**C**ellular **A**dhesion **M**olecule-1* (ICAM-1), ***E**ndothelium **L**eukocyte **A**dhesion **M**olecule 1* (ELAM-1) i ***P**latelet **E**ndothelial **C**ell **A**dhesion **M**olecule-1* (PECAM-1).

La sobreexpressió de molècules d'adhesió facilita la unió del sistema inmune a la BHE i la seva migració al parènquima cerebral (Feuerstein et al, 1998), com s'ha demostrat en estudis histopatològics (García & Kamiyo, 1974; Hallenbeck et al, 1986; Dereski et al, 1992; Ritter et al, 1998), bioquímics (Barone et al, 1991) i de marcatge radiactiu de leucòcits amb In^{111} (Pozzilli et al, 1985; Dutka et al, 1989).

A la infiltració inmunitaria, s'alliberen més radicals lliures (produits per la iNOS), proteases (ex. Metaloproteinases de matriu, MMPs) (Akopov et al, 1996) i també els seus inhibidors (ex. Inhibidors tisulars de metaloproteinases, TIMPs). Aquesta infiltració incrementa el dany tissular en fase aguda però també participa als fenòmens de neuroreparació posteriors a l'ictus (Wang et al, 1998).

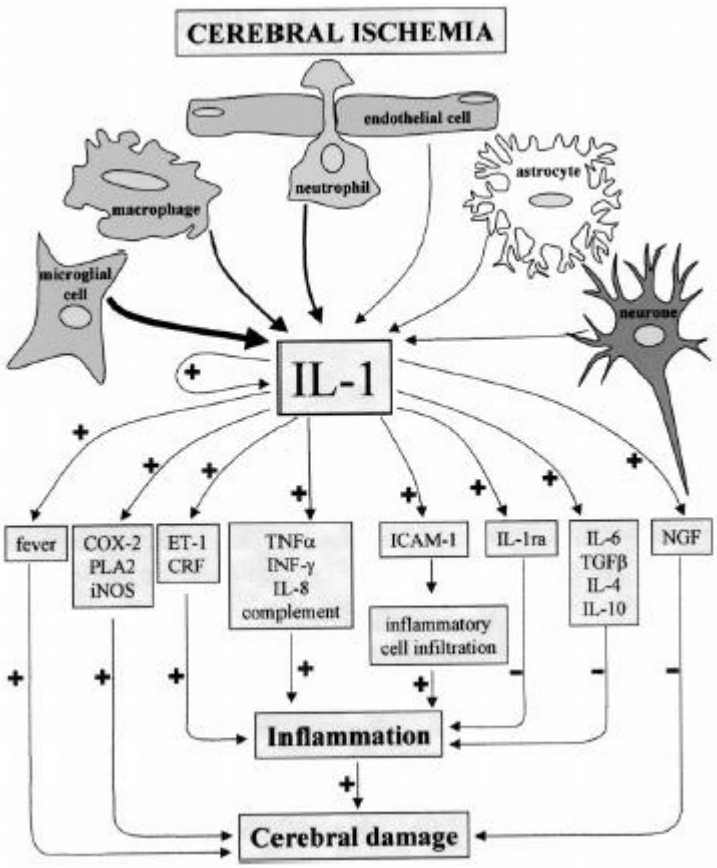


Figura 5. Producció i efectes de la IL-1 durant la isquèmia cerebral. Extreta de Touzani et al, 1999

La IL-1β és la isoforma més abundant de IL-1 al cervell; s'ha detectat la seva expressió constitutiva en neurones, astròcits, oligodendròcits i endotel·li (Touzani et al, 1999). Com hem dit, la seva expressió s'incrementa durant la primera hora d'isquèmia cerebral i participaria al desencadenament de la resposta inflamatòria, afavorint la infiltració leucocitària.

També podria influir a la temperatura corporal, el fluxe cerebral i la neovascularització, entre d'altres. Globalment, s'ha associat la IL-1β amb un increment del dany isquèmic, perquè la seva administració en models animals provocava el creixement de l'infart mentre la seva inhibició el reduïa (Touzani et al, 1999; Banwell et al, 2009). No obstant, la funció beneficiosa o perjudicial de la IL-1 depèn de la seva localització, concentració i entorn (Rothwell et al, 1997). **(figura 5)**

Finalment, es produeix el remodelament tissular i la plasticitat neuronal a llarg termini, on intervenen mediadors com TGF-β (***T**ransforming **G**rowth **F**actor* β), osteopontina o factors de creixement proangiogènics i neurotròfics.

En tots aquests processos inflamatoris també participen el receptor de membrana CD40 i el seu lligand CD40L. Durant la fase aguda de l'ictus, la transcripció de CD40 s'incrementa

als macròfags i els limfòcits B i T (Kassner et al, 2009) i s'incrementa la disponibilitat de CD40 a la membrana dels monòcits (Garlichs et al, 2003). L'expressió de CD40 també es troba elevada a les plaques ateroscleròtiques simptomàtiques (Schumacher et al, 2001).

En condicions normals, l'endotel·li expressa CD40 a baix nivell, però la seva expressió augmenta en resposta a TNF-α ó INF-γ (Hollenbaugh et al, 1995; Omari & Dorovini-Zis, 2003). L'activació de l'endotel·li pel CD40L soluble o per la unió de limfòcits T i B, macròfags, basòfils i eosinòfils que expressen CD40L, provoca l'expressió de mol·lècules d'adhesió (ICAM-1, VCAM-1 i E-selectina) i la síntesi de citoquines (IL-1, IL-4, IL-6), GM-CSF i TNF-α (Hollenbaugh et al, 1995; Karmann et al, 1996; Déchanet et al, 1997), regulant la infiltració cel·lular a la regió danyada (Omari & Dorovini-Zis, 2003).

Al sistema immune, CD40 indueix la producció de IL-1α, IL-1β, TNF-α, IL-6 i IL-8 en monòcits; IL-12, TNF-α i NO en macròfags (Grewal and Flavell, 1998) i IL-10 i TNF-α en limfòcits T, desequilibrant el ratio Th1/Th2 i afavorint la formació de nuclis germinals de limfòcits B, així com el canvi d'isotip a les immunoglobulines (Chabot et al, 2001).

Les plaquetes activades s'uneixen als leucòcits via P-selectina - PSGL-1, però el CD40 dels leucòcits també pot agregar-se amb el CD40L plaquetar, tal i com s'ha observat en models animals d'isquèmia focal (Ishiwaka et al, 2005; Yilmaz et al, 2006). En aquest sentit, l'expressió de CD40L s'incrementa a les plaquetes dels ictus aterotrombòtics (Cha et al, 2002) i la interacció CD40 - CD40L s'ha proposat com el lligam entre la inflamació i la trombosi (Grau & Lichy, 2004) **(figura 6)**.

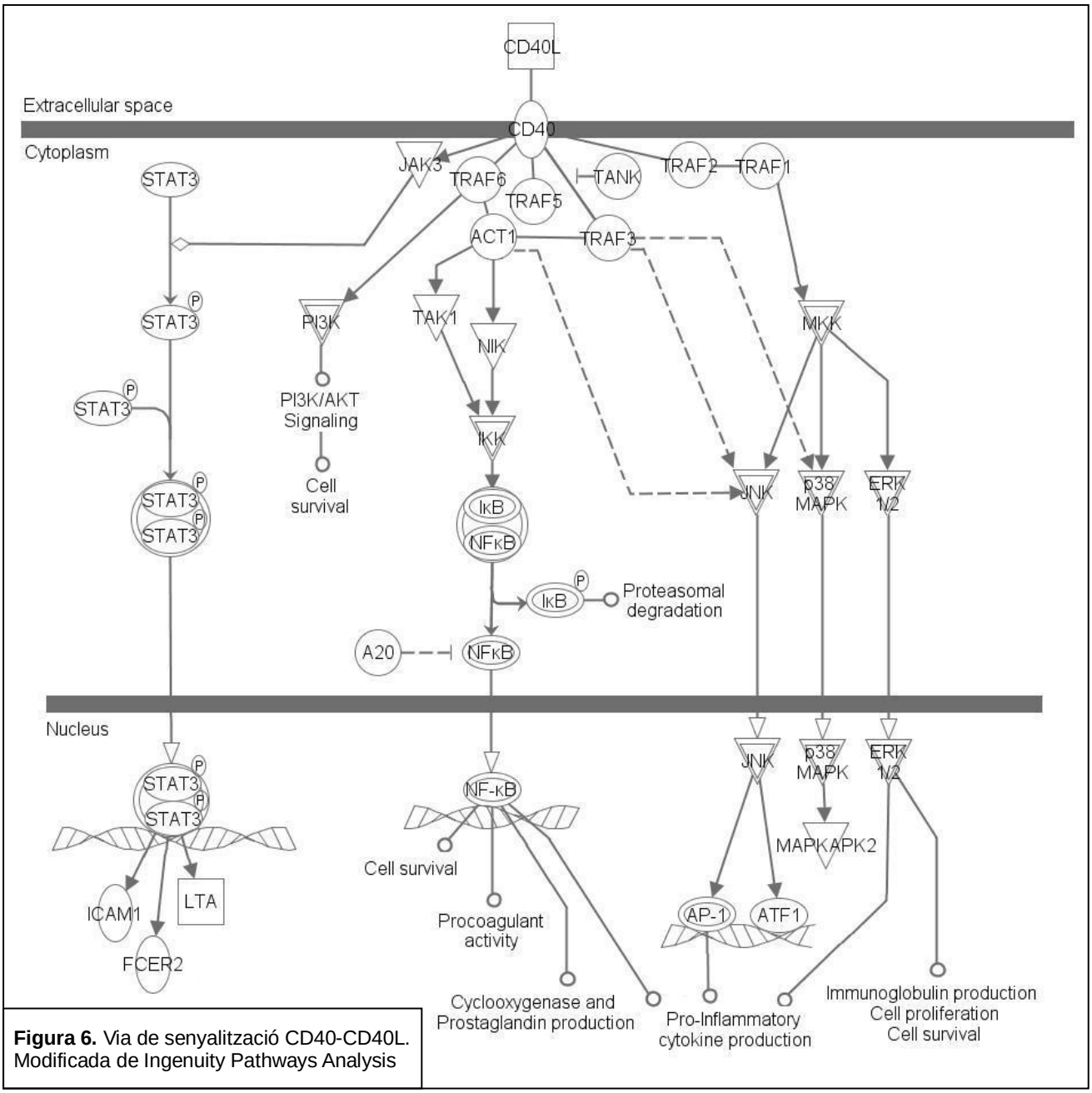


Figura 6. Via de senyalització CD40-CD40L. Modificada de Ingenuity Pathways Analysis

2.4 Mecanismes de mort cel·lular durant l'ictus

La mort isquèmica es produeix pels processos de necrosi (principalment al nucli de l'infart) i apoptosi (principalment a la zona periinfart ó penombra). Els factors que determinen la prevalença de la necrosi o apoptosi són la intensitat i duració de la isquèmia, el grau de maduresa neuronal i els nivells d'ATP i Ca^{2+} intracel·lulars (Lipton, 1999).

La necrosi es produeix de forma indiscriminada i desordenada. Les cèl·lules necròtiques s'identifiquen per la desintegració de la membrana plasmàtica a conseqüència de l'edema

cel·lular, alliberant els continguts citoplasmàtics i mantenint la membrana nuclear (Brown and Brierley, 1972) que contribueix a una reacció inflamatòria posterior. La fragmentació de l'ADN es produeix de forma tardana mitjançant serin-proteases. Per contra, l'apoptosi és un procès de mort regulada (Kerr et al, 1972), modulat per Bax, Bak i Bad, on s'alliberen proteïnes apoptogèniques mitocondrials com el citocrocom C, Smac/DIABLO, factor inductor d'apoptosi (AIF), HtrA2/Omi i procaspases 2, 8, 9 i 10 (caspases iniciadores). El citocrom C, el factor 1 d'activitat proteasa apoptòtic (Apaf-1) i la procaspasa-9 formen l'apoptosoma, que activa la caspasa 9 i aquesta activa les caspases efectores (3, 6 i 7) i l'endonucleasa depenent de caspases, encarregada de fragmentar l'ADN a les regions internucleosomals. La cèl·lula s'encongeix, la cromatina es condensa i apareixen els cossos apoptòtics; és només a les darreres etapes quan s'altera la integritat de la membrana citoplasmàtica **(Figura 7)**.

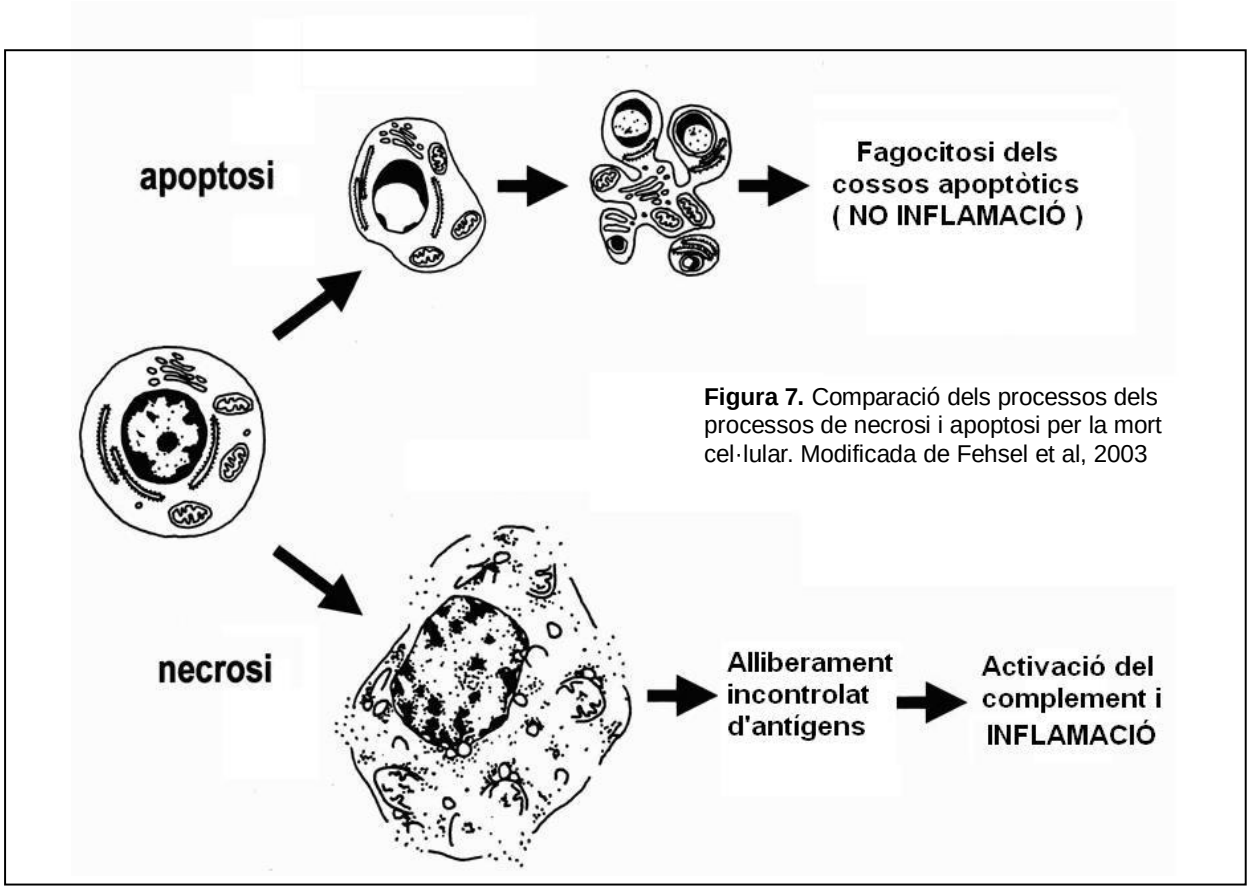


Figura 7. Comparació dels processos dels processos de necrosi i apoptosi per la mort cel·lular. Modificada de Fehsel et al, 2003

L'apoptosi també pot estar provocada per l'activació de receptors de mort com Fas-Fas L

o els pertanyents a la familia del TNF. L'activació de Fas provoca la unió de la proteïna adaptadora amb domini de mort associada a caspases (FADD) i la caspasa 8, iniciant-se el procés apoptòtic. La unió de TNF als seus receptors de mort provoca l'alliberament de SODD (***S**ilencer **O**f **D**eath **D**omain*) i el reclutament de TRADD (***TNFR-A**ssociated **D**eath **D**omain*), FADD i la caspasa 8.

Tot i que clàssicament la necrosi i l'apoptosi s'han descrit com fenòmens clarament diferenciats, s'han descrit morfologies intermitges ocasionades per la isquèmia, el que s'ha denominat necrapoptosi (Jaeschke and Lemasters, 2003). D'altra banda, existeixen altres formes de mort cel·lular programada no apoptòtiques, com l'autofagia o la mort mediada per PARP1 *(**P**oly **A**DP-**R**ibose **P**olymerase 1)* que també s'observen durant la isquèmia **(Figura 8)**.

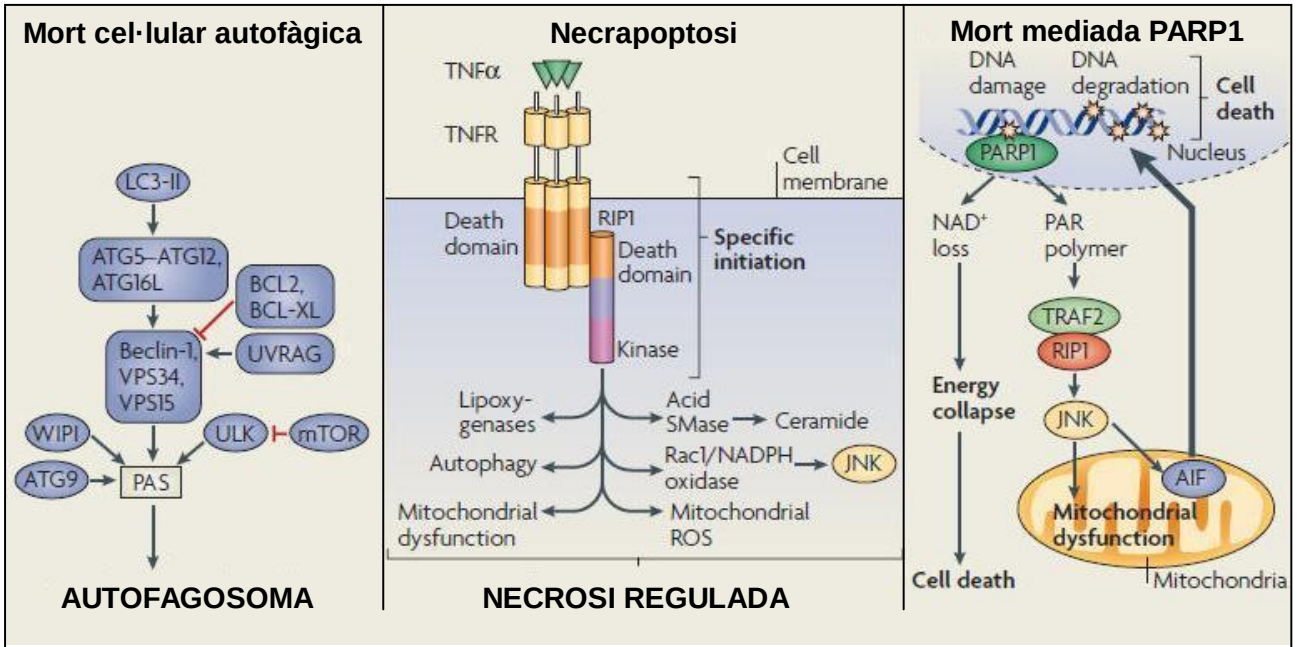


Figura 8. Nous processos de mort cel·lular associats a la isquèmia cerebral. Modificat de Degterev & Yuan, 2008

3 Hemostàsia, coagulació i fibrinòlisi

L'hemostasia és un sistema molt finament regulat que manté la sang fluida en condicions fisiològiques, però davant una lesió vascular permet reaccionar molt ràpidament per frenar l'hemorràgia. L'hemostasia inclou un sistema coagulatiu, un d'anticoagulatiu i un altre fibrinolític, tots composats d'activadors, zimògens, cofactors e inhibidors (Spronk et al, 2003). Existeix un balanç entre un mecanisme procoagulant, que afavoreix l'hemostasia i la trombosi i els mecanismes anticoagulant i fibrinolític que eviten la formació de trombus intravasculars. Aquest equilibri ve determinat pels nivells dels factors de la coagulació i fibrinolítics i el seu grau d'activació / inhibició (Clowes et al, 2005).

Com factors protrombòtics podríem citar la pèrdua o alteracions a l'endoteli, l'activació plaquetar (ja sigui pel vWF o altres agonistes), l'activació de la coagulació i la reducció del fluxe sanguini. Els mecanismes antitrombòtics inclouen factors de membrana o alliberats per l'endoteli (ex. Trombomodulina i heparan-sulfat), inhibidors dels factors de coagulació (ex. Inhibidor de la vía del Factor Tisular (TFPI)), aclariment dels factors de coagulació activats pel fetge o disrupció i dissolució dels trombus pel fluxe sanguini i el sistema fibrinolític.

La majoria dels factors de la coagulació se sintetizen al fetge. Els factors FII, FVII, FIX, FX i les proteïnes C i S necessiten la carboxilació dels residus d'àcid glutàmic, un procés on intervé la vitamina K, per la seva síntesi complerta (Rodríguez Bueno, 2007). Aquesta carboxilació els dóna gran afinitat pels fosfolípids. D'aquesta manera, les reaccions en fase fluida són molt poc eficients, millorant enormement quan els complexes estan adherits a les membranes plasmàtiques. La necessitat de formar complexes proteics redueix la propagació de les reaccions de coagulació lluny del punt d'iniciació.

De fet, la carboxilació per part de la proteïna K és una diana terapèutica per l'anticoagulació mitjançant warfarina, aprovada per la FDA als anys 50. Aquest tractament és efectiu i segur, pero afecta també altres proteïnes amb possibles efectes beneficiosos com la ***M**atrix **G**lycoplatelet **P**rotein* (MGP), una proteïna de matriu que inhibeix el dipòsit de calci (Luo et al, 1997; Proudfoot et al, 1998; Yagami et al, 1999; Schurgers et al, 2007) per inhibició de les BMPs (Yagami et al, 1999; Boström et al, 2001; Sweatt et al, 2003; Schurgers et al, 2007) i podria reduir la progressió de l'aterosclerosi (Shanahan et al, 1994; Farnazeh-Far et al, 2001; Danziger, 2008). Així, els ratolins KO per MGP presenten intenses calcificacions arterials i moren per hemorràgies durant els primers mesos d'edat (Luo et al, 1997), mentre la presència de mutacions de pèrdua de funció en humans causen la síndrome de Keutel, caracteritzada per estenosis pulmonars i calcificacions dels cartílags, entre d'altres (Munroe et al, 1999).

Ara bé, la carboxilació hepàtica dels factors de coagulació és causada per les filoquinones (vitamina K1), mentre la carboxilació perifèrica de MGP és produïda per les menoquinones (vitamina K2) (Danziger, 2008). Aleshores, la inhibició específica de la vitamina K1 mantindria els efectes beneficiosos del tractament sense afectar la carboxilació perifèrica.

3.1 La coagulació

Quan l'endoteli s'activa en resposta a la IL-1, el TNF o la trombina, internalitza la trombomodulina i sintetitza factor tisular (FIII) i l'inhibidor de l'activador del plasminògen (PAI-1) (Sidelmann et al,. 2000), redueix la secreció de prostaciclina i s'incrementa la del factor activador plaquetar i multímers vWF d'alt pes molecular, que contribueixen a l'adhesió plaquetaria a la paret del vas lesionat. L'agregació plaquetar sense requerir la formació de trombina es coneix com hemostasi primària (Baumgartner, 1973).

La coagulació sanguinia **(figura 9)** engloba un seguit de reaccions bioquímiques que es propaguen escalonadament entre els successius complexos enzimàtics (Macfarlane, 1964). Cada zimògen és activat per una serin-proteasa depenent de vitamina K i cofactors que s'hi uneixen a la superficie de la membrana fosfolipídica de les plaquetes, endoteli vascular o altres cèl·lules circulants, especialment els monòcits activats (Spronk et al, 2003). La lisi del pèptid d'activació del zimògen provoca un canvi conformacional del domini catalític que deixa accessible el centre actiu i li permet catalitzar la següent reacció. D'aquesta forma, la velocitat i eficiència de la reacció s'incrementa de manera exponencial (Macfarlane, 1964). Aquestes reaccions són fortament depenents dels ions Ca^{2+} i els fosfolípids, a excepció de la interacció entre la trombina (FIIa) i el fibrinògen (Clowes et al, 2005).

La composició dels coàguls varia segons la localització on s'originen: el trombus arterial es forma en un ambient de fluxe ràpid i es composa principalment d'agregats de plaquetes units a filaments de fibrina (Baumgartner, 1973), mentre que el trombus venós es forma en regions de baix flux i es composa d'hematies amb una gran quantitat de fibrina i un baix contingut en plaquetes (Hirsh et al, 2001). La composició del coàgul, en particular la riquesa en fibrina, afecta la capacitat de lisi dels trombus i s'ha proposat com un predictor d'eficàcia del tractament trombolític a l'ictus isquèmic (Kim et al, 2006).

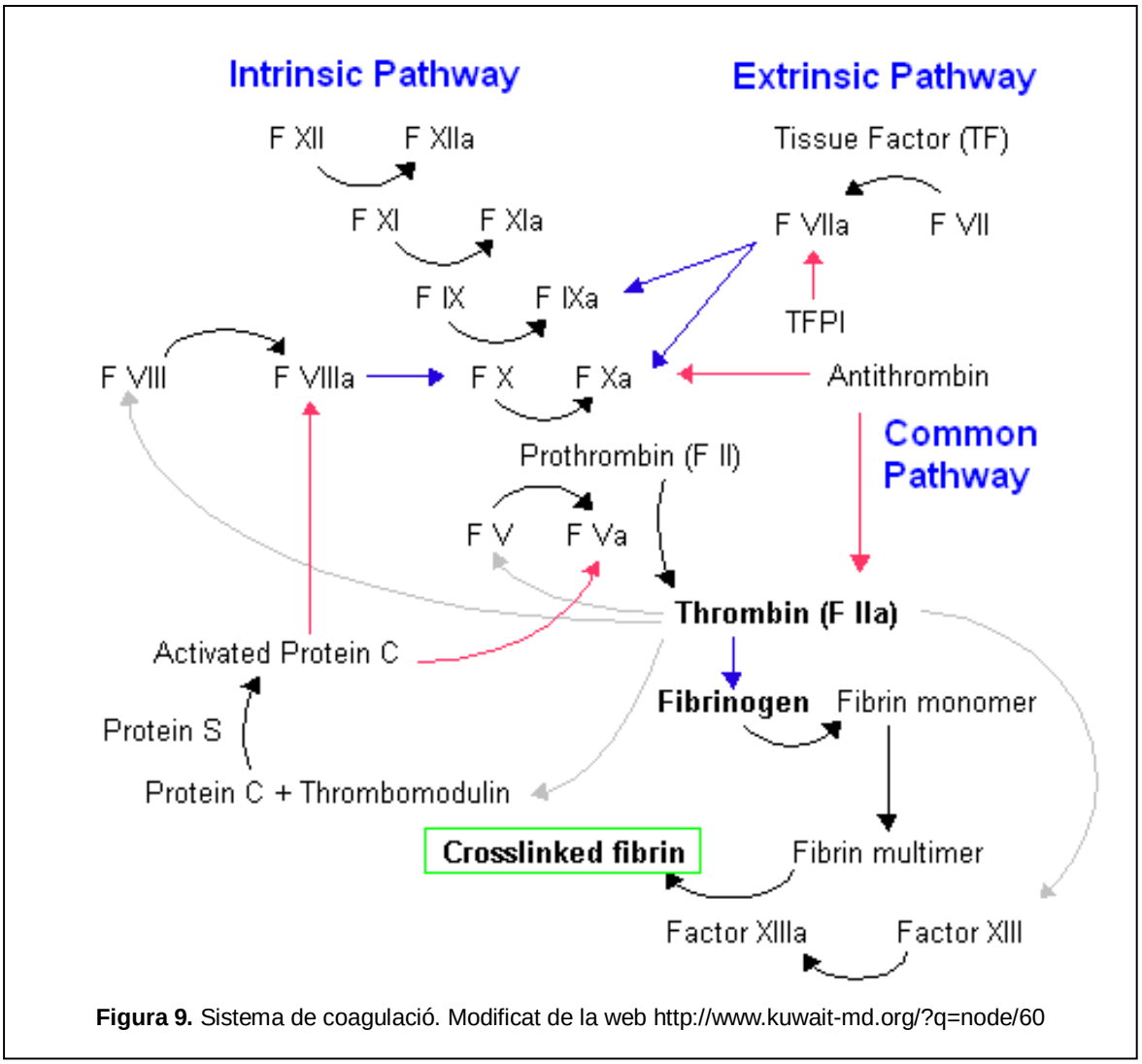


Figura 9. Sistema de coagulació. Modificat de la web http://www.kuwait-md.org/?q=node/60

3.1.1 Via extrínseca

La via extrínseca **(figura 9)** és la via principal de coagulació sanguínea "in vivo" en resposta a una lesió (Spronk et al, 2003). Aquesta via la inicia el FIII (Mackman, 2004), una proteïna integral de membrana que s'exposa a la sang quan es produeix una lesió vascular. Aleshores, el factor VII (FVII) s'hi uneix i s'activa ràpidament (FVIIa). El complex FVIIa/FIII activa els factors IX (FIX) i X (FX) a FIXa i FXa (Mackman, 2004). El factor IXa s'uneix al FVIIIa i el factor X, activant el factor Xa de forma 50 vegades més eficient que el complex FIII-FVIIa-FX, ja que el TFPI o l'antitrombina inhibeixen aquest últim (Broze, 1995). El FXa que resta unit a la superfície cel·lular pot combinar-se amb el FVa per produir petites quantitats de trombina i promoure l'activació i adhesió plaquetar, amplificant la senyal procoagulant inicial i l'activación dels factors FV, FVIII y FXI (Spronk et al, 2003).

La deficiència dels factors d'aquesta via provoca desordres hemorràgics greus com la hemofilia tipus A (dèficit FVIII), la hemofilia tipus B (dèficit FIX) o sagnats moderats com les del FVII o el FX (Peyvandi et al, 2008).

Relacionada amb la deficiència de FVIII també trobem la malaltia de von Willebrand, provocada per l'absència (tipus I i III) o la disfunció d'aquest cofactor (tipus II) (Ahmad et al, 2009). L'associació del vWF amb el FVIII l'estabilitza i n'evita la seva degradació, pero també participa a l'agregació plaquetar (Spiel et al, 2008). Com als casos anteriors, aquesta malaltia també es caracteritza per sagnats repetitius.

Per contra, els nivells elevats de vWF s'han associat al risc de malalties trombòtiques com l'infart de miocardi, cardiopatia isquèmica i nefropatia diabètica (Lacquemant et al, 2000; Klemm et al, 2005). Els nivells de vWF estan regulats principalment pel grup sanguini ABO (Ostarvik et al, 1985; Souto et al, 2000), tot i que també hi influeixen altres variants del grup sanguini (Ostarvik et al, 1989), al gen del vWF (Keightley et al, 1999; Harvey et al, 2000; Lacquemant et al, 2000; Klemm et al, 2005) o en altres locus (Souto et al, 2003; Smith et al, 2010).

3.1.2 Via intrínseca

La via intrínseca o de contacte **(figura 9)** és un mecanisme alternatiu d'activació del sistema de coagulació. S'inicia per l'activació del FXII (FXIIa), que activa el FXI (FXIa) i el FXIa activa el FIX (FIXa) (Spronk et al, 2003). En aquest procès participen el kininògen d'alt pes molecular i la pre-kalikreína com a cofactors.

La funció fisiològica d'aquesta via no està establerta, ja que no juga un paper important a

la coagulació iniciada per una lesió. Les deficiències dels seus components no estan associades a problemes hemorràgics, excepte en el dèficit del FXI, que provoca un sagnat moderat (O'Connell, 2003), mentre els nivells elevats de FXIa s'ha associat a un risc elevat de patir trombosis arterial (Minnena et al, 2000).

La deficiència del FXII, per contra, s'ha relacionat amb trombosi arterial i venosa i avortaments repetitius (Halbmayer et al, 1992; Pauer et al, 2003; Kuhli et al, 2004), el que podria explicar-se pel seu rol dual al sistema fibrinolític com activador del plasminògen (Levi et al, 1991). A més, el FXII també participa a la cascada de les quinines i l'activació del sistema del complement **(figura 10)**. La seva sobreactivació és causant de l'angioedema hereditari tipus III, mentre la deficiència del seu inhibidor C1q s'ha associat amb els tipus I i II (Cichon et al, 2006), sembla que degut a l'activació de la via de les quinines (Kaplan, 2008).

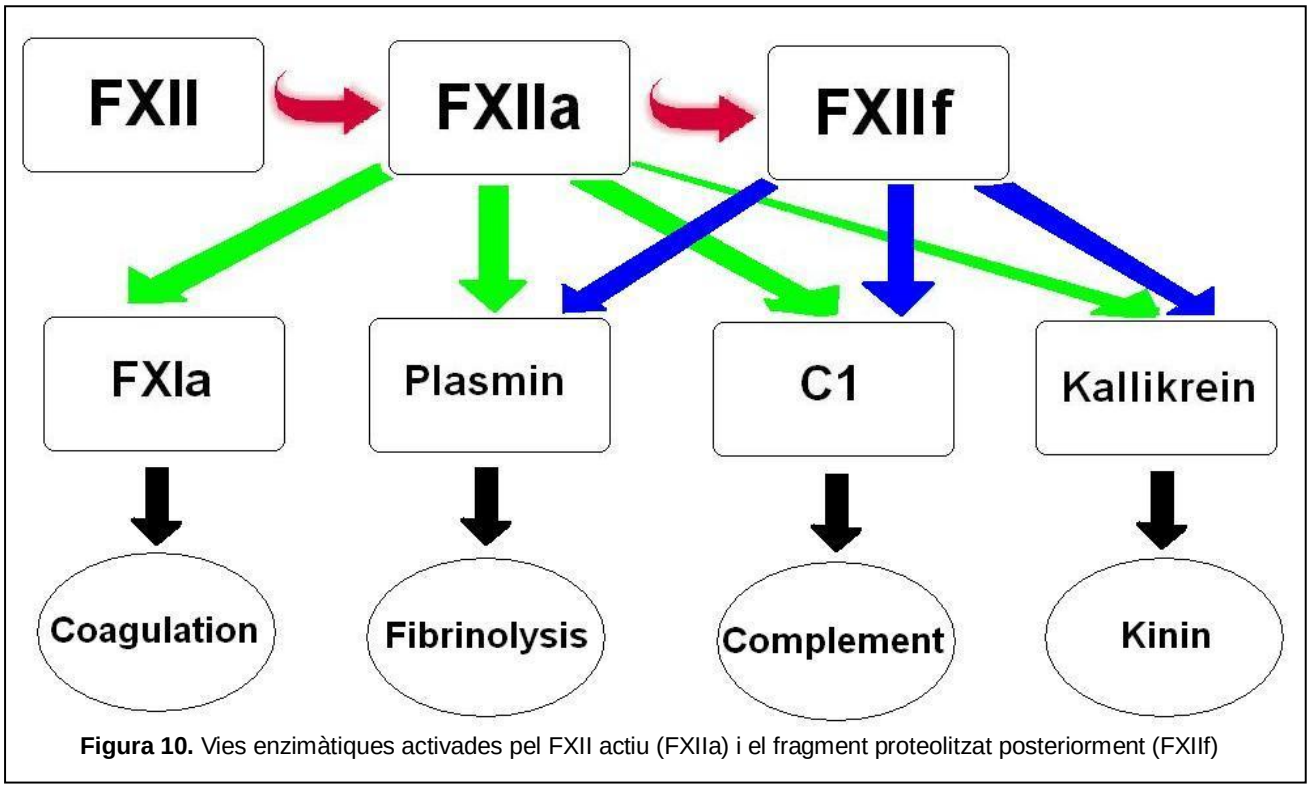


Figura 10. Vies enzimàtiques activades pel FXII actiu (FXIIa) i el fragment proteolitzat posteriorment (FXIIf)

L'activitat del F12 varia segons els nivells d'estròg ens, IL-6, els nivells d'anticossos anti-

FXII i diverses variants genètiques, el que provoca que només existeixi una moderada correlació entre els nivells plasmàtics de la proteïna i la seva activitat coagulativa, mesurada pel mètode APTT (Bach et al, 2008).

3.1.3 Via comuna

La via comuna de la coagulació **(figura 9)** s'inicia per l'activació del FX pel FIXa en presència del FVIIIa, fosfolípids i ions Ca^{2+}. El FVIII es troba a la sang formant un complexe no covalent amb el vWF, que estabilitza la seva activitat coagulant i el localitza als punts d'adhesió plaquetar.

La fase final de la cascada de la coagulació es la formació de trombina (FIIa), catalitzada pel FXa i el FVa (Dahlbäck, 2000). La trombina converteix el fibrinògen en fibrina que polimeritza formant el trombus **(figura 9)**. A més, retroalimenta la via per activació del FIX, FV, FVIII i FXIII.

El fibrinògen està format per tres parells de polipèptids (α, β i γ) units per ponts disulfur. La trombina hidrolitza la molècula de fibrinògen a les cadenes α (Arg_{16}-Gly_{17}) i β (Arg_{14}-Gly_{15}), alliberant els fibrinopèptids A i B. Els extrems amino-terminals α i β reaccionen amb l'extrem carboxi-terminal de la γ, formant-se una fibra de 2 hebres amb els monòmers mig solapats, que s'engrosseix progressivament. Els polímers de fibrina i s'estabilitzen per acció del factor XIII (FXIII) en unions covalents Lys-Gln, incrementant la resistencia química, mecànica i a la fibrinòlisi del coàgul (Ariëns et al, 2000). El resultat final és una malla de fibrina hemostática i estable.

3.2 L'anticoagulació

Els mecanismes d'anticoagulació es classifiquen en 2 tipus:

- Inhibidors dels factors de coagulació activats: antitrombina, cofactor II de l'heparina, TFPI, inhibidor de la C1-esterasa i α1-antitripsina
- Reguladors dels cofactors activats: trombomodulina, proteïna C i proteïna S

3.2.1 Inhibidors dels factors de coagulació activats

L'antitrombina, el cofactor II de l'heparina, la α1-antitripsina i l'inhibidor C1-esterasa pertanyen al grup de les serpines (***SER****in* ***P****rotease* ***IN****hibitor*), amb elevada homologia estructural i un mecanisme d'acció similar (unió com a fals substrat) per inhibir les serin-proteases (Carrell et al, 1987; Carrell, 2004).

La unió del factor activat i l'inhibidor forma un complex de transició inestable. El factor proteolitza un enllaç peptídic a la molècula de l'inhibidor i es genera un complex intermedi que, segons les condicions de reacció, pot estabilitzar-se en un complex covalent estable o poden dissociar-se quedant l'inhibidor inactivat i la proteasa activa. Per aquest motiu, l'eficiència de la inhibició dels enzims per les serpines es relativament baixa, molt menor de la relació 1:1 teòrica. No obstant, alguns cofactors poden accelerar i afavorir la formació dels complexes estables factor activat:inhibidor.

L'antitrombina és el principal inhibidor de la trombina (FIIa) i FXa, però també dels factors FIXa, FXIa, FXIIa, kallikreina i el complex FVIIa-FIII, així com la proteïna C activada (PCa). L'antitrombina forma un complex ternari amb l'heparàn-sulfat i la trombina. La trombina trenca l'enllaç Arg_{393}-Ser_{394} al centre actiu de l'antitrombina, provocant un canvi conformacional que atrapa la trombina. El complex trombina-antitrombina s'uneix a la vitronectina i són internalitzats (Faioni, 1994).

El cofactor II de la heparina té un 25% d'homologia amb l'antitrombina i inhibeix específicament la trombina. La seva activitat també s'incrementa notablement en presència de glicosaminoglicans, però l'estímul més potent és el dermatan-sulfat enlloc de l'heparan-sulfat (Faioni, 1994).

L' α-1-antitripsina és un inhibidor de proteases d'ampli espectre, que actua tant sobre la coagulació (ex. Trombina i FXIa) com l'anticoagulació (proteïna C activada) i la fibrinòlisi (plasmina), a més d'altres fenòmens inflamatoris (principalment, l'elastasa provinent dels neutròfils) (Rodríguez Bueno, 2007).

L'inhibidor C1q esterasa actua a l'inici de la via de coagulació intrínseca sobre el FXIIa, FXIa i les kallikreines. La seva funció biològica principal és la inhibició de la vía del complement, a l'inici de la via clàssica d'activació. La seva deficiència o disfunció causa l'angioedema hereditari tipus I i II (Cichon et al, 2008).

L'inhibidor depenent de Proteïna Z (IPZ) també pertany a la familia de les serpines. Inactiva el FXIa i, en presència de proteïna Z, inhibeixen el FXa sobre la superfície fosfolipídica. La deficiència d'IPZ pot desencadenar trombosis, mentre la manca de proteïna Z també pot provocar un sagnat excessiu en intervencions quirúrgiques (Rodríguez Bueno, 2007).

El TFPI viatja unit a lipoproteïnes (LDL i VLDL) i un 5-10% en forma lliure. També és secretat de l'endoteli i les plaquetes quan s'activen per acció de la trombina. L'activitat anticoagulant es produeix en 2 etapes utilitzant 2 centres actius diferents: primer s'uneix 1:1 de forma reversible amb el FXa i aquest complex binari alhora s'uneix al binomi FVIIa-

FIIIa en un complex quaternari.

3.2.2 Reguladors dels cofactors activats

La trombomodulina és una proteïna integral de membrana que s'uneix a la trombina mitjançant els seus dominis EGF-like, provocant-li un canvi conformacional que la inhibeix i, a més, afavoreix el reconeixement del binomi trombina-antitrombina per la proteïna C, a la qual activa (PCa). Minoritàriament, la proteïna C pot activar-se pel factor Xa i la plasmina (Varadi et al, 1994).

La PCa s'uneix als fosfolípids de membrana i degrada els factors Va i VIIIa, un procés en el qual la proteïna S i el factor V actuen com a cofactors. La proteïna S lliure capaç d'unir-se a la proteïna C està modulada per la unió entre la proteïna S i la proteina reguladora del complement C4b. La PCa és lentament neutralitzada per l'inhibidor de la proteïna C, l'α-2-macroglobulina i l'α-1-antitripsina.

3.3 La fibrinòlisi

La fibrinòlisi és el mecanisme pel qual es lisa la fibrina del trombus, en un procés anàlog al de coagulació. La reacció final és la conversió del plasminògen en plasmina, la qual pot hidrolitzar el coàgul de fibrina i solubilitzar-lo (Makris, 2005) **(figura 11)**.

La formació de plasmina està catalitzada endógenament per la uroquinasa activadora del plasminògen (u-PA) i l'activador tissular del plasminògen (t-PA) (Lijnen & Collen, 1982). La u-PA és una proteïna de superficie cel·lular responsable de l'activació del plasminògen a nivell tisular (Mondino & Blasi, 2004), mentre el t-PA és una proteïna soluble responsable de l'activació del plasminògen a nivell intravascular (Yepes et al, 2004). Ambdós activadors proteolitzen el plasminògen entre els residus Arg_{560}-Val_{561}, formant 2 cadenes

de plasmina unides per 2 ponts disulfur.

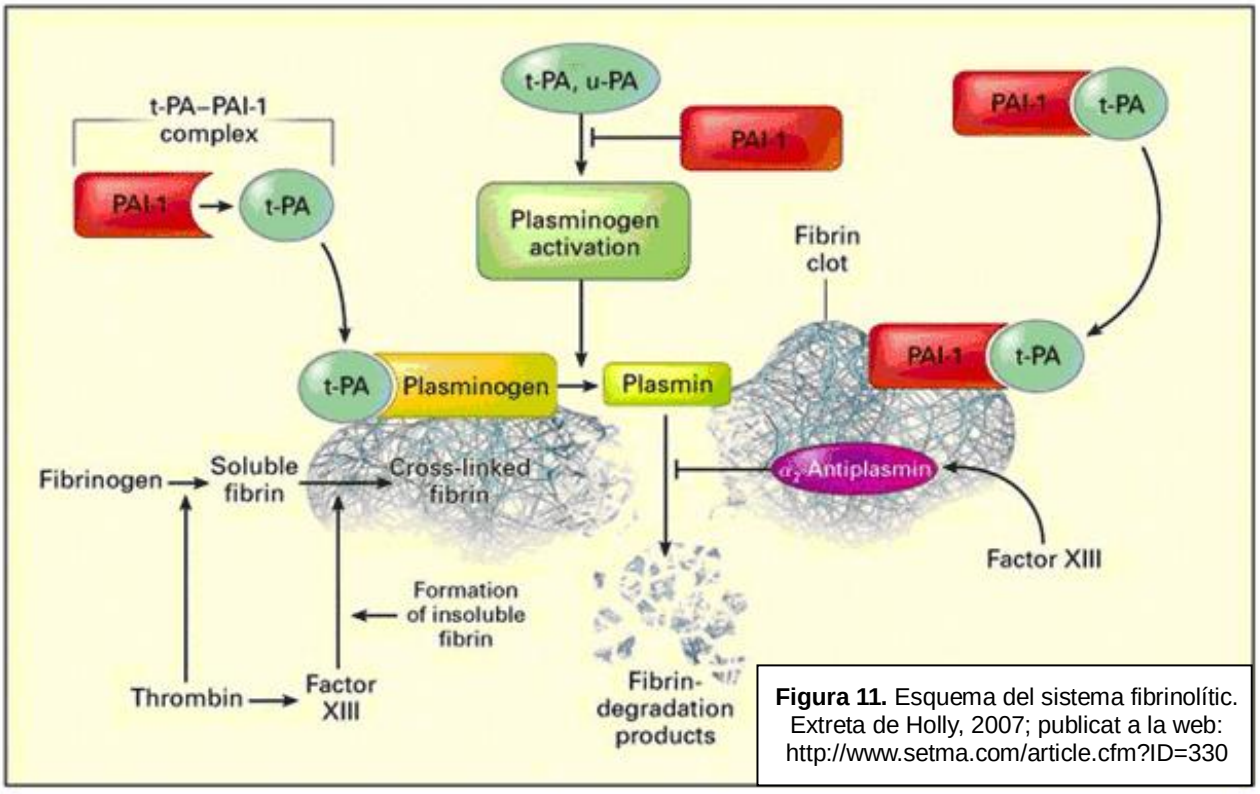


Figura 11. Esquema del sistema fibrinolític. Extreta de Holly, 2007; publicat a la web: http://www.setma.com/article.cfm?ID=330

La fibrinòlisi comença amb la secreció del t-PA desde l'endoteli vascular al torrent circulatori. En presència de fibrina, el plasminògen i el t-PA s'hi adhereixen a la superficie (unint-se als residus lisina) i el plasminògen és transformat en plasmina, protegida de la inactivació per l'α-2-antiplasmina en tenir ocupat el centre actiu (Makris, 2005). Per contra, la plasmina lliure, generada al plasma, será ràpidament neutralitzada per l'α-2-antiplasmina i l'α-2-macroglobulina (Preissner, 2004). Finalment, la plasmina unida a fibrina la hidrolitza en diferents fragments solubles. La fibrina parcialment degradada exposa residus lisina que són aceptors de noves molècules de plasminògen, les quals són transformades en plasmina pel t-PA, produint-se una fibrinòlisi progressivament accelerada (Fleury et al, 1993).

El t-PA regula el mecanisme fibrinolític en exercir la seva acció als dipòsits de fibrina, mentre que al plasma la proteòlisi està limitada per la baixa afinitat de l'activador pel plasminògen i la ràpida inhibició de la plasmina formada. El procés fibrinolític és, per tant, desencadenat per l'acció de la fibrina i queda confinat a la seva superficie (Wiman & Collen, 1978).

3.4 L'anti-fibrinòlisi

La trombina protegeix els coàguls de la fibrinòlisi en augmentar la secreció de PAI-1 endotelial i activar una carboxipeptidasa plasmàtica, el TAFI (***T****hrombin* ***A****ctivable* ***F****ibrinolysis* ***I****nhibitor*) (Bajzar et al, 1995). Altres inhibidors de l'acció del t-PA són el PAI-2 i el PAI-3, mentre la plasmina pot ésser inhibida directament per l'α2-antiplasmina i l'α2-macroglobulina (A2M).

3.4.1 Inhibició de la pro-fibrinòlisi

El TAFI és una carboxipeptidasa B de síntesi hepàtica, que circula unida als residus lisina del plasminògen mitjançant el seu propèptid. És activat pel complexe trombina-trombomodulina i proteolitza els residus lisina C-terminals a la malla de fibrina parcialment digerida per la plasmina, prevenint la unió del plasminògen, la plasmina, o el t-PA a la fibrina inhibint secundàriament la fibrinòlisis (Bazjar et al, 2000). La plasmina regula l'activitat del TAFI mitjançant proteòlisi als residus 302, 327 i 330, inactivant el TAFI. El TAFI activat restant circula unit no covalentment a l'A2M i s'elimina per aclariment renal.

L'inhibidor de l'activador del plasminògen tipus 1 (PAI-1) és el principal inhibidor del t-PA i u-PA. És una serpina de síntesi hepàtica, endotelial i als megacariocits, que es troba acumulat als grànuls "α" plaquetars o circulant al plasma en complexe amb la vitronectina. La seva activació es espontània i reversible, la majoria del PAI-1 circulant es troba en

forma activa. S'uneix en complexes 1:1 a l'u-PA i el t-PA, que són internalitzats pel receptor LRP (***L****DL* ***R****elated* ***P****rotein*) per a la seva degradació. El seu rol fisiològic és neutralitzar l'excés de t-PA existent al torrent circulatori i impedir la dissolució de la fibrina al tap hemostàtic. Durant la fase inicial de la coagulació, les plaquetes activades i les cèl·lules endotelials alliberen PAI-1 als punts de lesió vascular, una cop realitzada l'agregació plaquetària. L'activitat de PAI-1 és sensible a la inflamació, ja que és degradat per l'elastasa dels granulòcits i les MMPs (Rodríguez Bueno, 2007).

L'inhibidor de l'activador del plasminògen tipus 2 (PAI-2) és un inhibidor del u-PA i, en menor grau, del t-PA. L'alliberen els macròfags activats a la zona de la lesió. La seva concentració plasmàtica augmenta notablement durant l'embaràs per la seva producció a la placenta. El PAI-2 s'uneix a la malla de fibrina per acció del FXIIIa. Quan l'u-PA s'uneix al PAI-2, es produeix un canvi conformacional que incrementa l'afinitat de l'u-PA pel LRP, internalitzant-se per la seva degradació. A diferència de PAI-1, no es produeix contacte entre PAI-2 i la molècula de LRP (Rodríguez Bueno, 2007).

El PAI-3 té activitat anti-fibrinolítica, per la seva capacitat d'inhibició del u-PA, però també anticoagulant, per la seva unió a PCa, trombina (FIIa), FXa, FIXa i kallikreina (Rodríguez Bueno, 2007). El PAI-3 és d'origen endotel·lial i regula l'acció de la PCa. A més de la seva inhibició directa, regula la producció de PCa dissociant el complex trombina (FIIa) – trombomodulina, alliberant-se trombina inactivada.

La seva concentració plasmàtica és reduïda i els seus efectes són més importants en orina i líquid seminal, on s'ha vist relacionada amb problemes d'infertilitat. La seva capacitat inhibitòria requereix de la unió a trombomodulina o heparàn sulfat (Rodríguez Bueno, 2007).

3.4.2 Antifibrinòlisi directa

L' α2-antiplasmina és el principal inhibidor fisiològic de la plasmina lliure, tot i que també pot inhibir la PCa. És una serpina de síntesi hepàtica, que circula unida als residus lisina del plasminògen (60%) o en forma lliure (40% restant). A més de segrestar part del plasminògen circulant, es fixa covalentment a la fibrina per acció del FXIIIa, impossibilitant l'acció de t-PA i u-PA. La seva proteòlisi a l'extrem N-terminal (residus 12-13) incrementa la seva afinitat per la fibrina i capacitat inhibitòria, mentre la proteòlisi C-terminal (residus 438-439) la redueix.

L'A2M és el segon inhibidor principal de la plasmina, després de l' α2-antiplasmina. És un inhibidor general de proteases, que també actua sobre altres serin-proteases com la PCa, t-PA, FXIIa, FXa, trombina (FIIa) i kallikreina. De fet, és responsable del 25% de l'activitat antitrombínica. La seva expressió s'incrementa durant l'embaràs i els primers anys de vida, el que pot compensar temporalment el dèficit d'altres proteïnes com l'antitrombina (Rodríguez Bueno, 2007).

De síntesi ubicua, circula a elevades concentracions al plasma com un tetràmer formada per enllaços no covalents entre dímers creats per la unió antiparal•lela de ponts disulfur (residus 255 i 408). Els tetràmers formen una estructura en forma de "H" o doble gàbia amb dues cavitats grans al centre de la mol·lècula, on es localitza el domini "bait" (aa. 667-705), una regió amb seqüències diana per nombroses proteases (Mortesen et al, 1981). Després de la unió a la proteasa i la seva proteòlisi al domini "bait", l'A2M pateix un petit canvi conformacional que exposa la regió d'enllaç tioèster (residus 949 i 952) vers la proteasa. L'atac nucleofílic per part d'una amina primària de la proteasa provoca un gran canvi estructural, la gàbia es tanca i atrapa la proteasa al seu interior, inhibint-la

irreversiblement (Salvesen & Barret, 1980; Salvesen et al, 1981). Aquesta estructura és més compacta, té una mobilitat electroforètica superior i és coneguda com A2M* o *fast*-A2M (Branson et al, 1984) **(figura 12)**.

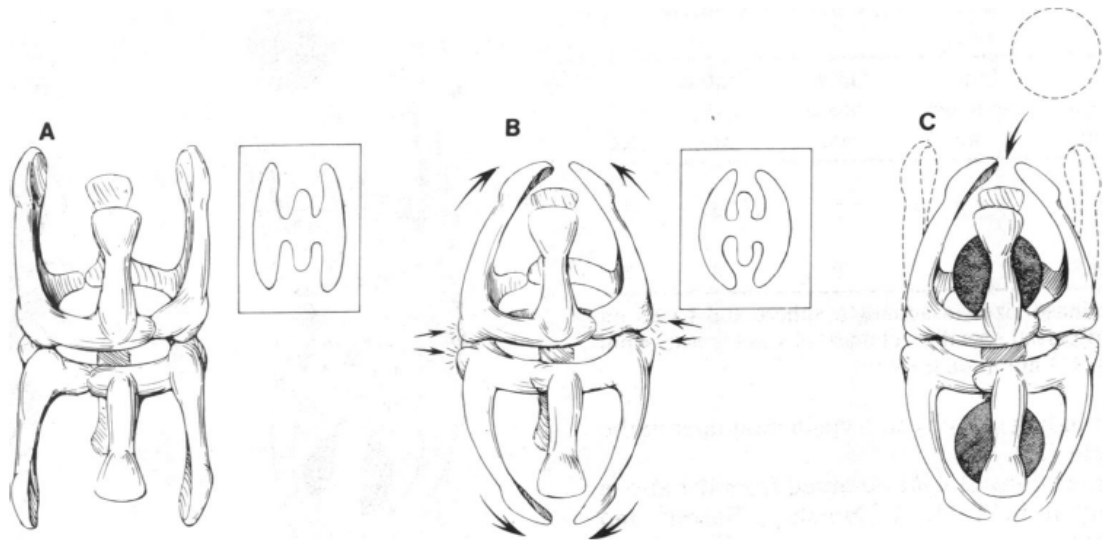


Figura 12. Mecanisme d'activació de l'A2M. Forma nadiva (A), activada (B) i activada unida a proteasa (C). Extreta de Feldman et al, 1985

Amb el canvi conformacional, s'exposen els domini "RBD" (***R**eceptor **B**inding **D**omain*, aa. 1314-1451) que permet la internalització del complexe pel receptor LRP (Kaplan & Nielsen, 1979; Imber & Pizzo, 1981). És un procés ràpid que es complerta en un període de 2 a 5 minuts (Imber & Pizzo, 1981). Dins la cèl·lula, el complexe és transportat en endosomes que s'acidifiquen i es produeix una degradació per via lisosomal (Kaplan & Nielsen, 1979). Aquesta activitat anti-proteasa evitaria una destrucció excessiva del teixit durant la resposta inflamatòria de l'ictus, on s'incrementa la concentració d'A2M (Fletcher et al, 1976). La internalització d'A2M pot activar el receptor GRP78 i provocar canvis als nivells de calci, inositol fosfat i cAMP (Misra et al, 1993; Misra et al, 1994). Així, l'A2M podria actuar com un sensor de l'activitat proteolítica (Chu et al, 1994).

L'A2M també pot unir-se a nombrosos factors de creixement mitjançant un altre domini

d'unió (aa. 718-733) (Rodríguez Bueno, 2007). De fet, a més de la seva activitat anti-proteasa, l'A2M regula l'activitat de nombrosos factors de creixement: FGF, NGF, PDGF, TGF-β1 i TGF- β2 i mol·lècules inflamatòries: TNFα, IFNγ, IL-1β i IL-6 (Borth & Luger, 1989; Matsuda et al, 1989). Algunes d'aquestes mol·lècules (PDGF, TGF-β1, TGF-β2, i IL-6) s'uneixen a la forma nativa d'A2M, que funcionaria com una proteina transportadora que les protegiria de la seva degradació proteolítica o del filtratge glomerular (Matsuda et al, 1989). Altres citoquines, com TNFα i IL-1β, s'uneixen a la forma activa A2M* i són ràpidament eliminades del torrent sanguini (Borth & Luger, 1989).

Finalment, A2M participa a la resposta immune afavorint la presentació d'antígens: el complexe Antígen-A2M* permet obtenir produccions d'anticòs molt superiors (Chu et al, 1994; Cianciolo et al, 2001) o generar una resposta immunitària amb quantitats d'antígen molt inferiors (Chu & Pizzo, 1993; Liao et al, 2002).

4. Epidemiologia i factors de risc vascular

Els factors de risc vascular poden classificar-se segons la capacitat que tenim d'actuar sobre ells, ja sigui mitjançant fàrmacs o canvis de comportament (modificables i no modificables). Dins els modificables trobarem la hipertensió, diabetis, dislipèmia, fibrilació auricular i altres cardiopaties, entre d'altres. Dins els no modificables classificarem l'edat, el sexe, la raça, el pes en el moment del naixement i els factors genètics **(taula).**

FACTORS DE RISC VASCULAR	
MODIFICABLES	NO MODIFICABLES
Hipertensió	Edad
Diabetis	Sexe
Habits tòxics (tabac, alcohol, drogues)	Raça
Dislipèmia	Fibrilació auricular i altres cardiopaties
Obesitat i sedentarisme	

L'edat avançada és el factor de risc vascular més conegut; a partir dels 55 anys el risc d'ictus es duplica cada dècada i es triplica a partir dels 80 anys (Sacco et al, 1997). La influència del sexe a la incidència d'ictus varia segons l'edat. En general, és més elevada en homes, però existeixen dos grups d'edat on la tendència s'inverteix: les dones de 35-44 anys, relacionant-se amb l'embaràs i l'ús de contraceptius orals, i en dones més grans de 85 anys, per la mort anterior dels homes amb factors de risc vascular (Goldstein et al, 2010; Kelly-Hayes, 2010).

La raça o etnia també influeix a la prevalença d'ictus en població nord-americana: mentre a la població caucàsica és d'un 2.3%, als afro-americans és del 4%, en asiàtics del 1.6%, 2.6% en hispans i 6% en població indígena americana (Lloyd-Jones D et al, 2010). El baix pes en nèixer, indicat en altres estudis epidemiològics, pot ésser una variable confusora perquè ve condicionat per la regió geogràfica i el nivell social familiar; són necessaris més

estudis per confirmar la seva associació amb un elevat risc d'ictus (Kelly-Hayes, 2010).

Per últim, les cardiopaties en un 41% dels malalts d'ictus (Martí-Vilalta et al, 1999). La fibrilació auricular, l'arritmia més prevalent, multiplica per 5 el risc d'infart cardioembólic (Wolf et al, 1998; Lloyd-Jones et al 2010). Altres arritmies, com el flúter auricular, la fibrilación-flúter o la síndrome del sinus malalt també incrementen el risc d'embolisme cerebral (Wood et al, 1997; Mattioli et al, 1997). Les valvulopaties, especialment l'estenosi mitral, es relacionen amb l'infart embòlic; la coexistència de fibrilació auricular i estenosi mitral multiplica per 18 el risc d'ictus isquèmic (Bogousslavasky et al, 2000). Les malalties coronàries també incrementen el risc d'infart cerebral, del 0,7% al 4,7% dels afectats d'infart de miocardi patiran un ictus durant les quatre setmanes següents (Wolf et al, 1998). D'altra banda, la síndrome d'apnea obstructiva greu del son multiplica per 3,6 el risc d'ictus isquèmic després d'un primer episodi coronari (Calvin et al, 2009).

Entre els factors de risc modificables, el més comú es la HTA. Els individus hipertensos i pre-hipertensos (presió arterial superior a 120/80 mmHg) tenen el doble de risc que els normotensos de patir un ictus durant la seva vida (Lloyd-Jones D et al, 2010).

La diabetes mellitus s'associa de forma independent amb la malaltia cerebrovascular, especialment de tipus aterotrombòtic, amb un **R**isc **R**elatiu (RR) de 1,8 en homes i de 2,2 en dones (Barrett et al, 1988).

El tabaquisme duplica el risc d'ictus, fins i tot els fumadors passius tenen un 25% més de risc d'ictus isquèmic, ***O**dds **R**atio* (OR) 1.25 (IC95% 1.16-1.36) (Bogousslavasky J et al, 2000; Lee & Forey, 2006). El risc sembla incrementarse amb el nombre de cigarrets: la OR és de 2.1 (IC95% 1.5-3.3) per un consum fins a 21 cigarrets diaris i augmenta fins

9,1(IC95% 3.2-26) per consums superiors als 40 cigarrets diaris (Bhat et al, 2008). El consum d'alcohol superior a 60 gr/dia incrementa el RR en 1.69 (IC95% 1.34-2.15), tot i que un consum moderat es relaciona amb un risc menor, RR 0,72 (IC95% 0.57-0.91) (Reynolds et al, 2003). El un estudi cas-control, el consum abusiu de drogues suposava un increment notable del risc d'ictus amb OR 6.5 (IC95% 3.1-13.6) (Kaku & Lowenstein, 1990), tot i que caldrien estudis prospectius per confirmar la magnitud de l'efecte.

La relació entre la dislipèmia i la isquèmia cerebral no es troba tan ben definida com a la cardiopatia isquèmica. Tot i així, els nivells de LDL >130mg/dL i de HDL <35mg/dL s'han relacionat amb el risc l'ictus isquèmic en diversos estudis (Gorelick et al, 1997). S'estima que el 22% del total de morts per malaltia cardiovascular a Espanya són atribuibles al sobrepes (índex de massa corporal ≥ 25) (Banegas et al, 2003). Complementariament, s'ha atribuit un efecte protector a l'activitat física, probablement pels seus efectes sobre la glucèmia, perfil lipídic i presió arterial (Bogousslavasky et al, 2000).

Altres factors, com la migranya, estats pro-inflamatoris, infeccions o la homocisteina, lipoproteína A o la fosfolipasa A2 plasmàtiques elevades, requereixen estudis epidemiològics més extensos per determinar si són veritables factors de risc d'ictus.

El coneixement dels factors de risc modificables permet una acció terapèutica eficient per la prevenció primària (Goldstein et al, 2010) i secundària (Furie et al, 2010) dels events cerebrovasculars. L'enregistrament dels factors de risc no modificables identifica aquells individus on el tractament preventiu ha d'ésser més agressiu, ja que tenen un risc més elevat de patir la malaltia (Goldstein et al, 2010).

5. Component genètic de l'ictus

L'herència genética influeix notablement a la malaltia cerebrovascular; ja que la història familiar d'ictus en un familiar de primer grau incrementa entre 1.5 i 2.5 vegades el risc d'ictus (Kiely et al, 1993) i la prevalença d'ictus en bessons univitelins és 5 cops la dels bivitelins, confirmat en 2 estudis independents incloent 1000 i 2700 parells de bessons (Brass et al, 1992; Bak et al, 2002). Són nombrosos els locus que s'han identificat relacionats amb el risc d'ictus mitjançant estudis de lligament o estudis d'associació, ja siguin de gens candidats o de genoma complert **(figura 13).**

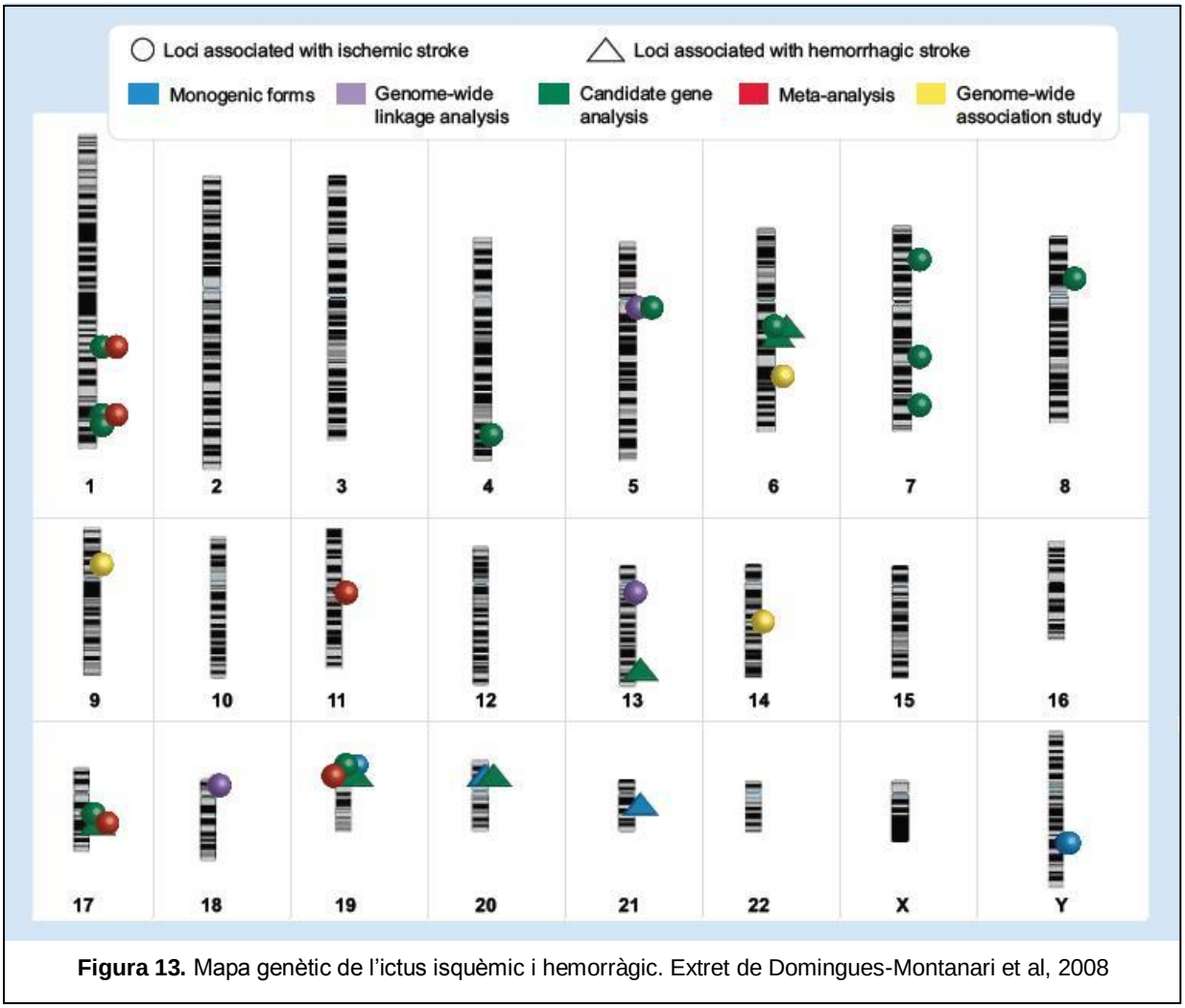


Figura 13. Mapa genètic de l'ictus isquèmic i hemorràgic. Extret de Domingues-Montanari et al, 2008

5.1 Estudis de lligament

Mitjançat estudis de lligament en 100 families amb ictus d'Islandia, es va associar la

Fosfodiestearasa 4D (*PDE4D*) amb el risc d'ictus (Gretarsdottir et al, 2003). La PDE4D podria participar a la progressió de l'aterosclerosi (Gretarsdottir et al, 2003), ja que degrada el cAMP, un transductor de senyals que regula la proliferació de les VSMC (Fukumoto et al, 1999). Els nombrosos intents de replicació posterior mitjançant estudis d'associació han tingut resultats gairebé sempre negatius, indicant que podria tractar-se d'un factor de risc específic de població islandesa (Bevan et al, 2008; Domingues-Montanari et al, 2010).

També en població islandesa, un haplotip format per 4 polimorfismes del gen ALOX5AP duplicaba el risc d'event vascular (infart de miocardi o ictus) (Helgadottir et al, 2004). La proteïna codificada per *ALOX5AP* és una activadora de la 5-lipoxigenasa, participa a la síntesi de leucotriens, participaria al desenvolupament de l'aterosclerosi mitjançant els seus efectes proinflamatoris i la producció de ROS (Dixon et al, 1990). No obstant, la replicació d'aquests resultats no és consistent i podrien ésser depenents de la població analitzada (Bevan et al, 2008; Domingues-Montanari et al, 2010). A la població ibèrica en particular, sembla que *ALOX5AP* sí s'associa a un risc més alt d'ictus (Domingues-Montanari et al, 2010).

5.2 Estudis de gens candidats

Els estudis de gens candidats, basats en el coneixement racional de les vies metabòliques associades a l'ictus, han identificat numeroses variants polimòrfiques associades amb el risc de patir la malaltia, però cap d'elles s'ha replicat de forma consistent, probablement degut al nombre reduit de mostres analitzades, l'heterogeneitat en el disseny dels estudis o que són falsos positius. Aquesta heterogeneitat també dificulta la capacitat de meta-analitzar les dades, arribant-se a conclusions sugestives però no definitives (Casas et al, 2004; Stankovic & Majkic-Singh, 2010).

Els polimorfismes més estudiats en aquest camp són polimorfismes funcionals en vies de coagulació i trombosi, com el FV Leiden (Arg506Gln; rs6025), en hipertensió com l'**E**nzim **C**onversor de l'**A**ngiotensina (ECA) (I/D; rs1799752), metabolisme de la homocisteïna per acció de la **M**etil **T**etra-**H**idro**F**olat **R**eductasa (MTHFR) (C667T; rs1801131) ó metabolisme lipídic com el receptor LRP (C766T; rs1799986) o l'Apolipoproteïna E (alels E2, E3 i E4, haplotips de rs429358 i rs7412) (Casas et al, 2004).

Els nous estudis en gens candidats requereixen homogeneitat al fenotip analitzat, un tamany mostral i una potència estadística suficients per resistir una correcció per test múltiples (ex. Bonferroni o *False Discovery Rate*), la replicació dels resultats en una cohort independent i, si és possible, determinar els efectes funcionals de les variants associades en mostres o models rellevants.

5.3 Estudis d'associació de genoma complet

La seqüenciació del genoma humà provisional l'any 2000 i complerta l'any 2003 (Lander et al, 2001; Venter et al, 2001) i la posterior determinació del mapa de lligament a 4 poblacions model (caucàsics de Utah d'origen europeu; població xinesa Han; japonesos de Tokio i Yoruba d'Ibadan, Nigèria) del projecte internacional HapMap (The International Hapmap Consortium, 2005) va permetre el desenvolupament de plataformes de genotipatge massives que identificaven 100.000, 500.000 o un milió de variants puntuals de tipus SNP distribuides per tot el genoma. Aquestes plataformes van donar lloc als estudis d'associació de genoma complert o ***G**enome-**W**ide **A**ssociation **S**tudies* (GWAS).

L'estudi més rellevant de GWAS va ésser el publicat pel ***W**elcome **T**rust **C**ase-**C**ontrol **C**onsortium* (WTCCC), on es van comparar 2.000 casos de 7 malalties comunes amb un

clar component genètic (trastorn bipolar, malaltia coronària, malaltia de Crohn, HTA, artritis reumatoide, diabetes tipus 1 i diabetes tipus 2) amb 3.000 controls sans compartits, identificant-se nous gens o regions cromosòmiques associades a moltes de les patologies estudiades (WTCCC, 2007).

El gran aventatge d'aquesta mena d'estudis és que no cal una hipòtesi previa o un coneixement extens de les vies implicades al fenotip a estudiar. En canvi, donat l'elevat nombre de variants analitzades i l'alt risc de falsos positius, calen uns tamanys mostrals molt grans (milers de casos i controls) per obtenir senyals significatives ($p<10^{-8}$), han de controlar-se aspectes com la estratificació de la població i són projectes que requereix una gran despesa econòmica i una alta capacitat de computació **(figura 14)**.

L'any 2007 s'inicià l'era dels GWAS dins del camp de l'ictus isquèmic. El primer estudi va genotipar 400000 SNPs en 249 pacients y 268 controls en població caucàsica nord-americana, sense trobar cap variant associada amb el risc d'ictus després de la correcció de Bonferroni, probablement pel baix nombre de mostres analitzades (Matarín et al, 2007). Tot i així, una de les variants al gen *KCNK17*, que mostrava una tendència, s'ha replicat posteriorment en població espanyola (Domingues-Montanari et al, 2010).

El mateix any, es van identificar el SNP rs9943582 al gen *AGTRL1* en un cas-control involucrant 2224 individus i 52608 variants polimòrfiques, que doblaba la incidència d'ictus en un estudi poblacional involucrant 1659 japonesos seguits durant un període de 14 anys (Hata et al, 2007).

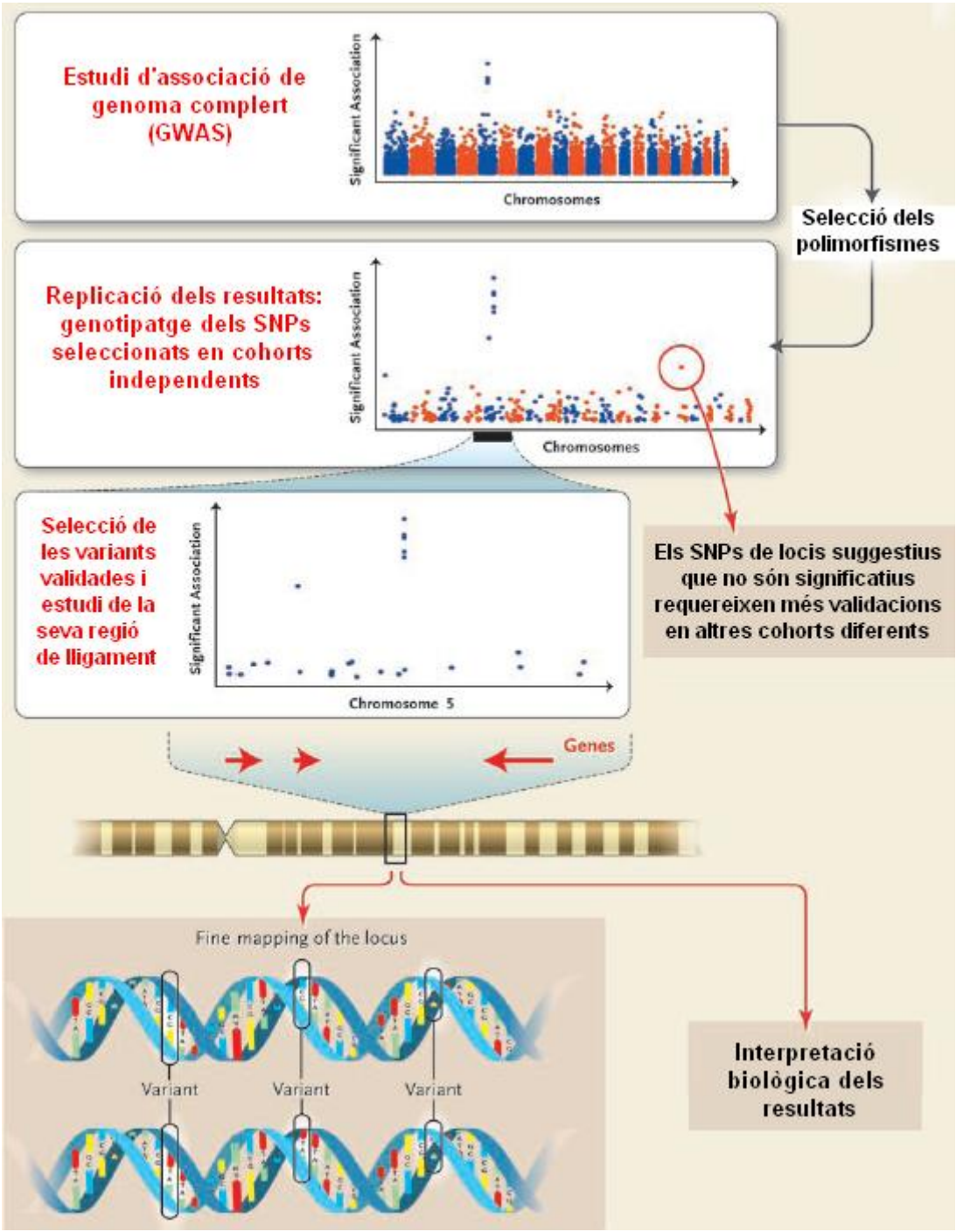


Figura 14. Diagrama de les diferents fases d'un estudi GWAS. Modificat de Hardy & Singleton, 2009

El consorci CHARGE (***C**ohorts for **H**eart and **A**ging **R**esearch in **G**enomic **E**pidemiology*) va indentificar un SNP a la regió 12p13, entre els gens *NINJ2* i *WNK1*, que s'associava a l'ictus (total e isquèmic) en un estudi poblacional amb 1200 casos i 20000 controls, validat internament en 4300 individus caucàsics i 650 afro-americans (Ikram et al, 2009). Els resultats no es van confirmar en un metaanàlisi posterior incloent 8637 casos i 8733

controls o 278 casos d'una cohort de 22054 participants (International Stroke Genetics Consortium, 2010), ni en 3 casos controls en població sueca amb 3606 pacients i 2528 (Olsson et al, 2010).

Dos variants a la regió 4q25 (rs2200733 i rs10033464) circundant el gen *PITX2*, prèviament relacionades amb la fibrilació auricular (Gudbjartsson et al, 2007), es van associar també amb l'ictus d'etiologia cardioembòlica en població islandesa en un estudi GWAS on s'analitzaren 1661 casos i 10815 controls, validant-se en 2 series de 2224 casos / 2583 controls i 2327 casos / 16760 controls (Gretarsdottir et al, 2008). Aquests resultats han estat confirmats independentment en 4199 casos i 3750 controls en població caucàsica (Lemmens et al, 2010).

Una variant al gen *ZFHX3* (rs7193343) també es va associar amb fibrilació auricular en població islandesa en un estudi GWAS involucrant 2385 casos i 33752 controls. L'anàlisi per subgrups va indicar que s'associaba a ictus isquèmic i especialment al d'etiologia cardioembòlica (Gudbjartsson et al, 2009).

Finalment, la variant rs1333049, que representa un interval de 58Kb entre els gens *CDKN2A* i *CDKN2B* a la regió 9p21, és factor de risc d'ictus isquèmic, infart de miocardi, enfermetat arterial perifèrica o mort sobtada (Matarin et al, 2008; Wahlstrand et al, 2009). Sembla que els processos trombòtics estarien provocats per un elevat risc d'aterosclerosi (Burd et al, 2010) i una major reactivitat plaquetar (Musunuru et al, 2010). Es desconeix el mecanisme a nivell subcel·lular que provoca aquest fenotip, però sembla que és degut a regulació d'expressió gènica mitjançant el RNA no codificant ANRIL (Pasmant et al, 2010).

L'efecte de les variants esctructurals al genoma, com les variants del nombre de còpies o

***C**opy **N**umber **V**ariants* (CNVs), encara no s'ha pogut determinar de forma concloent. L'únic estudi de CNV en ictus va incloure 263 casos i 275 controls (Matarin et al, 2008) i no va trobar cap nova variant relacionada amb un risc més elevat de patir ictus isquèmic. D'altra banda, un estudi massiu comptabilitzant 2000 casos de 8 malalties comunes (sense incloure l'ictus) i 3000 controls tampoc va trobar noves variants associades a cadascuna de les malalties (WTCCC, 2010). No obstant, les plataformes utilitzades no eren específiques per CNVs sino per genotipatge de SNPs i la seva resolució encara no és prou elevada per extreu-re'n conclusions definitives.

5.4 Estudis d'associació per etiologies específiques

Donada l'heterogeneitat etiològica de l'ictus isquèmic, probablement relacionada amb els diferents mecanisme patogènics involucrats, i la impossibilitat de trobar un gen o variant polimòrfica consistentment relacionada amb l'ictus, s'han iniciat estudis cas-control específics per les diferents etiologies.

Així, l'any 2007 la variant rs2230500 (1425 G>A ó Val374Ile) del gen *PRKCH*, es va relacionar amb l'ictus lacunar, presumiblement per influir al desenvolupament d'ateromatosis (Kubo et al, 2007). Van genotipar-se 52608 Tag SNPs en 188 casos i 188 controls. Es van seleccionar les 1098 variants més significatives ($p<0.01$) i es van genotipar en uns altres 1848 individus, detectant-se una associació amb el gen *PRKCH*. Finalment, es va seqüenciar el gen *PRKCH* en 96 individus, identificant-se la variant funcional rs2230500, que alteraba l'activitat de la proteïna en estudis "in vitro" amb cultius cel·lulars.

També en població japonesa, s'ha estudiat l'ictus aterotrombòtic en un estudi genotipant 500.000 SNPs en 992 casos i 5349 controls (Yamada et al, 2009). Es van seleccionar 100

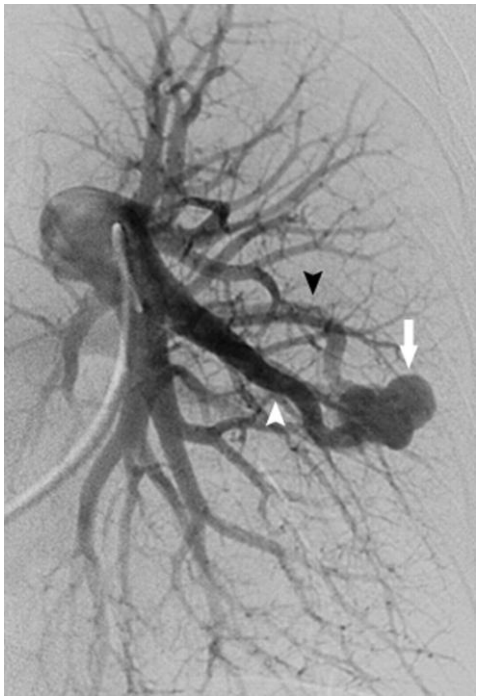
SNP per la validació en 4000 nous individus. Finalment 3 SNPs van associar-se al risc d'ictus: rs1671021 en *LLG2*, rs753307 en *RUVBL2* i rs9615362 en *CELSR1*). Els efectes vindrien originats per les mutacions Ile2107Val (rs4044210) i Thr2268Ala (rs6007897) al gen *CELSR1* i Phe479Leu (rs1671021) al gen *LLG2.*

5.5 Malalties monogèniques causants d'ictus

A més d'aquestes petites influències genètiques (generalment OR<2) sobre la tolerància a la isquèmia i/o els seus factors de risc, existeixen numeroses malalties monogèniques que cursen amb ictus o poden presentar infarts cerebrals. A més de per la freqüència d'ictus, aquestes malalties també es poden classificar pel territori vascular afectat (petit o gran vas) (Razvi & Bone, 2006).

D'entre les malalties que poden presentar ictus, podem destacar la malaltia de Rendu-Osler-Weber o Hemorràgia-Telangectàsia Hereditària (HHT) (MIM 187300). El diagnòstic es realitza per la presència d'un mínim de 3 dels 4 criteris clínics de Curaçao: epistaxis, telangectàsies, malformacions arteriovenoses (MAV) i història familiar de la simptomatologia. A nivell neurològic, la malaltia pot cursar amb hemorràgies cerebrals per la ruptura d'una MAV o amb ictus de tipus embòlic, originat a la fístula pulmonar.

Els gens mutats són l'endoglina (*ENG*, al cromosoma 9q33) (McAllister et al, 1994) o el receptor d'activina tipus quinasa 1 (*ALK-1* al cromosoma 12q11) (Johnson et al, 1996). Les mutacions puntuals en aquests gens provoquen el funcionament deficient de la proteïna i la malaltia per haploinsuficiència. S'ha observat una correlació entre mutacions a l'endoglina, la presència de fístules pulmonars i l'ictus isquèmic **(figura 15).**



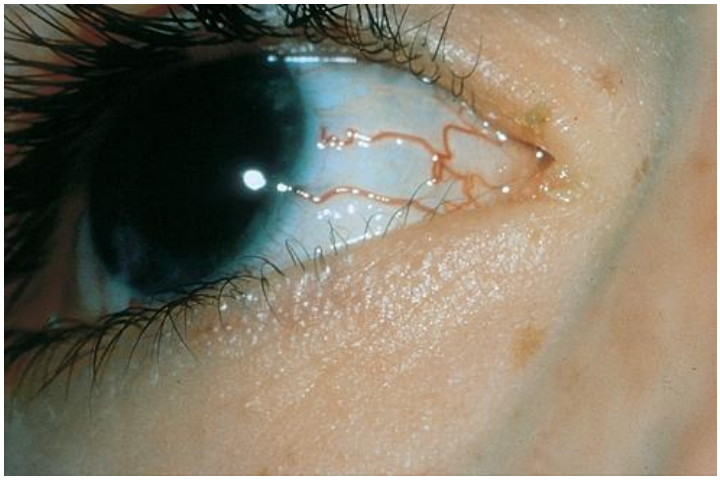


Figura 15. Malformació arteriovenosa pulmonar (esquerra) amb la MAV (fletxa blanca gran), l'artèria irrigant (blanca petita) i la vena (negre petita), extreta de Jaskolka et al, 2003).

Hemorràgia Telangectàsia Hereditària a l'ull (dreta). Extreta de Kane et al, 2004.

També és interessant la malaltia de Fabry (MIM 301500), pel fet de disposar de tractament efectiu. La patololgia és causada per la disfunció o deficiència de l'enzim α-galactosidasa (gen *GLA*, regió Xq22) (Eng et al, 1993), que provoca un acúmul d'esfingolípids globotriaosilceramida (Gb3) als lisosomes, notòriament a les cèl·lules endotel·lials vasculars, que poden acabar degenerant en dolicoectasis i produint la oclusió del vas. És la segona malaltia més prevalent d'entre les malalties lisosomals de dipòsit de lípids (Meikle et al, 1999). La simptomatologia neurològica apareix a la forma clàssica o greu (activitat α-galactosidasa<1%), mentre defectes parcials (activitat 1-30%) presenten síndromes parcials d'afectació cardíaca i/o renal (Mendióroz et al, 2006).

La simptomatologia general inclou insuficiència renal, cardiopatia (insuficiència mitral, miocardiopatia hipertròfica, cardiopatia isquèmica), angioqueratomes cutanis, hipoacusia progressiva, problemes gastrointestinals i opacitat corneal (*còrnia verticilata*). A nivell neurològic, els símptomes inclouen dolor paroxístic a les extremitats (crisis de Fabry), hipohidrosia, dolor pospandrial, hemiparèsia, vertigen, diplopia, disartria, nistagmus,

cefalea i atàxia. A nivell radiològic, s'ha descrit la calcificació dels ganglis de la base i l'aparició d'infarts lacunars en territori posterior. L'afectació del pulvinar en seqüències T1 s'ha considerat un signe patognomònic de la malaltia (Takanashi et al, 2003) **(figura 16)**. L'aparició dels infarts lacunars podria estar provocada per una hipertensió nefròpena, juntament amb l'oclusió per dipòsit de les artèries perforants, tot i que l'afectació cardíaca també podria causar ictus d'origen embòlic (Mendióroz et al, 2006).

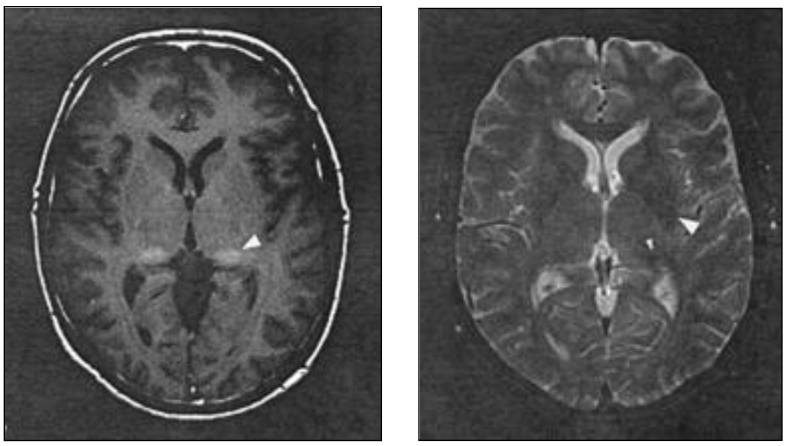


Figura 16. Hiperintensitat del pulvinar a la seqüència T1 i T2, patognomònica dels malalts de Fabry (fletches blanques). Extret de Takanashi et al, 2003.

El tractament és per remplaçament enzimàtic amb infusió de la proteïna recombinant comercialitzada per les companyies Genzyme i Shire (Blom D et al, 2003). Tot i ésser efectiu i atacar la causa de la patologia, no té efectes retroactius; els danys provocats pels dipòsits de Gb3 són irreversibles. S'ha de fer atenció al patró facial d'aquests malalts, que ens pot fer sospitar de la malaltia i aconseguir un diagnòstic precoç **(figura 17)**.

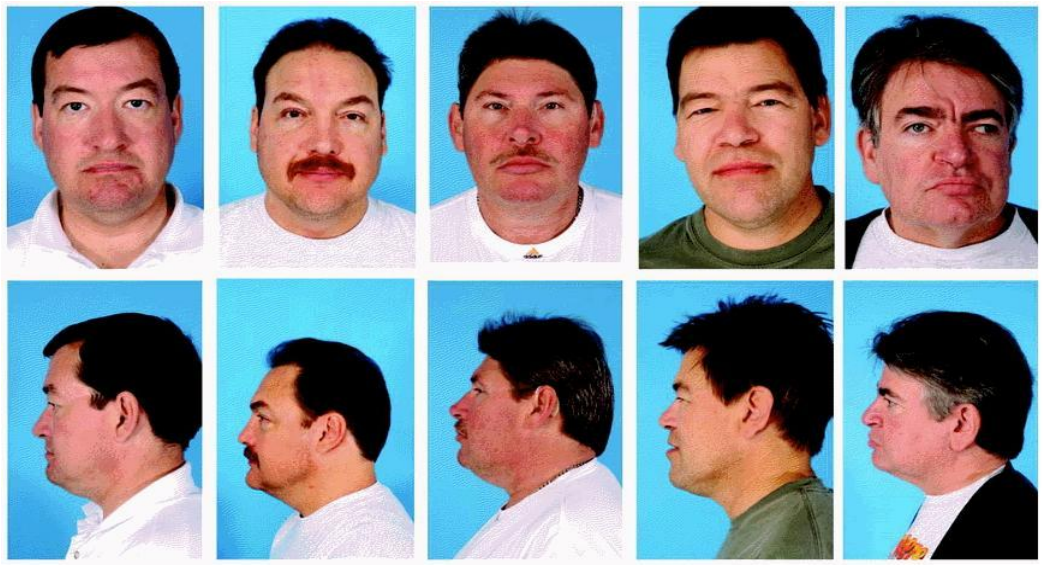


Figura 17. Patró facial dels malalts de Fabry. Extret de Ries et al, 2006.

D'entre les malalties que cursen amb ictus, la malaltia cerebrovascular hereditària més prevalent i causa principal de demència vascular és la síndrome de CADASIL *(**C**erebral **A**utosomal **D**ominant **A**rteriopathy with **S**ubcortical **I**nfarcts and **L**eukoencephalopathy)*. La síndrome de Maeda o CARASIL *(**C**erebral **A**utosomal **R**ecessive **A**rteriopathy with **S**ubcortical **I**nfarcts and **L**eukoencephalopathy)*, va ésser considerada inicialment una forma recessiva de la mateixa síndrome, però tant el curs de la malaltia, l'origen genètic i la via metabòlica alterada ho han descartat (Hara et al, 2009).

6. La síndrome de CADASIL

La CADASIL (MIM 125310) es una angiopatia sistèmica, d'herència autosòmica dominant, caracteritzada per migranya (normalment amb aura severa i perllongada) i infarts lacunars repetitius que evolucionen cap a la demència vascular. Els pacients de CADASIL perden la capacitat de caminar sense assistència entre 56 i 64 anys d'edat, postració entre els 59 i 69, i moren entre 61 i 74 principalment per problemes respiratoris, com és usual als pacients demenciats (Opherk et al, 2004). La qualitat de vida d'aquests pacients no només està afectada pels infarts recurrents, també per altres símptomes desabilitants com trastorns de l'ànim, migranya i epilèpsia (Davous, 1999; Desmond et al, 1999). Encara que l'epidemiologia no és coneguda, la prevalença de CADASIL a l'oest d'Escòcia és de 1.98 per cada 100.000 adults, 4.14 considerant els portadors de mutació predits (Razvi et al, 2006).

A la Resonància Magnètica, presenten leucoaraiosi prominent afectant usualment la càpsula externa i la part anterior dels lòbuls temporals. A les imatges de resonància es veu una alteració clara després dels 35 anys, éssent un bon marcador de sospita de CADASIL (O'Sullivan et al, 2001). Els infarts lacunars es localitzen a la càpsula externa, ganglis de la base i part anterior del lòbul temporal, en contrast amb el corpus callós i la major part de les regions infratentorials (excepte la protuberància) (Singhal et al, 2005). Les microhemorràgies es troben al tàlem, ganglis basals, unió cortical-subcortical i estructures profundes. La seva prevalença i localització varia entre cohorts (Lesnik Oberstein et al, 2001; Dichgans et al, 2002; Viswanathan & Chabriat, 2006).

Existeixen diversos símptomes infreqüents: hemorràgies intracerebrals (Ragoschke-Schum et al, 2005; Choi et al, 2006; Werbrouck et al, 2006), encefalopatia (Requena et al, 1999; Le et al, 2002; Feuerhake et al, 2002; Schon et al, 2003; Kleinig et al, 2007), retard

mental (Desmond et al, 1998; Kotorii et al, 2001; Ávila et al, 2007) i anomalies retinianes o del nervi òptic, causades possiblement per problemes circulatoris (Schon et al, 2003; Cumurciuc et al, 2004; Haritoglou et al, 2004; Rufa et al, 2005). D'altres signes i símptomes només s'han identificat en una única familia i cal demostrar si formen part del fenotip CADASIL (del Río-Espínola & Mendióroz et al, 2008).

6.1 Diagnòstic de CADASIL

Quan un metge detecta un pacient altament suggestiu de CADASIL, es poden realitzar 3 proves diagnòstiques **(figura 18)**:

1. Prova genètica, per detectar mutacions als exons 2 al 24 del gen *NOTCH3*. Només cal una mostra de sang, és el métode més cómode pel pacient (Escary et al, 2000).

2. Immunohistoquímica en biòpsia de pell amb l'anticòs monoclonal (1E4) del domini extracel·lular del receptor Notch3 (Joutel et al, 2001; Lesnik Oberstein et al, 2003).

3. Microscopia electrònica d'una biòpsia de pell similar per detectar directament l'acumulació de GOM (Baudrimont et al, 1993).

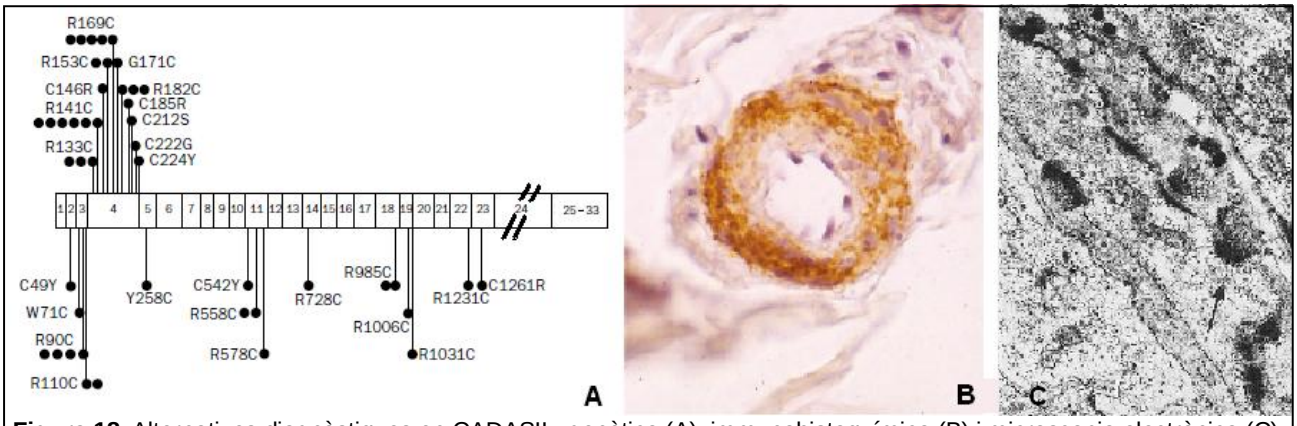


Figura 18. Alternatives diagnòstiques en CADASIL: genètica (A), immunohistoquímica (B) i microscopia electrònica (C). Extretes de Bergmann et al, 1996; Joutel et al, 1997 i Joutel et al, 2001.

Aquestes 3 proves són les eines principals per arrivar al diagnòstic, i es poden combinar

per optimitzar el procés. La genètica és el *"gold standard"* quan es seqüencia el gen complet, perquè la sensiibilitat i l'especificitat properes al 100%, però no és eficient. La solució usual és la reducció del nombre d'exons analitzats, amb la conseqüent reducció de sensibilitat (Joutel et al, 2001). L'anticòs és molt efectiu, amb una sensibilitat superior al 90%, i una especificitat al voltant del 98% (Joutel et al, 2001; Lesnik Oberstein et al, 2003). En una malatia orfe, només pot ésser considerada com una primera prova complementària. La microscopia electrònica és molt específica (100%) però la seva sensibilitat és reduida (57%), tot i que depèn del nombre d'artèries examinades (Malandrini et al, 2007), pot ésser utilitzat com un test confirmatiu.

Si totes aquestes proves són negatives, pot seqüenciar-se els exons 25 i 26 de *NOTCH3*, involucrats en una altra malaltia de petit vas provocada per la sobreactivació de Notch3 (Fouillade et al, 2008). Si no es troba res, una opció és estudiar el mRNA en biòpsia muscular per estudiar canvis d'expressió o mutacions intròniques que modifiquin el procés d'edició *splicing*.

6.2 Tractament de CADASIL

Quan s'arriba al diagnòstic, es recomana un examen neurològic, psicomètric i resonància magnètica cerebral (incloent seqüències T2, FLAIR i Gradient Echo). En aquest moment, s'ha de reevaluar la medicació: no hi han tractaments efectius provats en CADASIL, però sí contraindicacions específiques.

Basant-nos en la simptomatologia, proposem distingir 3 estadis de la malaltia: estadi lleu, caracteritzat per símptomes invalidants (migranya i epilèpsia); estadi moderat, representat pels infarts recurrents i transtorns de l'ànim; i estadi greu, quan els pacients desenvolupen deteriorament cognitiu **(Figura 19)**.

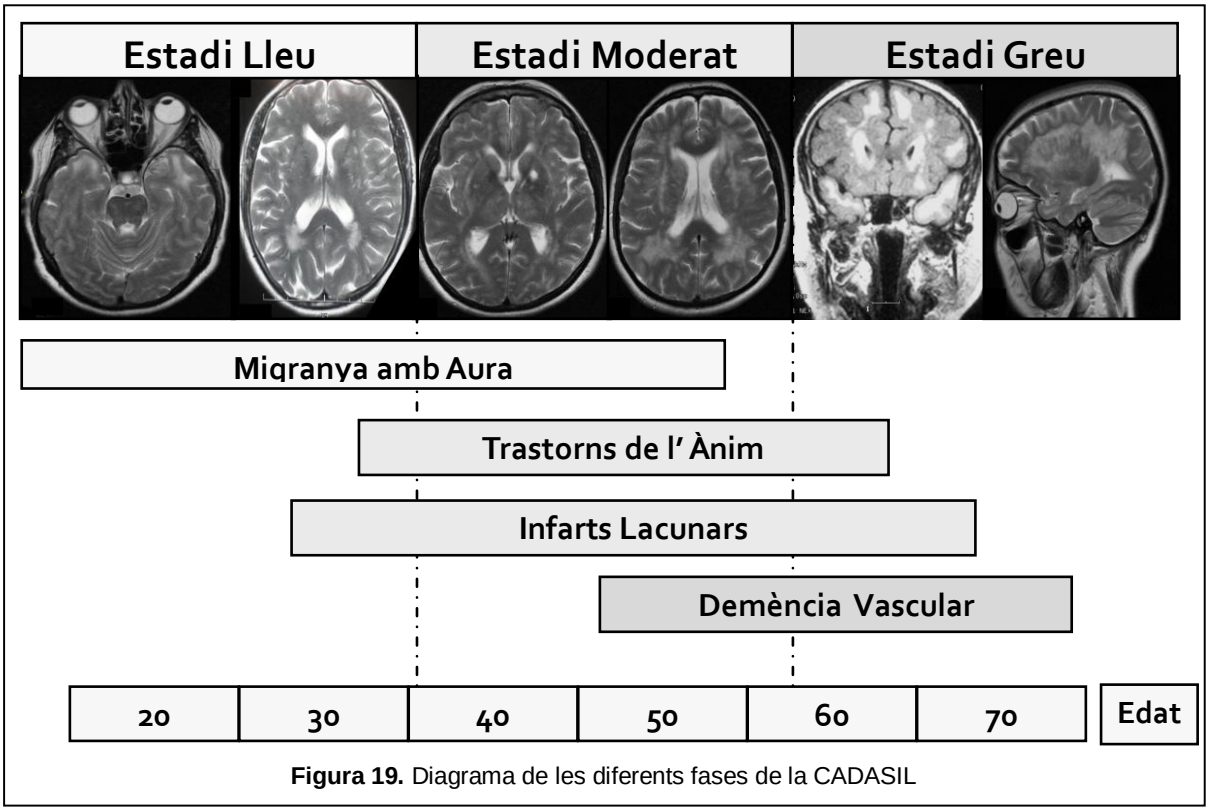


Figura 19. Diagrama de les diferents fases de la CADASIL

6.2.1 Estadi lleu

En aquest punt, els pacients són joves i sovint desconeixen ésser portadors de la mutació, reben el diagnòstic amb la confirmació d'un familiar.

Les crisi de migranya haurien de controlar-se amb simples analgèsics orals, com paracetamol o anti-inflamatoris no esteroideus i anti-emètics quan calgui. Els triptans (Ferrari et al, 2001) no es recomanen, i els ergots queden contraindicats pels seus efectes secundaris vasculars.

El tractament profilàctic pot considerar-se per reduir el nombre de crisis o aura persistent, com a la migranya comuna. Els profilàctics habituals de primera línea s'han d'administrar amb cura pels seus efectes en l'ànim i la cognició. El valproat sòdic (Shaygannejad et al, 2006) pot ésser útil en presència d'epilèpsia.

L'acetazolamida, un inhibidor de l'anhidrasa carbònica, és prometedora en CADASIL, probablement per l'increment de la perfusió cerebral a les regions corticals oligohèmiques (Chabriat et al, 2000; Forteza et al, 2001). Per contra, el tractament amb 80mg d'atorvastatina durant 8 setmanes no va millorar la vasoreactivitat cerebral en 24 pacients de CADASIL (Peters et al, 2007).

Abans de prescriure cap fàrmac, els pacients haurien de mantenir un estil de vida regular, controlant la son, la dieta, l'exercici i el stress. Tot i que l'eficàcia del magnesi, riboflavina i coenzima Q10 és reduïda, la seva seguretat els converteix en teràpies alternatives (Young et al, 2003).

6.2.2 Estadi moderat

La majoria de pacients de CADASIL es diagnostiquen en aquesta fase, quan presenten infarts lacunars, generalment sense factors de risc vascular. En aquest punt, ens hem de centrar en prevenir els nous ictus i reduir la progressió de la malaltia.

Un 85% dels pacients de CADASIL pateixen atacs isquèmics transitoris (AIT) i infarts (Chabriat et al, 1995), usualment infarts lacunars típics amb elevades taxes de recurrència, produint una invalidesa severa. Com els infarts són petits, normalment no es beneficien de la teràpia amb t-PA, però l'han de rebre quan es compleixen criteris clínics. Als infarts de gran vas, s'ha de fer un estudi ultrasonogràfic complet de les artèries cervicals i intracranials, així com un examen cardíac i de l'arc aòrtic per definir la font de l'émbol. Cal indicar que l'angiografia està contraindicada en CADASIL perquè pot provocar nous ictus (Dichgans et al, 1997).

En prevenció primària, cap tractament o mesura s'ha provat en pacients asimptomàtics de CADASIL; tots els factors de risc vascular s'han de controlar estrictament, especialment el tabaquisme (Singhal et al, 2004) i la hipertensió (Holtmannspotter, 2004; Peters et al, 2006; Viswanathan et al, 2006). Donades les similituds dels models animals de CADASIL amb els d'hipertensió essencial, s'està estudiant el tractament profilàctic amb hipotensors en CADASIL, tot i els seus possibles efectes deleteris sobre la perfusió cerebral.

No sabem si els antiagregants són efectius per la prevenció secundària de l'ictus, caldrien assaigs clínics específics. Dosis petites d'aspirina (50-325mg), triflusal, clopidogrel o dipiridamol poden ser útils, tot i que s'ha descrit una hemorràgia cerebral ocasionada pel consum d'aspirina (Oh et al, 2006).

Després de cada ictus, els malalts de CADASIL han de rebre rehabilitació multidisciplinar, perllonganda fins que es detecti una millora perceptible. La fisioteràpia i la teràpia ocupacional també poden ésser útils. Els inhibidors de la recaptació de la serotonina poden ser útils per la depressió, modulant a més la síndrome pseudobulbar (Lauterbach & Schweri, 1991).

6.2.3 Estadi greu

En aquesta fase, els pacients es troben greument discapacitats i la teràpia ha de centrar-se en mantenir la capacitat cognitiva i ajudar els pacients i els seus familiars a sobreposar-se a les seves limitacions.

El primer assaig clínic específic en CADASIL avaluant l'eficàcia i seguretat del donepezil, un inhibidor de la colinesterasa, en 168 malalts deteriorats cognitivament, va tenir resultats negatius, tot i que algunes funcions executives milloraven, com la velocitat de

processament i l'atenció (Dichgans et al, 2008). Altres inhibidors de la colinesterasa, com la rivastigmina i la galantamina poden ser útils pel dèficit colinèrgic (Roman, 2005; Keverne et al, 2007).

El tractament suportiu (ajuda pràctica, suport emocional i consell) és necessari per les famílies afectades. Altres mesures com evitar les úlceres de postració, gastrostomia, i fisioteràpia respiratòria també són útils.

6.2.4 Embaràs i consell genètic

Les complicacions cerebrovasculars isquèmiques durant la gestació i el puerperi són rares (8.1 de cada 100,000 embaràs) (Grosset et al, 1995). Els símptomes neurològics i la preeclàmpsia semblen comunes en CADASIL, particularment en dones més grans de 30 anys (Roine et al, 2005). Al 82% dels casos van ser les primeres manifestacions de CADASIL. La preeclampsia no és sorprenent degut als problemes hemodinàmics i endotelials (Roberts et al, 1989), el tractament amb L-arginina pot ésser una bona teràpia per la hipertensió gestacional (Peters et al, 2008).

Els malalts de CADASIL han de rebre un consell genètic adequat, per poder prendre les decisions personals i mèdiques amb informació suficient. El diagnòstic prenatal (Milunsky et al, 2005) i el diagnòstic preimplantacional (Konialis et al, 2007) poden ésser considerats com noves opcions reproductives. Pel que fa als individus asimptomàtics, s'han de seguir les recomanacions de la malaltia de Huntington, amb una entrevista per determinar si estan preparats per l'impacte del diagnòstic.

6.3 Anatomia Patològica

Encara que la simptomatologia clínica és majoritàriament neurològica, l'arteriopatia CADASIL és sistèmica, afectant les artèries del cervell, múscul, nervis perifèrics, pell, intestins, fetge, ronyó i cor (Ruchoux et al, 1995; Bergmann et al, 1996). També s'alteren els polisacàrids del teixit cerebral, cor, ronyó, fetge i pulmó (Brulin-Fardoux et al, 2003).

Una característica patognomònica de la malaltia és l'aparició d'acúmuls d'un material granular electrodens (GOM) que s'intercala amb les cèl·lules de múscul llis vascular (VSMC), detectables per microscopia electrònica i òptica. La densitat del material disminueix amb la distància respecte les VSMC i pericits; els grànuls mesuren 10 - 15 nm de diàmetre, formant acúmuls que poden superar 1 mm (Ruchoux et al, 1995). Els canvis apareixen entre les 14 setmanes i els 9 anys, ja que un fetus afecte de 14 setmanas no presentava afectacions (Lesnik Oberstein et al, 2008), però sí una nena de 9 anys (Ruchoux, dades no publicades).

La composició dels GOM és desconeguda: són d'origen celular, como es dedueix del tricròmic de Masson. Conté polisacàrids (Bergmann et al, 1996) i un baix contingut en sulfats i fosfats (Lapoint et al, 2000). Els seus components definits són el domini extracel·lular de Notch3 (Ishiko et al, 2006) i l'elastina (Caronti et al, 1998).

A nivell cerebral, s'aprecia una dilatació del sistema ventricular i l'eixamplement dels espais perivasculars a les seccions coronals (Lapoint et al, 2000). La substància blanca presenta desmielinització, coloració groguenca i augment de la consistència (Ruchoux et al, 1995; Bergmann et al, 1996) i degeneració axonal amb la tinció de Bodian (Lapoint et al, 2000). Les lesions profundes solen ser més grans, per la menor densitat vascular del teixit (Ruchoux et al, 1995). Per últim, s'ha observat apoptosi neuronal, afectant a les

capes 3 i 5 del còrtex, riques en neurones colinèrgiques (Mesulam et al, 2003) i s'ha comprobat en 2 estudis independents (Gray et al, 2007; Keverne et al, 2007).

A nivell vascular, les grans artèries cerebrals pateixen canvis menors. A les artèries de menor calibre, la tinció hematoxilina-eosina evidencia la destrucció de la capa mitja arterial, amb un nombre menor de VSMC en procés de degeneració. Les cèl·lules musculars llises no vasculars presenten morfologia normal (Ruchoux et al, 1995).

Després de la degeneració de la túnica mitja, és comú la duplicació o escissió de la làmina elástica. L'engrossiment intimal progressiu podria conduir, en fases avançades, a l'estenosis i oclusió de l'artèria (Miao et al, 2004). També s'han observat agregats perivasculars focals de limfòcits o histocits, en ocasions associats amb necrosi fibrinoide i trombus de fibrina (Lapoint et al, 2000).

Per últim, a nivell ocular es detecten anomalies vasculars, amb engrossiment dels vasos, fibrosi perivascular i pèrdua de VSMC i pericits, pero sense obliteració del lúmen. L'endoteli es desprèn i presenta vacuoles intracel·lulars i alteraciones mitocondrials. També es detecten mucopolisacàrids als vasos, desmielinització i rarefacció del nervi óptic.

6.4 NOTCH3, funció i funcionament

La CADASIL està causada per mutacions al gen *NOTCH3* del cromosoma 19 (Joutel et al, 1996). S'han descrit més de 150 mutacions puntuals, localitzades a les repeticions EGF (***E****pidermal* ***G****rowth* ***F****actor*) del domini extracel·lular del receptor, afectant exclusivament a residus de cisteina.

La familia de gens *NOTCH* codifiquen receptors transmembranals implicats al procés de creixement i diferenciació cel·lular durant el desenvolupament (Artavanis-Tsakonas et al, 1999). Són heterodímers, amb un domini C-terminal intracel·lular amb activitat senyal-transductora i un domini N-terminal extracel·lular amb la regió d'unió a lligand i l'activitat reguladora. Es troben molt conservats al llarg de l'evolució, el que ha permès el desenvolupament de models animals en mosca (de la Peña et al, 2001), nemàtode, peix zebra o ratolí (Ruchoux et al, 2003; Lundkvist et al, 2005; Monet-Lepretre et al, 2008; Joutel et al, 2010). El receptor Notch3 va aparèixer tardanament a l'evolució i només pot estudiar-se en vertebrats. Les úniques cèl·lules madures que mantenen activa l'expressió de *NOTCH3* són les VSMC i els pericits, les afectades primàriament a CADASIL.

Els ratolins *knock-out* (KO) de *NOTCH3* han permès identificar per microarrays les vies metabòliques on participa: desenvolupament del mesoderm i muscular, contracció muscular i estructura cel·lular (Arboleda-Velásquez et al, 2008). Notch3 també està implicat a la diferenciació arteriovenosa (Villa et al, 2001; Domenga et al, 2004) i regula el tonus miogènic a les artèries de petit calibre (Belin de Chantemèle et al, 2008). Els problemes de vasoreactivitat dels KO són específics de les arterioles cerebrals, mantenint-se intactes les capacitats contràctils de les artèries aorta i caròtida (Arboleda-Velásquez et al, 2008; Belin de Chantemèle et al, 2008). El dèficit de Notch3 també comporta una sensibilitat superior a la isquèmia i un increment de les taxes de despolarització isquèmica a l'àrea periinfart (PID) (Arboleda-Velásquez et al, 2008).

El procés de síntesi i senyalització del receptor es regula a múltiples nivells (Baron et al, 2002) **(Figura 20)**: a nivell d'expressió (1); a la N-glicosilació, que determina la capacitat d'unió al lligand DSL (3); el tipus de lligand disponible (5); el procés d'activació (6,7); el punt de tall de γ-secretasa (8), que condiciona l'estabilitat del fragment intracel·lular i la

ubiquitinació d'aquest domini, que controla la seva capacitat d'endocitosi al nucli (9) (Dziewulska & Rafalowska, 2008). La degradació del fragment intracel·lular és per via lisosomal enlloc del proteosoma, com s'ha descrit per Notch1 i Notch4 (Jia et al, 2009).

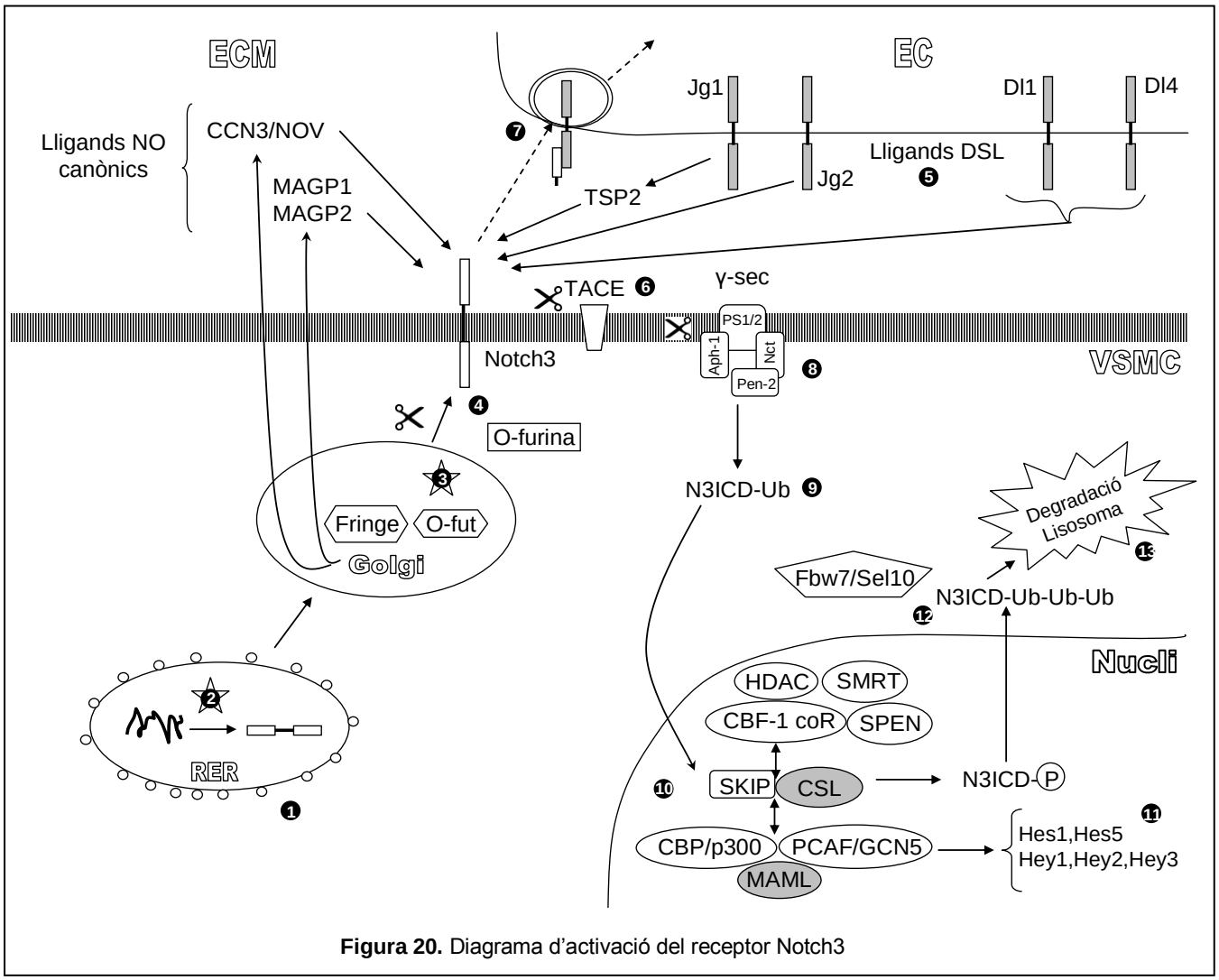


Figura 20. Diagrama d'activació del receptor Notch3

Molècules de la matriu extracel·lular poden modular la senyal dels lligands DSL (Meng et al, 2009). Dels lligands no canònics podem destacar CCN3, MAGP-1 i MAGP-2, que provoquen una senyalització autocrina (D'Souza et al, 2008).

Les mutacions de CADASIL podrien afectar la vía clàssica del receptor des de la traducció fins el segon tall proteolític d'activació (1-7). S'han proposat la formació de ponts bisulfur (2) (Dichgans et al, 2000) i la glicosilació per Fringe (3) (Arboleda-Velasquez et al, 2005)

com els processos afectats per la mutació.

6.5 Etiopatogènia

Podem dividir el procès patogènic de CADASIL en 3 parts: processos subcel·lulars que provoquen la degeneració de VSMC i pericits, processos supracel·lulars o de teixit, que expliquin com la mort de VSMC origina les lesions neurològiques i causes de l'especificitat de les lesions a nivell neurològic, éssent una arteriopatia sistèmica.

6.5.1 Nivell subcel·lular

La CADASIL s'ha considerat una malaltia autoimmune, per la morfologia dels GOM similar als dipòsits d'inmunoglobulines i la detecció d'infiltrats perivasculars i necrosi fibrinoide.

També es va postular que la causa era l'acumulació d'elastina (present als GOM) i l'increment de fibres elàstiques, com al pseudoxantoma elàstic, perquè s'ha descrit elastogènesi alterada en fibroblasts de pacients de CADASIL (Caronti et al, 1998).

Estudis extensos del múscul i la funcionalitat mitocondrial sense trobar anormalies (Chabriat et al, 1995), pero la identificació de diversos malalts amb fibres vermelles-esquinçades va suggerir la disfunció mitocondrial com a causa de la malaltia. No obstant, no s'ha realitzat un estudi molecular suficient. Els afectats de CADASIL tenen una taxa més elevada de variacions al DNA mitocondrial i es van detectar disfuncions a la cadena respiratòria OXPHOS d'un model en mosca (de la Peña et al, 2001).

Altres autors han proposat la glicosilació per Fringe, perquè en un model "*in vitro"* el patró de N-glicosilació s'alterava per mutacions CADASIL (Artavanis-Tsakonas et al, 2005). No obstant, l'ús de mutacions murines ortòlogues en CADASIL és poc extrapolable, perquè el

ratolí transgènic *knock-in* generat per la mutació R142C murina no presentava signes de la malaltia (Lundkvist et al, 2005), a diferència dels que contenien el gen humà (Ruchoux et al, 2003; Monet-Lepretre et al, 2008) o de rata (Joutel et al, 2010).

Les mutacions en *NOTCH3* podrien provocar una disfunció del receptor, alterant-se les vies metabòliques on participa, en homologia a la malaltia de Fabry. La teoria de pèrdua de funció es fonamenta als problemes de processament i/o funcionalitat provocats per diverses mutacions (no totes) en cultius cel·lulars, la implicació de Notch3 en la regulació del tonus miogènic i vies antiapoptòtiques, el menor increment de PDGF-Rβ en resposta a l'activació de Notch3 en VSMC de malalts de CADASIL (Jin et al, 2008) i la sensibilitat elevada dels seus fibroblasts i limfòcits a l'estrès oxidatiu (Formichi et al, 2009).

El gens humans amb les mutacions R90C i C428S mantenen la seva expressió i activitat en ratolins KO (Monet et al, 2008; Monet-Lepretre et al, 2008), el que descartaria la pèrdua de funció. A més, no s'ha observat apoptosi a les arterioles dels ratolins KO i el tonus miogènic de les artèries cerebrales del ratolí R90C s'incrementa (Lacombe et al, 2005), mentre que al ratolí KO es redueix (Arboleda-Velásquez et al, 2008; Belin de Chantemèle et al, 2008). Inclús s'ha associat la pèrdua de funció del receptor amb una evolució més lenta de la malaltia (Monet-Lepretre et al, 2008).

En cas de guany de funció, no s'ha establert la funció tòxica adquirida. S'han proposat l'agregació tòxica de Notch3 en forma de GOM (com Alzheimer i Parkinson, amb β-amiloide i tau o α-sinucleína) i l'activació perllongada de la resposta ***U****nfolded* ***P****rotein* ***R****esponse* (UPR; com en diabetis i esclerosi lateral amiotròfica) (Ihalainen et al, 2007). Aquest model es fonamenta a l'acumulació de Notch3 als GOM (Ishiko et al, 2006) i l'agrupació de les mutacions en segments poc conservats del receptor, que no són

essencials pel seu funcionament (Donahue & Kosik, 2004).

La degeneració de la túnica mitja vascular abans de detectar-se GOM al teixit arterial sembla descartar la toxicitat dels GOM com a causa primària de la mort cel·lular (Ruchoux et al, 2003; Monet-Lepretre et al, 2008).

L'alteració de la resposta UPR es basa en un estudi de proteòmica amb un cultiu de cordó umbilical de CADASIL i cinc controls sans **(Figura 21)**. En aquest model, les mutacions de Notch3 provocarien l'estrés del **R**etícle **E**ndoplasmàtic **R**ugós (RER), que entraria en un cicle d'oxidació-reducció dels ponts bisulfur del receptor mutat. L'estrés del RER generaria ROS causant la disfunció mitocondrial, una potent activació del proteosoma i l'entrada en apoptosi de la cèl·lula. De forma paral·lela, alteracions a la polimerització de microtúbuls i filaments d'actina explicarien les alteracions microestructurals observades a les VSMC. Els GOM apareixerien per la disfunció de la seva recaptació de la membrana per UCH-L1. El model UPR no està exent de limitacions:

- A nivell teòric, no s'estableix una cadena causal entre les mutacions de Notch3 i els nivells de 6 de les 11 proteïnes (ex. UCH-L1) ni es mencionen les altres 24 proteïnes alterades.

- A nivell tècnic, no es van replicar els resultats per *western blot* ni es van estudiar les vies d'activació clàssiques de la UPR.

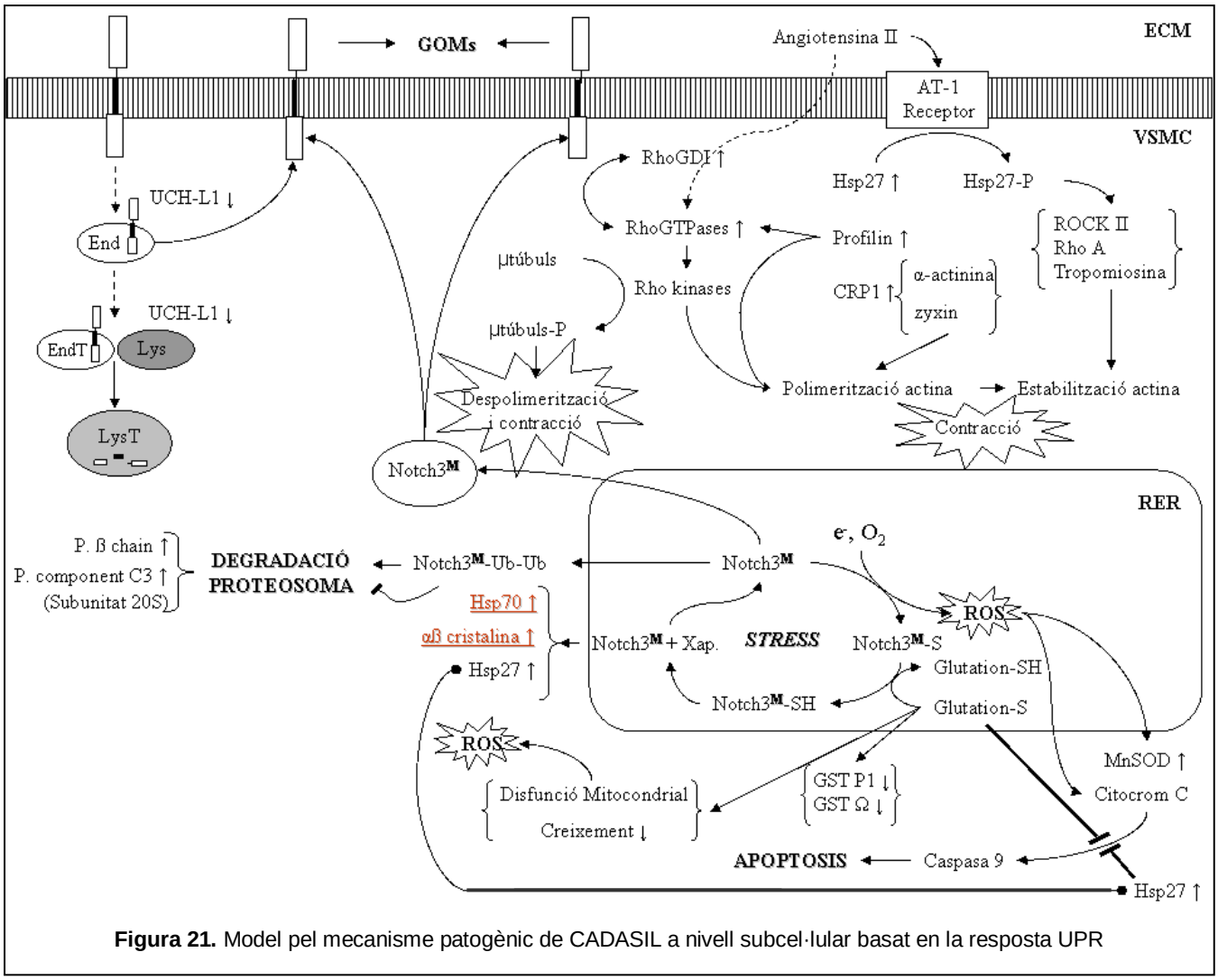


Figura 21. Model pel mecanisme patogènic de CADASIL a nivell subcel·lular basat en la resposta UPR

La presència de les xaperones αβ-cristalina i Hsp70 a les VSMC dels pacients (Rubio et al, 1997), la degradació retardada dels receptor Notch3 mutat (Takahashi et al, 2009) i la inhibició del proteosoma pel pèptid senyal de Notch3 (Zhang et al, 2007) podrien interpretar-se com indicis independents d'aquest mecanisme. Ara bé, la degradació de Notch3 és via lisosomal així que la inhibició del proteosoma no explica la seva acumulació (Jia et al, 2009).

6.5.2 Nivell teixit

Aquest apartat descriu com la degeneració cel·lular conduiria a la simptomatologia neurològica. Si seguim el model d'UPR, la reactivitat de les VSMC es reduiria per disfunció del sistema renina-angiotensina **(figura 21)**, que explicaria el tonus miogènic

exacerbat. Aquest fenòmen va acompanyat de baixa capacitat proliferativa i un estat cel·lular proapoptòtic (Ihalainen et al, 2007) que empitjoraria amb la pèrdua de VSMC i pericits i la tortuositat creixent de les artèries penetrants. Finalment, es perdria qualsevol tipus de vasoreactivitat i la circulació cerebral variaria segons la pressió arterial sistèmica (Okeda et al, 2002). Com a resposta adaptativa, la pressió arterial i les seves oscilacions circadianes disminueixen i es produeix un engrossiment de l'adventicia i la íntima per reforçar la paret arterial i evitar l'aparició de sagnats.

La vasoreactivitat cerebral alterada conduiria a un estat d'oligoèmia, reducció del consum metabòlic i de producció de neurotransmissors, com s'observa per tomografia d'emissió de protons i espectroscopia per resonància magnètica. La substància blanca profunda, irrigada per artèries de petit calibre i gairebé sense circulació colateral, seria la zona més afectada: els espais perivasculars s'eixamplen i s'observa una leucoaraiosi severa (Cumurciuc et al, 2006; Liem et al, 2009). Existeixen 2 hipòtesi per l'aparició de les lesions isquèmiques:

- L'estretament dels vasos per l'acumulacío de GOM i componentes de la matriu extracel·lular, que comporta una reducció del lúmen i condueix a la oclusió arterial (Miao et al, 2004)

- Vasoreactivitat deficient i infarts d'origen hemodinàmic, degut a les necessitats metabòliques canviants de l'encèfal i les variacions a la pressió sistèmica (Ruchoux et al, 2000).

Els infarts lacunars provoquen la mort de neurones colinèrgiques per toxicitat retrògrada axonal. Existeix una correlació entre els ictus lacunars i les escales de deteriorament cognitiu, tot i que les lesions isquèmiques inicials no tindrien repercusió funcional per la

reorganització del còrtex motor. Segons les regions cerebrals afectades, podrien explicar els transtorns de l'ànim (Chabriat et al, 1995), transtorns motors (Reddy et al, 2002), disexecutius i deteriorament cognitiu (Royall, 2008).

L'origen dels atacs epilèptics i la migraña amb aura, que apareixen abans de la isquemia podria ésser la despolarització peri-infart (PID) (Strong et al, 2002), representant una mateixa disfunció però amb llindars d'acció diferents. En primer lloc, la disfunció elèctrica donaria lloc a l'aura migranyosa o els atacs epilèptics (segons la intensitat i localització).

En aquest sentit, l'aura migranyosa està causada per la PID i Notch3 modifica la taxa de PID (Arboleda-Velasquez et al, 2008) **(Figura 22)**.

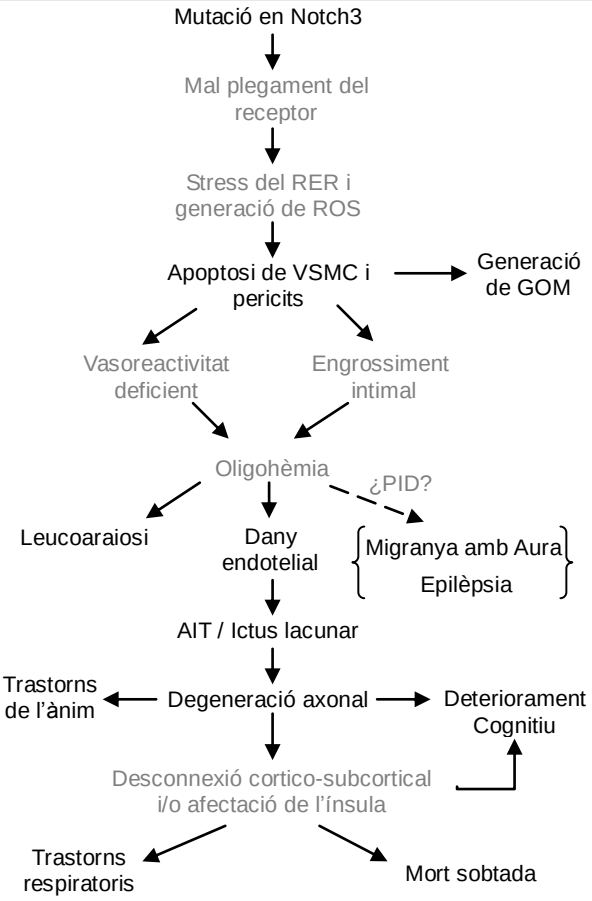


Figura 22. Model pel mecanisme patogènic de CADASIL

6.5.3 Nivell organisme

Les causes per la especificitat neurològica de les lesions a la CADASIL, éssent una arteriopatia sistèmica. Podem trobar diverses explicacions que podrien coexistir:

- La particular morfologia cerebrovascular: la presència de BHE (Ruchoux et al, 2002), la integritat de la qual està regulada pels pericits (Armulik et al, 2010) i la proporció menor d'adventicia i VSMC als vasos cerebrals respecte les artèries extracraneals (Rafalowska et al, 2004).

- La limitada capacitat regenerativa neuronal en edat adulta.
- Les elevades necessitats metabòliques de l'encèfal: constitueix un 2% del pes corporal però consumeix fins un 20% de l'energia, éssent l'òrgan més sensible a la isquèmia i al dèficit de glucosa.
- La funció variable de les artèries segons el seu diàmetre i localització. En ratolins KO i CADASIL, la vasoreactivitat cerebral estava alterada (Lacombe et al, 2005; Arboleda-Velásquez et al, 2008; Belin de Chantemèle et al, 2008) mentre les grans artèries extracranials no mostraven canvis (Arboleda-Velásquez et al, 2008; Belin de Chantemèle et al, 2008).

També s'ha observat un gran nombre de morts súbites en estadis terminals (Opherk et al, 2004), que podria estar relacionada amb l'elevada taxa d'arritmies d'aquests pacientes. L'explicació més plausible sembla una mort súbita d'origen central, mediada per una desconexió cortical-subcortical, lesions a la ínsula o la protuberància, ja que no s'han trobat deficiències consistents al funcionamient cardíac en CADASIL (Cumurciuc et al, 2006; Rufa et al, 2007; Piccirillo et al, 2008).

És important determinar el mecanisme causant de l'arteriopatia per identificar-ne dianes terapèutiques i fer un tractament efectiu de la CADASIL. Per exemple, els inhibidors de G-secretasa sembla evitar l'agregació de Notch3 en cultius cel·lulars, però caldrà veure la seva utilitat en models animals i pacients per evitar la malaltia (Takahashi et al, 2010).

7. La síndrome de Maeda (CARASIL)

La síndrome de Maeda o CARASIL (MIM 600142) va ésser descrita per primer cop l'any 1965 en població japonesa (Maeda, 1965). Fins ara, només s'ha descrit uns 20 casos en families asiàtiques, exclusivament al Japó (Fukutake et al, 1999; Yanagawa et al, 2002; Arima et al, 2003; Nakazato et al, 2008; Hashida et al, 2009) i un cas recent de Xina (Zheng et al, 2009).

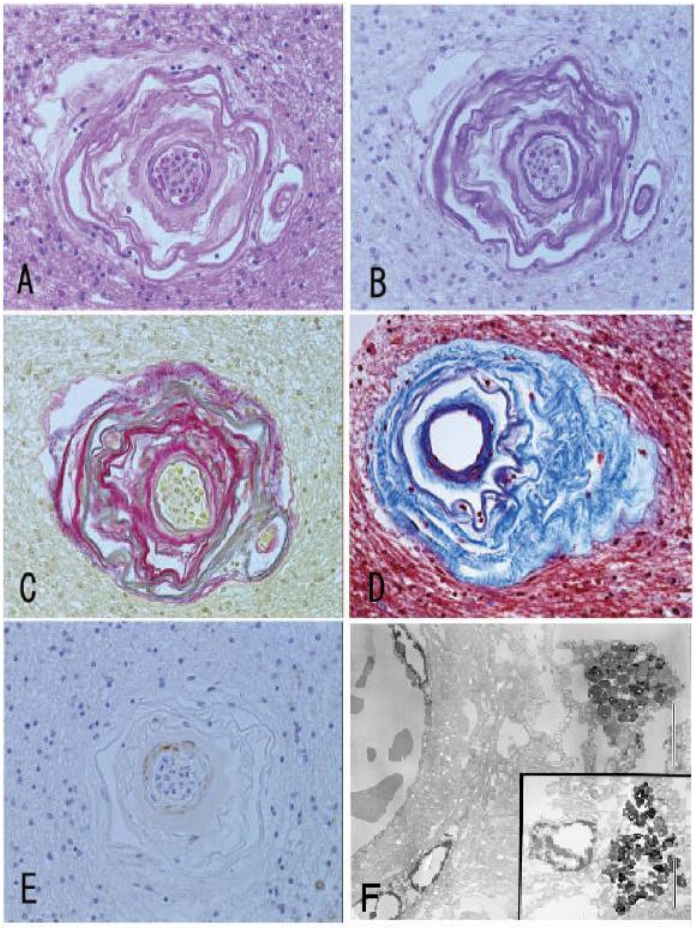


Figura 23. Tincions d'anatomia patològica dels CARASIL: (A) Hematoxilina-Eosina, (B) tinció d'Àcid Periòdic de Schiff, tincions d'elàstiques de Gieson (C) i Tricròmic de Masson (D). Immunohistoquímica per α-actina com a marcador de VSMC (E); extret d'Arima et al, 2003. Inclusions citoplasmàtiques a la microscopia electrònica (F); extret de Yanagawa et al, 2002.

És una malaltia de petit vas caracteritzada per leucoencefalopatia no hipertensiva, alopècia i espondilosi (Fukutake et al, 1995). D'inici entre els 20 i 30 anys, els malalts pateixen un dèficit motor progressiu i deteriorament cognitiu i moren en 10 anys (Fukutake, 1995). Normalment mostren herniacions dels discs vertebrals i alopècia, però aquesta última pot restar absent en dones (Yanagawa et al, 2002). Els signes radiològics inclouen infarts lacunars subcorticals i leucoencefalopatia difusa homogènia precoç, preservant les fibres en U (Fukutake et al, 1999).

Un estudi patològic de dos cervells de CARASIL, va mostrar les artèries eixamplades centrífugament, amb desaparició de les VSMC i la matriu extracel·lular, tant a substància

blanca com a les meninges (Oide et al, 2007). Un altre estudi mostrava duplicació de la làmina elàstica, engrossiment de la íntima fibrosa i reducció del lúmen del vas (Arima et al, 2003). També s'han descrit inclusions citoplasmàtiques semblants a lisosomes dins les VSMC del cervell d'una altra pacient (Yanagawa et al, 2002) **(figura 23)**.

Inicialment, des del punt de vista clínic, es va considerar que CADASIL i CARASIL constituien síndromes semblants que es diferenciaven pel patró d'herència, dominant a la primera i recessiu a la segona. Ambdues presenten el mateix patró de lesions a la neuroimatge, els infarts lacunars repetitius i el deteriorament cognitiu. Ara bé, la forma recessiva és més agressiva, tot i que els malalts no pateixen migranya i els infarts clínics són menys freqüents. A més, els afectats de CARASIL no presenten GOM als vasos (Oide et al, 2007; Yanagawa et al, 2002).

A nivell molecular, aquesta malaltia s'assembla més al Rendu Osler o HHT, perquè ambdues estan causades per disfuncions de la via del TGF-β, però mentre la HHT és causada per una activitat insuficient (haploinsuficiència, calen 2 copies funcionals per mantenir-se sa), a CARASIL trobem una activació excessiva.

El gen mutat en CARASIL, situat al cromosoma 10q23, codifica per la proteasa HTRA1 (Hara et al, 2009). *HTRA1* s'expressa en nombrosos teixits (Nie et al, 2003), especialment a les capes madures de l'epidermis i moderada a l'endoteli, VSMC, fibroblasts i osteoblasts (de Luca et al, 2003). És una serin-proteasa secreteda (Gray et al, 2000) que inhibeix múltiples substrats com:

- la familia TGF-β, emprant els dominis proteasa i el *linker* anterior (Oka et al, 2004)
- el col·làgen, regulat per la unió al domini PDZ (Murwantoko et al, 2004).

Els dominis PDZ regulen la selecció dels sustrats (Runyon et al, 2007), com indica el seu requeriment per reduir la mineralització depenent de ***B**one **M**ineralization **P**roteins* (BMP) dels osteoblasts (Hadfield et al, 2008). Sembla que la majoria de les mutacions es localitzen al domini amb activitat proteasa, encara que no afecten directament el centre actiu de l'enzim **(figura 24)**.

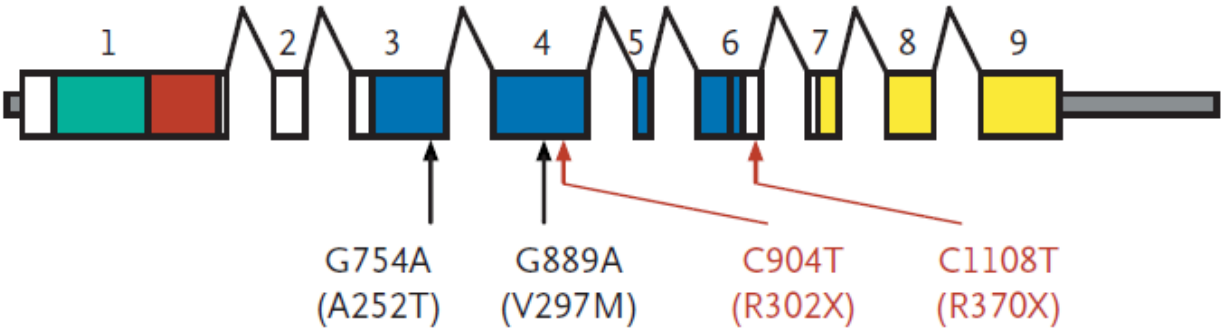


Figura 24. Distribució de les mutacions CARASIL: domini insulin-like growth factor binding protein (verd), domini inhibidor de serin-proteases tipus Kazal (vermell), domini proteasa trypsin-like (blau), domini PDZ (groc) y regions UTR (gris). Extret de Hara et al, 2009.

8. Tractament de l'ictus isquèmic

Els tractaments d'elecció per l'ictus isquèmic són les teràpies destinades a la restauració del flux sanguini (trombolítics, sonotrombolisi i dispositius mecànics), tot i que també s'estan assajant tractaments neuroprotectors i neurorreparadors.

8.1 Els fàrmacs trombolítics

El principi de la trombolisi es la dissolució del coàgul enzimàticament mitjançant la conversió del plasminògen en plasmina. La primera publicació sobre el seu ús a l'ictus isquèmic data de 1963 (Meyer et al, 1963), però no va ser fins la dècada dels 80 que es van dur a terme els primers assaigs clínics amb uroquinasa (u-PA) en infart cerebral. Els trombolítics es van aprobar a principis dels 90 pel seu ús en infart de miocardi i es va intentar generalitzar el seu ús a les oclusions cerebrovasculars. S'han utlitzat com a fàrmacs els activadors endògens u-PA i el t-PA (alteplasa); precursors o modificacions d'aquestes com pro-uroquinasa (pro-uPA), reteplasa (rPA), lanoteplasa (nPA) o tenecteplasa (TNK); i proteïnes activadores d'altres organismes com estreptoquinasa (SK), estafiloquinasa (SAK), desmoteplasa (DSPA) o ancrod (Macrez et al, 2009).

8.1.1 Estreptoquinasa (SK)

L'estreptoquinasa forma part de la primera generació de fàrmacs trombolítics. Extreta del streptococus beta-hemolític (*Streptococcus pyogenes*), és una proteïna de 47 kDa que forma un complex amb el plasminògen al plasma. Ja que s'extreu de bacteris, la seva producció és senzilla, però durant la infusió del fàrmac es produeix una reacció inflamatòria subclínica.

Es van realitzar un estudi pilot i tres assaigs clínics: el ***M**ulticenter **A**cute **S**troke **T**rial-**I**taly* (MAST-I) al 1995, el ***M**ulticenter **A**cute **S**troke **T**rial-**E**urope* (MAST-E) i l'***A**ustralian **S**treptoK**K**inase trial* (ASK) al 1996. Els tres estudis van mostrar resultats negatius. De fet, els assaigs MAST-E i MAST-I van finalitzar prematurament degut a la mortalitat superior del grup tractat.

L'assaig MAST-E (1996) va incloure 310 pacients amb infarts de fins a 6 hores d'evolució on s'administrava 1.5 mU d'estreptokinasa en infusió contínua d'una hora. La mortalitat a 10 dies i la freqüència d'hemorràgies van ésser superiors al grup tractat, sense diferències de pronòstic als 6 mesos. Els assaigs MAST-I i ASK van obtenir resultats similars en grups de 622 i 340 pacients, respectivament.

8.1.2 Estafiloquinasa (SAK)

L'estafiloquinasa a més de promoure la conversió de plasminògen a plasmina, també digereix les IgG i el factor del complement C3b, inhibint la fagocitosi. No obstant, el seu origen bacterià (*Staphilococcus aureus*) provoca una reacció immune subclínica durant la seva infusió (Warmerdam et al, 2002). Quan la variant SAK42D es va provar als assaigs ***C**ollaborative **A**ngiographic **P**atency **T**rial **O**f **R**ecombinant **S**taphylokinase (*CAPTORS) i CAPTORS-2 per la trombolisi en pacients d'infart de miocardi es va registrar una freqüència elevada d'hemorràgies (Armstrong et al, 2000; Armstrong et al, 2003) i no es va arribar a probar en infart cerebral. S'està investigant en mutants de l'estafiloquinasa que no presenten resposta inflamatòria, encara en fase preclínica amb models animals (He et al, 2010).

8.1.3 Uroquinasa (UK)

La uroquinasa constitueix amb l'estreptoquinasa la primera generació de trombolíitics. Malgrat ésser efectius, aquests fàrmacs eren poc específics pel plasminògen unit a la malla de fibrina. Com el plasminògen circulant i el del coàgul es troben en equilibri, la reducció del plasminògen circulant suposava un alliberament del plasminògen unit al trombus i la reducció de l'actividad del fàrmac (Verstraete et al, 2000).

S'han dut a terme diferents assaigs clínics amb uroquinasa administrada per via intraarterial (Fujishima et al, 1986; Zeumer et al, 1993). L'últim assaig clínic realitzat, l'estudi AUST (***A**ustralian **U**rokinase **S**troke **T**rial*) va terminar prematurament per la retirada de la companyia farmacèutica Serono. Només es van incloure 16 pacients, i el tractament amb uroquinasa intraarterial per les oclusions al territori vertebrobasilar va demostrar ésser segur i efectiu (Macleod et al, 2005).

8.1.4 Desmoteplasa (DSPA)

La desmoteplasa s'extreu del rat-penat vampir mexicà (*Desmodus rotundus*). Existeixen 4 isoformes en estat natiu: DSPAα1, DSPAα2, DSPAβ i DSPAγ. Les isoformes α són més grans (52 kDa) que la β (46 kDa) i la γ (44 kDa). El DSPAα1 té un 85% d'homologia amb el t-PA, el principal canvi estructural és l'absència del domini K2, que impedeix la seva digestió per plasmina. Com a conseqüència, el DSPAα1 té més especificitat per la fibrina i una vida mitja plasmàtica més elevada (Liberatore et al, 2003). A més, a diferència del t-PA, no provoca neurotoxicitat mediada pels receptors NMDA (Liberatore et al, 2003).

La DSPA va ésser provada l'any 2005 a l'assaig de fase II DIAS (***D**esmoteplase **I**n **A**cute **S**troke*), que va incloure 104 pacients amb infarts de fins a 9 hores amb desacoblament difusió-perfusió (DWI/PWI) >20%. Inicialment, volien provar-se 3 dosis de 25, 37.5 i 50 mg

en bolus. Donat el nombre elevat d'hemorràgies intracranials simptomàtiques, les dosis es van ajustar a 62.5, 90 i 122.5 ug/kg. La dosi més alta va tenir resultats favorables (60% millora al grup tractat vs. 22% al placebo), sense relació entre el temps d'administració del fàrmac i les hemorràgies iatrogèniques.

L'any 2006 es presenta l'estudi DEDAS (***D**ose **E**scalation of **D**esmoteplase for **A**cute ischemic **S**troke*) en 37 pacients, amb els mateixos criteris d'inclusió que l'estudi DIAS i les dosis de 90 i 125 ug/kg. La dosi de 125 ug/kg tenia un 35% de millora sobre placebo, tot i que la diferència no era estadísticament significativa.

Aquests resultats encoratjadors donaren lloc a l'assaig DIAS-2 de fase III, publicat l'any 2008), amb dosis de 90 i 125 ug/kg en 186 pacients amb desacoblament DWI/PWI en ictus d'entre 3 i 9 hores d'evolució. Els resultats van ésser negatius, amb un 21.2% de mortalitat al 3er mes al grup tractat vs. 6.3% al grup placebo. En un anàlisi posterior, es va determinar que 10/14 morts al grup tractat no estaven relacionades amb el tractament.

Aquests resultats donaren lloc als assaigs DIAS-3 (inici Febrer 2009) i DIAS-4 (inici Abril 2009), amb una dosi de 90 ug/kg en pacients de perfil similar al DIAS-2. El tamany final objectiu és de 320 pacients, els assaigs finalitzaran a mitjans de 2011.

8.1.5 Microplasmina

La microplasmina és el resultat d'una digestió autolítica de la plasmina, sense els 5 dominis *kringle* (K). És un polipèptid de 29 kDa format per 2 cadenes unides per ponts disulfur: 31 aminoàcids de l'extrem C-terminal de la cadena A i la cadena B sencera (230 aminòacids).

L'assaig clínic de fase II va començar a l'octubre de 2005 i va incloure 40 pacients tractats durant les 12 primeres hores de l'ictus amb 2, 3 ó 4 mg/kg (Thijs et al, 2009). El tamany mostral tan reduit no permetia extreure conclusions d'eficacia, però tots els grups van presentar una elevada taxa d'hemorràgies intracraneals (50%, 41% i 50%). Els nivells d'enolasa, S100B, MMP-2, MMP-9 i TIMP-1 es van mesurar durant l'assaig i es va observar la influència de les diferents dosis sobre cadascun d'aquests marcadors.

8.1.6 Ancrod

L'ancrod és una fracció purificada del verí de la serp pitó de Malàsia (*Calloselasma rhodostoma*). Estrictament, no és un fàrmac trombolític, elimina el fibrinopèptid A del fibrinògen, evitant la formació del coàgul i estimulant la secreció d'activadors del plasminògen endògens.

Els principals assaigs clínics evaluant-lo són el *north american **S**troke **T**reatment with **A**ncrod **T**rial* (STAT) i l'***E**uropean **S**troke **T**reatment with **A**ncrod **T**rial* (ESTAT). L'assaig STAT (1998) va incloure 500 pacients d'ictus de fins a 3 hores d'evolució als quals s'administrava Ancrod durant 72h fins reduir els nivells de 10.55 umol/L a 1.18-2.03 umol/L. El pronòstic funcional al 3er mes fou significativament millor al grup tractat, tot i l'increment d'hemorràgies. L'assaig ESTAT estudià el mateix tractament en 1222 casos d'ictus de 3-6h, sense observar-se diferències al 3er mes però amb un nombre superior d'hemorràgies.

Finalment, els assaigs ***A**ncrod **S**troke **P**rogram* (ASP I) i ASP II inclouen pacients de 3-6h d'evolució amb infusió continua només durant 2-3h. Ambdós estudis van terminar de forma prematura quan s'havien inclòs 277 i 311 pacients per manca d'eficàcia i l'empresa responsable va aturar-ne el desenvolupament.

8.1.7 Pro-uroquinasa (pro-uPA)

L'activador del plasminògen uroquinasa (uPA) és secretat al plasma com un zimògen de cadena simple (pro-uroquinasa) i convertit a dipèptid unit per ponts disulfur mitjançant l'acció de la plasmina, kallikreina, FXII activat o catepsina B.

Aquest fàrmac s'ha administrat principalment per via intra-arterial enlloc d'intravenosa, reduint-ne la dosi. L'any 1995, l'assaig de fase II PROACT-1 (***PRO****lyse in* ***A****cute* ***C****erebral* ***T****hromboembolism*) va incloure 46 pacients amb oclusió de l'artèria cerebral mitja per CT-angiografia de fins a 6 hores d'evolució. Es van provar les dosis de 6mg i 12 mg en combinació amb heparina intravenosa durant 4 hores. La recanalització en 2h va augmentar del 14% al 58%, però el bon pronòstic només del 10% al 12%, mentre les hemorràgies van passar del 7.1% al 15.4%.

L'estudi PROACT-2 (1999) va incloure 180 pacients d'ictus de fins a 6h d'evolució, tractats amb 9mg d'uPA i heparina (n=121), o només heparina (n=59). El tractament amb pro-uroquinasa va millorar significativament el pronòstic al 3er mes, però amb un alt nombre d'hemorràgies, el que va evitar la seva aprobació per la FDA. Actualment, s'ha substituit per l'administració intra-arterial de tPA.

8.1.8 Activador tisular del plasminògen (tPA)

El t-PA es una glicoproteína de 69 kDa, sintetitzada com un zimògen de cadena simple de 527 ó 530 aminoàcids, que contenen 34 ponts disulfur. Activat per la plasmina per proteòlisi entre l'Arg_{275}-Ile_{276}, quedant 2 cadenes: A i B (Schellinger, 2009). La cadena A o cadena pesada, conté 4 dominins **(figura 25)**:

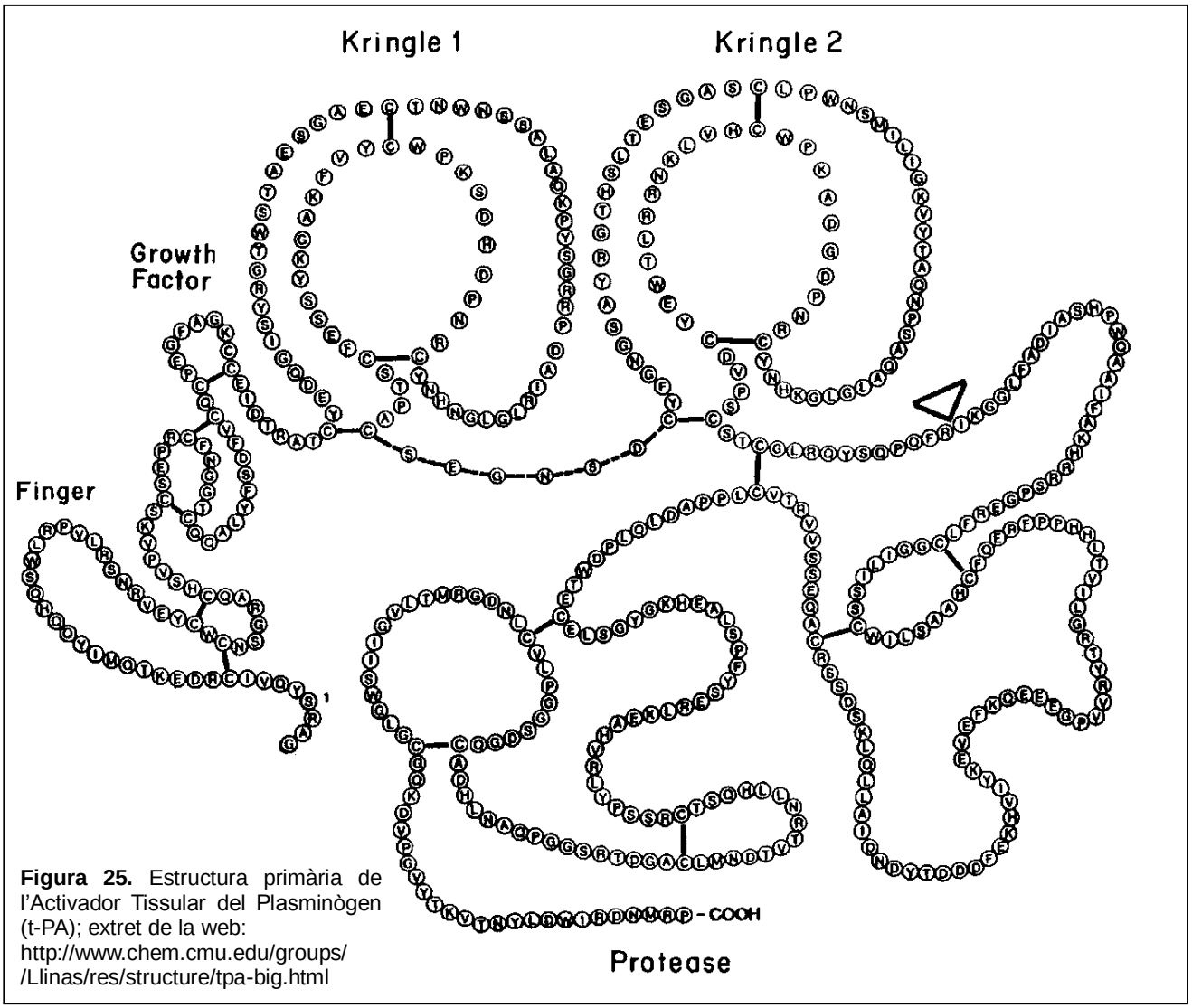


Figura 25. Estructura primària de l'Activador Tissular del Plasminògen (t-PA); extret de la web: http://www.chem.cmu.edu/groups/ /Llinas/res/structure/tpa-big.html

- Domini *finger* (F), consituït pels residus 6-43, és homòleg al primer domini de la fibronectina, amb gran afinitat per la fibrina.
- Domini EGF, format pels residus 44-92, semblant al domini F i homòleg al EGF humà i murí. Participa juntament amb el domini F a l'aclariment hepàtic del t-PA.
- Dominis *Kringle* (K1 i K2), homòlegs als dominis K del plasminògen (residus 93-176 i 176-275). Els dominis K2 i F formen la regió d'interacció amb la fibrina.
- La cadena B o cadena lleugera, formada pels últims 230 aminoàcids, conté el domini proteolític. El centre actiu està format per la His_{322}, Asp_{371} i Ser_{478}.

Els primers assaigs clínics amb t-PA per a l'ictus isquèmic van començar a la dècada dels 90, l'escalatge de dosi va arribar fins els 0.95 mg/kg, el seu tamany reduit no permetia extreure conclusions d'eficàcia però semblava un tractament segur. El 1995 es van publicar els assaigs del ***N**ational **I**nstitute of **N**eurological **D**iseases and **S**troke* (NINDS) i del ***E**uropean and **A**ustralian **C**ooperative **S**troke **S**tudy* (ECASS I).

L'assaig del NINDS va incloure 634 pacients tractats durant les 3 primeres hores (50% 0-90 minuts i 50% 90-180 minuts). S'administrava una dosi de 0.9 mg/kg, el 10% en bolus i la resta en infusió contínua durant una hora. La primera part de l'estudi va incloure 301 pacients i se'n mesurava l'evolució neurològica a les 24h mitjançant l'escala NIHSS (***N**ational **I**nstitute of **H**ealth **S**troke **S**cale*). A la segona part es va mesurar el pronòstic funcional al 3er mes de 333 pacients, utlitzant l'**I**ndex **B**arthel (BI), ***G**lasgow **O**utcome **S**cale* (GOS), NIHSS i escala **M**odificada de **R**ankin (mRS). No es van observar diferències a les 24h (part 1), però si al pronòstic funcional en qualsevol de les escales emprades (part 2), tot i que la taxa d'hemorràgies simptomàtiques augmentava d'un 0.6% al grup placebo a un 6.4% al grup tractat. Aquest resultat va promoure l'aprobació de l'ús del tPA per part de la FDA, l'únic trombolític capaç d'aconseguir-la.

A l'assaig ECASS I, es van administrar a 620 pacients amb fins 6h d'evolució una dosi de 1.1 mg/kg de tPA en el mateix règim de 10% bolus i 90% en infusió contínua d'una hora. Pacients amb dèficits molt lleus o severs (hemiplegia, pèrdua de consciència) o amb signes primerencs al CT afectant més del 33% del territori irrigat per l'artèria cerebral mitja eren exclosos. Aquest últim criteri va provocar l'augment de la taxa de violacions fins al 17.4%, fruit de la inexperiència. Es van observar diferències significatives a l'escala mRS al 3er mes, però no a la BI. No obstant, hi havia una tendència de mortalitat més elevada i una freqüencia d'hemorràgies clarament superior (19.8% vs 6.5%).

El 1998 es publicaren els resultats del ECASS II. La dosi de t-PA es va rebaixar a 0.9 mg/kg i l'objectiu primari era la millora al 3er mes a l'escala mRS, BI o NIHSS als 30 dies. La mortalitat en ambdós grups va ser similar (10.5% i 10.7%). No es van detectar diferències a l'escala mRS, només una tendència per la combinació mRS/BI i una diferència significativa per la NIHSS als 30 dies. Els resultats negatius dels estudis ECASS es troben influenciats per la freqüent millora del grup placebo (37.7%), molt superior al 26% de l'estudi NINDS.

L'estudi ***A**lteplase **T**hrombolysis for **A**cute **N**oninterventional **T**herapy in **I**schemic **S**troke* (ATLANTIS) va començar l'any 1991 per demostrar l'eficàcia del t-PA en infarts de fins a 6 hores d'evolució. Per motius de seguretat, l'any 1993 es va restringir fins a 5 hores mentre que després de l'aprobació de la FDA (1996), es va limitar a la franja de 3 a 5 hores. La part A va incloure 142 pacients, els resultats es van publicar en 1999. El grup tractat tenia una millora a l'escala NIHSS a les 24 hores (40% vs 21%) revertida als 30 dies (60% vs 75%), acompanyada d'un increment de les hemorràgies intracerebrals simptomàtiques (11% vs 0%) i mortalitat al 3er mes (23% vs 7%). La part B va incloure 613 pacients, mesurant el pronòstic al 3er mes per les escales BI, GOS i mRS. No es van detectar diferències i van augmentar les hemorràgies intracerebrals simptomàtiques (7% vs 1.1%).

El metanàlisi de les dades dels assaigs NINDS, ECASS i ATLANTIS indicava un benefici del t-PA durant les 4.5 hores d'isquèmia, potencialment fins les 6 hores (Hacke et al, 2004). Així, el projecte ECASS III (2008) va extendre la finestra terapèutica del t-PA fins les 4.5 hores **(figura 26)**. Es van incloure 821 pacients, mesurant el pronòstic al 3er mes per les escales mRS, GOS, BI i NIHSS. El grup tractat va presentar un millor resultat funcional (52.4% vs 45.2%), tot i presentar una taxa més elevada d'hemorràgies (2.4% vs

0.2%) sense canvis a la mortalitat (7.7% vs 8.4%).

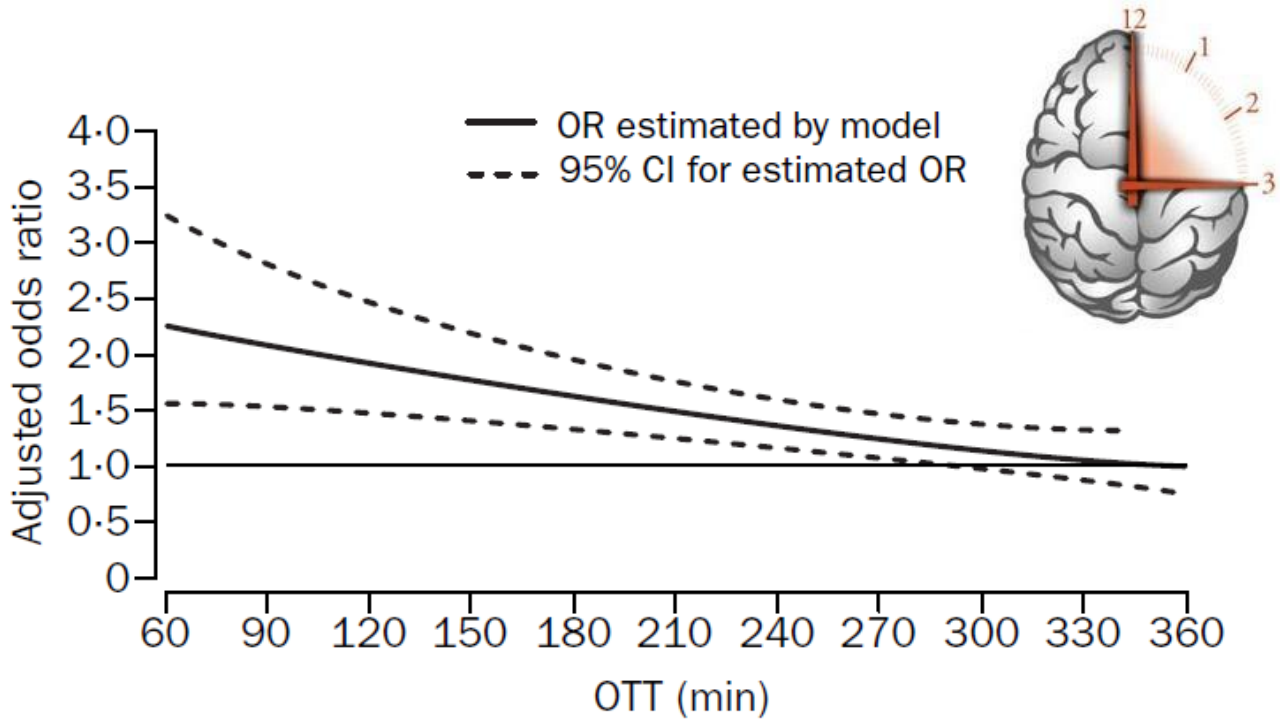


Figura 26. Benefici del tractament segons el temps transcorregut des de l'inici dels símptomes fins rebre t-PA. Modificat de Hacke et al, 2004.

Un cop aprobat, el fàrmac, es van realitzar 2 estudis de farmacovigilància. L'any 2000, es van presentar els resultats de l'estudi ***S**tandard **T**reatment with **A**lteplase to **R**everse **S**troke* (STARS) promogut per la FDA, que va incloure 389 pacients tractats segons criteris NINDS amb resultat positiu. Per contra, l'estudi de 3948 pacients a l'àrea de Cleveland va mostrar una mortalitat més elevada en pacients tractats amb t-PA (Katzan et al, 2000; Katzan et al, 2003), mentre un metaanàlisi acumulatiu mostrava que el tractament amb t-PA intravenós evitava una mort/dependència en un de cada set pacients tractats (Wardlaw et al, 2003).

Amb aquesta perspectiva, es van crear 2 registres: el ***C**anadian **A**lteplase for **S**troke **E**ffectiveness **S**tudy* (CASES) al Canadà i el ***S**afe **I**mplementation of **T**hrombolyisis in **S**troke - **MO**nitoring **ST**udy* (SITS-MOST) a Europa. Els resultats del CASES van publicar-se en 2005, amb resultat positiu en un total de 1135 pacients (Hill & Buchan, 2005).

L'estudi SITS-MOST, promogut per l'agència europea del medicament, va demostrar el benefici del t-PA en 6483 pacients (Wahlgren et al, 2007). La seva extensió ***I**nternational **S**troke **T**reatment **R**egistry* (SITS-ISTR) va incloure 664 pacients tractats al període 3-4.5h, sense canvis al pronòstic entre els períodes 0-3h i 3-4.5h, corroborant els resultats positius de l'estudi ECASS III (Ahmed et al, 2010).

Finalment, els assaigs s'han especialitzat en el tractament de pacients seleccionats mitjançant criteris radiològics (CT o DWI/PWI) a la franja 3-6h. Els estudis ***D**iffusion and perfusion imaging **E**valuation **F**or **U**nderstanding **S**troke **E**volution* (DEFUSE) amb 74 pacients i ***E**cho**P**lanar **I**maging **TH**rombolytic **E**valuation **T**rial* (EPITHET) amb 101 pacients han demostrat la seguretat i eficàcia del t-PA en pacients seleccionats per radiologia CT.

D'altra banda, en estudis guiats per resonància magnètica, es va demostrar l'efectivitat del tPA fins a les 6h en dues sèries de 56 i 174 pacients (Ribó et al, 2005; Thomalla et, 2006). La comparació directa de diferents criteris radiològics (CT<3h, MRI<3h i MRI<3h) en una serie de 382 pacients consecutius va mostrar una millora significativa als grups seleccionats per la diferència DWI/PWI, sense influir el temps d'inclusió (Kohrmann et al, 2006). Els resultats es van corroborar en un estudi multicèntric posterior de 1210 pacients; l'ús de la MRI va reduir considerablement el risc de sagnat (Schellinger et al, 2007).

Altres estudis més recents tenen criteris d'inclusió més limitats. Al *Third **I**nternational **S**troke **T**rial* (IST-3) el neuròleg aleatoritza els pacients en qui dubta si tractar o no, per determinar quines característiques influeixen a la resposta al t-PA.

Tot i els grans beneficis de l'administració del t-PA, un 60-70% dels pacients no recanalitzen l'artèria durant l'hora següent a l'administració del fàrmac i entre un 2-10%

dels pacients pateixen hemorràgiques cerebrals iatrogèniques associades amb una elevada mortalitat (Wahlgren et al, 2007). L'increment progressiu de la finestra terapèutica no ha de permetre la relaxació del personal d'assistència primària o les unitats d'ictus, perquè el benefici terapèutic es redueix amb el temps d'oclusió.

8.1.9 Tenecteplasa (TNK)

La tenecteplasa (TNK) és una molècula de t-PA modificada per mutagénesi dirigida en 6 residus dels dominis K1 y SP: Thr103Asp, Asp117Glu, Lys296Ala, His297Ala, Arg298Ala, Arg299Ala **(figura 27)**. Les mutacions al domini K1 afegeixen un nou punt de glucosilació (103) i n'eliminen un de carboxilació (117) reduïnt el seu aclariment hepàtic. La modificació dels aminoàcids 296-299 al domini proteasa incrementa la vida mitja de la TNK (20 minuts), incrementa l'especificitat per la fibrina i el fa més resistent a la inactivació per PAI-1.

La TNK es va provar en un estudi d'escalatge de dosi, 88 pacients van rebre un únic bolus de 0.1, 0.2, 0.4 ó 0.5 mg/kg durant les 3 primeres hores. No es van detectar diferències entre els pacients tractats amb TNK i els controls de l'estudi NINDS (Haley et al, 2005). En un estudi posterior sense aleatoritzar, es van comparar en pacients amb DWI/PWI > 20% a dosis l'administració de t-PA durant les 3 primeres hores (n=29) amb la de TNK a 0.1 mg/Kg, a les 3-9 hores (n=15). Els individus que van rebre TNK van mostrar una freqüència més alta de recanalització (Parsons et al, 2009).

L'assaig fase IIB es va iniciar el 2005 i va terminar el 2008, amb 112 pacients inclosos perquè el reclutament era massa lent (Haley et al, 2010). La dosi de 0.4 mg/kg es va descartar quan només s'havien aleatoritzat 73 pacients, però no es va poder triar la dosi ideal entre 0.1 i 0.25 al final de l'assaig (la dosi de 0.25 era superior, no significativament).

No hi havien indicis de superioritat del TNK sobre el tPA (Haley et al, 2010).

8.1.10 Reteplasa

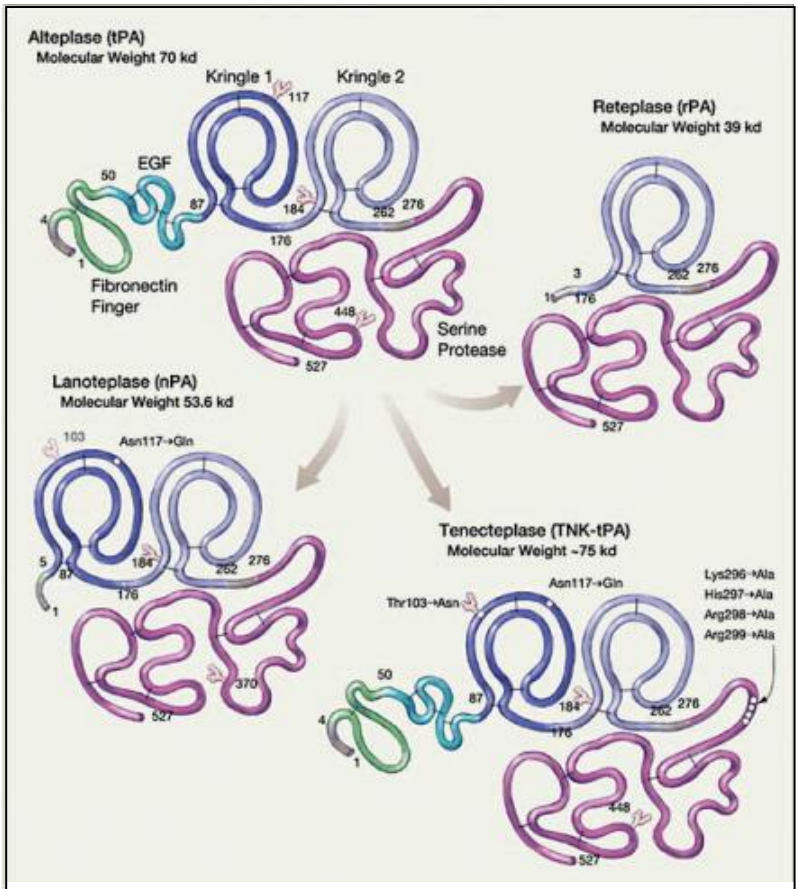


Figura 27. Nous trombolítics modificats del t-PA (Alteplase). Reteplase i Lanoteplase són formes truncades de vida mitja més llarga, mentre Tenecteplase (TNK) presenta diverses modificacions puntuals amb el mateix objectiu. Extret de Llevadot et al, 2001

La reteplasa (rPA) és una forma truncada del t-PA generada en *Escherichia coli*, consta només d'una cadena no glicosilada de 355 aminoàcids (39 kDa) **(figura 27)**. La seva unió a fibrina i la seva eliminació hepàtica es troben significativament reduïdes, amb una vida mitja plasmàtica a 14-18 minuts. Aquest fàrmac s'ha probat en pacients d'ictus de fins a 9 hores d'evolució, amb índex elevats de recanalització (Qureshi et al, 2001).

També s'ha fet un escalat de dosi en combinació amb antagonistes de les glicoproteïnes IIb/IIIa plaquetars al període 3-24h, seleccionant-los per MRI (assaig ROSIE) o CT scan (assaig ROSIE-CT), però no va oferir els resultats esperats. Els 2 assaigs es van terminar prematurament al 2008 i els resultats no van ésser publicats.

8.1.11 Lanoteplasa, Manoteplasa i Pamiteplasa

La lanoteplasa (nPA) és una altra forma truncada de t-PA que manté els dominis Kringle (amb una mutació puntual Asp117Glu) i serin-proteasa **(figura 27)**. Es va probar en una

dosi de 120 kU/kg a l'assaig de fase 2 InTIME d'infart de miocardi, aconseguint taxes més elevades de recanalització que l'alteplasa als 60 i 90 minuts. Els resultats preliminars de fase 3 mostraven una eficàcia similar, però una incidència més elevada de complicacions hemorràgiques sistèmiques i intracerebrals (Bhana & Spencer, 2000).

La pamiteplasa és un altre trombolític de tercera generació que presenta una delecció al domini Kringle 1 i una mutació puntual Arg275Glu, incrementant la vida mitja plasmàtica del trombolític. Aquest fet permet aconseguir la mateixa activitat trombolítica amb una tercera part de la dosi equivalent de t-PA en models animals. Es va comprobar als assaigs de farmacocinètica, mesurant els nivells del fàrmac per ELISA (Oikawa et al, 2000).

La monteplasa té una mutació al domini EGF que incrementa la seva vida mitja a 20 minuts, i pot ésser administrat com un únic bolus. Es va provar com a teràpia per a l'infart de miocardi a l'assaig ***CO****mbining* ***M****onteplase with* ***A****ngioplasty* (COMA). L'assaig va incloure 154 malalts: placebo, angioplastia i monteplasa + angioplastia on van demostrar la seva eficàcia independent dels nivells de PAI-1 (Inoue et al, 2002).

8.2 La sonotrombòlisi

La sonotrombòlisi és la insonació del trombus amb ultrasons mitjançant el Doppler Transcraneal (DTC) **(figura 28)**. Aquesta tècnica, utilitzada normalment per monitoritzar la dissolució del coàgul de forma no invasiva, pot utilitzar-se com a tractament complemetari del t-PA, incrementant-ne l'eficàcia a freqüències de 20 kHZ-2 Mhz i intensitats de 0.2-2.0 W/cm^2. Intensitats superiors a 2 W/cm^2 provoquen la vacuolització i denudació de l'endoteli, reoclusió arterial i lesions cutànies.

En models “in vitro”, l'aplicació d'ultrasons incrementa la temperatura del trombus i el

transport de tPA al seu interior, agilitzant la seva dissolució. Com més baixa és la freqüència de les ones, més energètica és la ona, millor penetra el crani i té més capacitat trombolítica. La combinació d'ultrasons i microbombolles (microesferes carregades d'un gas inert) pot dissoldre coàguls eficientment mitjançant l'estrés mecànic, en models "in vitro" i models animals sense necessitat de tractament trombolític. A més, s'han desenvolupat microbombolles encapsulades per fosfolípids o unides a antagonistes dels receptors Iib/IIIa plaquetars, incrementant el seu potencial terapèutic.

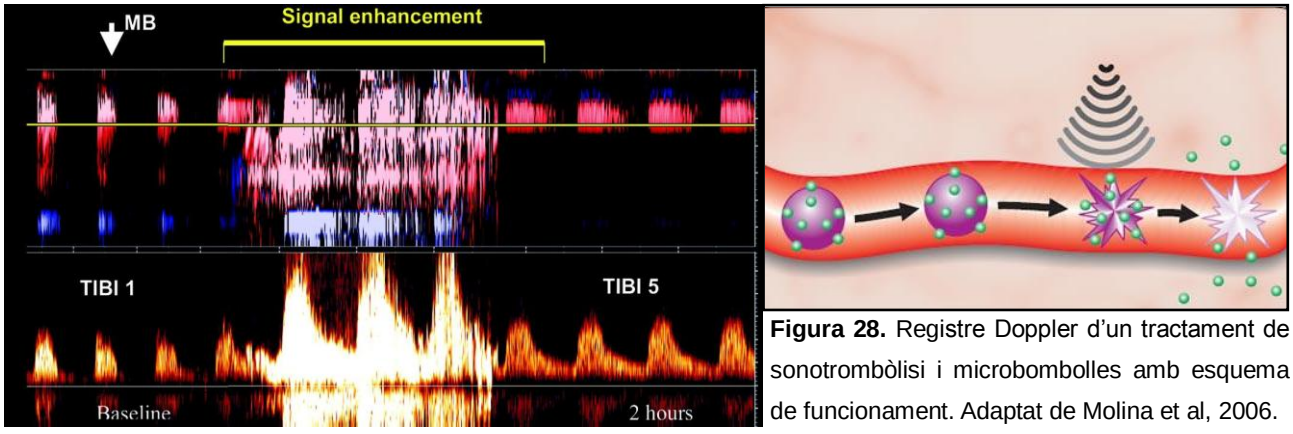


Figura 28. Registre Doppler d'un tractament de sonotrombòlisi i microbombolles amb esquema de funcionament. Adaptat de Molina et al, 2006.

El primer assaig clínic va ser el TRUMBI (***TR****anscranial low-frequency* ***U****ltrasound-****M****ediated thrombolysis in* ***B****rain* ***I****schemia*) l'any 2005. Es va comprobar l'eficàcia dels ultrasons en combinació amb el t-PA. La freqüència inicial era de 40 kHz però per molèsties als pacients es va modificar a 300 kHz amb una intensitat de 700 W/cm^2 durant 90 minuts. L'estudi es va detenir prematurament amb 26 pacients inclosos, degut al 35% d'hemorràgies cranials simptomàtiques.

L'any 2004, l'estudi de fase II CLOTBUST (***C****ombined* ***L****ysis* ***O****f* ***T****hrombus in* ***B****rain ischemia with* ***U****ltrasound in* ***ST****roke*) va incloure 126 pacients, dels quals 63 van rebre t-PA i una insonació de 2 MHz a 720mW/cm^2. El grup insonat presentava millor taxa de recanalització i millora neurològica, sense diferències a les reoclusions (12%) o

transformacions hemorràgiques simptomàtiques (4.8%). Per contra, en un estudi aleatoritzat amb Duplex Transcraneal, només es van incloure 25 pacients degut a l'elevat nombre d'hemorràgies cerebrals simptomàtiques (18% vs 5%) (Eggers et al, 2003). Per últim, s'ha combinat la teràpia amb ultrasons amb la trombolisi mecànica per disrupció.

8.3 Dispositius mecànics

Les tècniques endovasculars, generalment utilitzades com a teràpies de rescat, permeten l'ús de dispositius mecànics combinats amb baixes dosis de trombolítics, el que redueix el risc d'hemorràgies sistèmiques.

El catèter s'introdueix per l'artèria femoral fins la caròtida interna, d'on s'avançarà el microcatèter fins la oclusió, generalment localitzada al segment M1 de l'artèria cerebral mitja. En els ictus de l'artèria basilar, el catèter guia es situarà a l'artèria subclavia o a l'origen de l'artèria vertebral. S'atravessa el coàgul amb el microcatèter i la infusió del contrast permet veure la longitud del trombus.

Els dispositius mecànics no han estat provats en assaigs clínics, l'evidència es limita a sèries clíniques més o menys amples. Segons el mètode d'actuació, podem dividir-los en dispositius d'extracció o de disrupció.

8.3.1 Tècniques d'extracció

Dins els dispositius d'extracció, en tenim que capturen (Microsnare, Neuronet, MERCI) o que succionen (Neurojet, Penumbra) el coàgul **(figura 29)**. El dispositiu amb millor resultat és el MERCI, que combina una guia en forma d'espiral que atrapa el coàgul i un globus que es desplega a la caròtida ipsilateral, eliminant l'efecte de la pressió sanguínea que empeny el trombus distalment. Només cal tirar del dispositiu i extreu-re el coàgul, amb un

60-70% de recanalització. En pacients tractats fins a 8h va demostrar un millor pronòstic funcional, rebent l'aprobació de la FDA. També està aprobat des del 2008 el dispositiu Penumbra, que permet succionar petits fragments del trombus fins la seva eliminació.

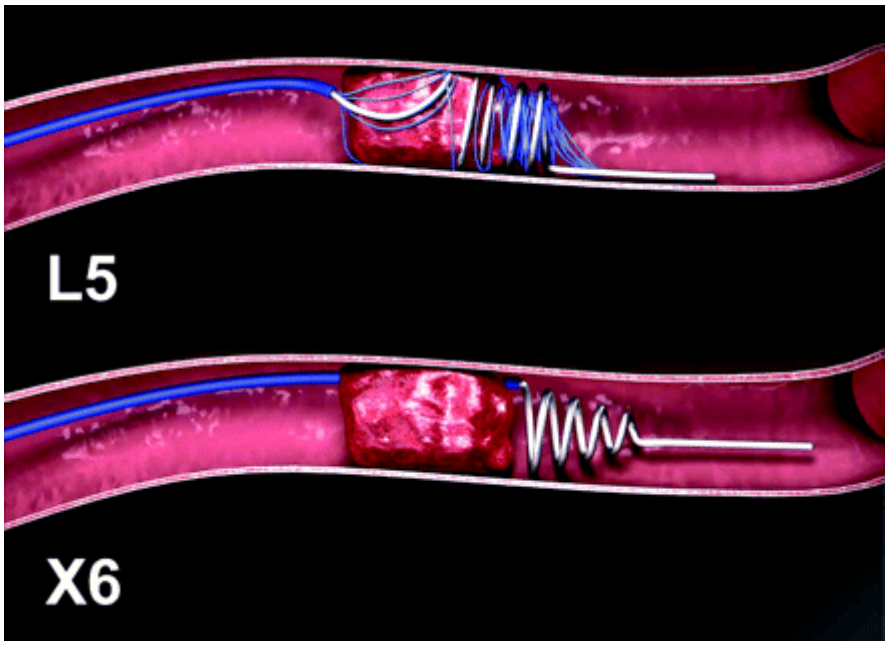


Figura 29. Esquema dels dispositius MERCI per a la extracció mecànica del coàgul. La variant L5 incorpora uns monofilaments adherits a l'espiral (part superior) present a la X5/X6 (inferior). Extret de l'assaig clinic MERCI (Smith et al, 2008).

8.3.2 Tècniques de disrupció

El seu objectiu és la fragmentació del coàgul, ja sigui per pas repetit de la via, fragmentació per láser (catèters EPAR) o acústica endovascular (EKOS MicroLysisUS). El dispositiu EKOS MicroLysisUS utilitza un microcatèter per emetre ultrasons (2.1 Mhz, 400mW/cm^2) directament a l'interior de l'artèria ocluida i ha estat provat amb i sense emissió.

Els assaigs ***I**nterventional **M**anagement of **S**troke* (IMS) I i IMS-II van comprobar l'eficàcia en 81 pacients que la trombolisi combinada intraarterial / intravenosa (n=55) era superior a la trombolisi intravenosa (n=26) durant les primeres 3 hores. La mortalitat es va situar al 16%, amb taxes de recanalització superior als assaigs previs PROACT-II i MERCI. L'assaig IMS-III es va iniciar el Juliol de 2006 i finalitzarà al 2011, amb un tamany mostral

de 900 pacients.

8.4 Tractaments neuroprotectors

Tots els tractaments que hem vist fins ara pretenien restablir el flux sanguini a la zona afectada. De forma complementària, podem intentar bloquejar els mecanismes de mort associada a la cascada isquèmica o potenciar les accions reparadores posteriors a l'infart. Aquesta idea és senzilla, pero l'obtenció de fàrmacs neuroprotectors útils a la pràctica clínica no està donant els resultats esperats. S'han probat més de 1000 compostos en models animals, dels quals 114 s'han emprat en assaigs clínics, tots negatius (O'Collins et al, 2006).

A les reunions STAIR (1999 i 2001) es van plantejar les possibles causes i solucions d'aquest fracàs: utilitzar models animals més adequats, amb finestres terapèutiques més llargues, controlant els efectes adversos i afegir noves mesures objectiu (pronòstic funcional a més del volum de l'infart). Els fàrmacs han de demostrar la seva eficàcia en diversos models d'isquèmia en diverses espècies animals.

Amb aquestes premises va evolucionar el compost NXY-059, que en models de rata i primat va ser un neuroprotector efectiu (Kuroda et al, 1999; Marshall et al, 2003). Els resultats preliminars de l'assaig SAINT també eren positius (Lees et al, 2006), però fracassaren al SAINT-2 (Diener et al, 2008). Un problema afegit és que el fàrmac ha d'arribar en concentracions suficients a la zona hipoperfundida de penombra i hauria de bloquejar diverses vies de mort cel·lular, una alternativa seria la seva administració coadjuvant amb el t-PA.

L'últim fàrmac en estudi és la citilcolina. L'assaig ICTUS promou l'administració d'una dosi

de 2000g intravenosa durant les primeres 24h d'oclusió de l'artèria cerebral mitja en pacients amb NIHSS≥8. L'objectiu és demostrar la recuperació global del pacient a les escales BI, mRS i NIHSS.

D'altra banda, s'ha observat que fàrmacs ja utilitzats a la pràctica clínica poden tenir efectes neuroprotectors. En aquest grup trobariem el clopidogrel, les estatines, l'eritropoietina, els inhibidors de l'angiotensina II, la minociclina i les tiazolidinediones.

8.5 Tractaments neurorreparadors

Aquestes teràpies, amb un ampli potencial terapèutic, encara estan en fase de desenvolupament al model animal. La potenciació de les vies reparadores després de la isquèmia s'ha enfocat a la infusió de factors de creixement o cèl·lules mare (principalment mesenquimals o endotelials). Els resultats en models animals són molt prometedors, però s'han de prendre amb cautela.

Els primers assaigs clínics en humans indiquen que la infusió de VEGF promou l'augment de cèl·lules progenitores endotelials (EPCs) circulants, com s'havia observat als models experimentals (Kalka et al, 2000). Els beneficis de la infusió de cèl·lules mare de mèdula ossia (BMSC) han estat demostrat en assaigs clínics d'infart de miocardi (Schachinger et al, 2004) i isquèmia cerebral (Tateishi-Yuyama et al, 2002; Bang et al, 2005). Finalment, la infusió de la línia cel·lular neuronal N Tera 2 (NT-2) en 12 pacients va demostrar la seguretat del tractament, però no semblava efectiu (Kondziolka et al, 2000).

Malgrat totes les alternatives que existeixen pel tractament de l'ictus isquèmic, la ràpida reperfusió per efecte del t-PA intravenós sembla la millor teràpia disponible.

9. Protocol del tractament amb t-PA

Com hem vist, el tractament trombolític amb t-PA és l'únic fàrmac aprobat per la fase aguda de l'ictus isquèmic. Segueix un protocol i criteris d'inclusió estrictes, el que provoca la seva administració només al 2-14% dels malalts (Litchman et al, 2009). La dosi de t-PA emprada és de 0.9 mg/kg fins un màxim de 90 mg. El 10% de la dosi s'administra en forma de bolus intravenós d'un minut i el 90% restant s'administra per infusió contínua durant una hora. L'administració del fàrmac ha d'iniciar-se durant les 4.5 horas després de l'inici dels símptomes. Després d'aquest període, el tractament amb t-PA està associada a un increment de la mortalitat (Hacke et al, 2004).

Els principals criteris d'exclusió són l'edat, la presència d'hemorràgies al CT o historial d'hemorràgies cerebrals i el temps de evolución des de l'inici dels símptomes. Alguns d'ells encara s'estan redefinint. Els resultats més recents indiquen que el tractament de pacients diabètics o amb INR<2 és segur, però no si han rebut heparina de baix pes molecular (LXII reunió de la SEN, 2010) **(Taula).**

Criteris d'exclusió

- Edat > 80 anys
- Símptomes menors o milloria evident (NIHSS<4) abans de l'inici de la infusió
- Ictus greu segons criteris clínics (NIHSS > 25) o de neuroimatge (hipodensitat > 33% ACM)
- Evolució dels símptomes superior a les 4.5 hores
- Crisis comicials a l'inici de l'ictus
- Pressió arterial sistòlica superior a 185 mm Hg, pressió arterial diastòlica superior a 110 mmHg o necessitat de mesures agressives per reduir la tensió arterial a aquests límits
- Ictus durant els tres mesos previs o ictus anterior concomitant amb diabetis mellitus
- Glucèmia per sota de 50 mg/dL o per sobre de 400 mg/dL
- Plaquetopènia, és a dir, recompte plaquetari per sota de 100.000 / mm^3
- Tractament amb anticoagulants orals

- Tractament amb heparina durant les 48 hores prèvies y TTPa elevat

- Hemorràgia intracranial a la tomografia computeritzada (CT)
- Símptomes suggestius d'hemorràgia subaracnoidea (encara que la CT sigui normal)
- Antecedents d'hemorràgia intracerebral o d'hemorràgia subaracnoidea per ruptura d'un aneurisma
- Historial de lesions al sistema nerviós central (aneurismes, neoplàsies, cirurgia intracraneal o medul·lar)

- Sagnat greu recent o manifest
- Diatesi hemorràgica coneguda
- Cirurgia major o traumatisme significatiu durant els tres mesos previs

- Retinopatia hemorràgica (ex. retinopatia diabètica)
- Antecedents de massatge cardíac, part o punció d'un vas sanguini no accessible durant els 10 dies previs
- Endocarditis bacteriana, pericarditis
- Pancreatitis aguda
- Malaltia ulcerativa gastrointestinal documentada els tres mesos previs, varius esofàgiques, malformacions vasculars intestinals conegudes
- Neoplàsia amb increment del risc de sagnat
- Malaltia hepàtica greu (insuficiència hepàtica, cirrosi, hipertensió portal, hepatitis activa)

9.1 Efectivitat del tractament amb t-PA

Com ja hem dit, l'objectiu del tractament amb t-PA és la recanalització de l'artèria en el menor temps possible, per restablir el flux sanguini a l'área isquèmica i salvar la zona de penombra. La recanalització del vas pot monitoritzar-se de forma no invasiva mitjançant el DTC, fixant el transductor a la regió temporal del pacient utilitzant una mena de casc que manté l'angle d'insonació constant **(figura 30)**. Per determinar la presència d'oclusió o recanalització s'utilitza l'escala ***T**hrombolyisis **I**n **B**rain **I**schemia* (TIBI) (Demchuk et al, 2001), derivada de l'escala angiogràfica d'infart de miocardi TIMI (***T**hrombolysis **I**n **M**yocardial **I**nfarction*) (Ganz et al, 1985). S'ha demostrat que la velocitat de lisi del

trombus es relaciona amb la millora neurològica inicial, el tamany de l'infart i el pronòstic del pacient (Christou et al, 2000; Molina et al, 2001; Alexandrov et al, 2001; Röther et al, 2002). En cas de no aconseguir-se la recanalització arterial durant la infusió del t-PA intravenós, els pacients podrien ésser elegibles per a teràpies de rescat (Ribó et al, 2006).

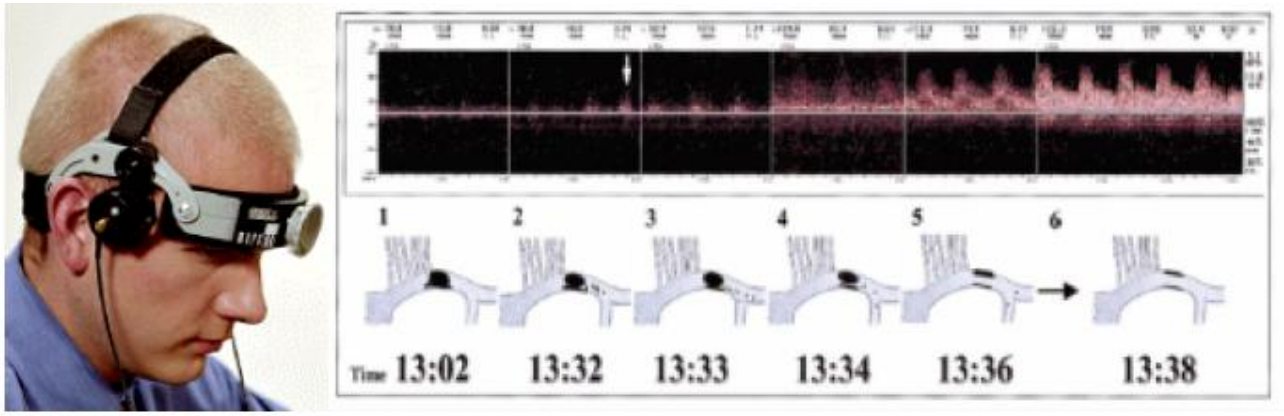


Figura 30. Monitorització de la recanalització arterial mitjançant Doppler Transcraneal. Extreta de Ribó (tesi doctoral)

Els predictors de recanalització arterial inclouen l'etiologia aterotrombòtica a la classificació TOAST, baixos nivells de PAI-1 (Ribó et al, 2004), no patir hiperglucèmia (Ribó et al, 2004) i α-2-antiplasmina (Marti-Fabregas et al, 2005). Recenment s'ha descrit que la densitat del coàgul també pot predir la recanalització, encara per confirmar (LXII reunió de la SEN, 2010).

9.2 Complicacions del tractament amb t-PA

Les complicacions del tractament trombolític inclouen, entre d'altres, l'edema intracerebral, la transformació hemorràgica i la reoclusió del vas cerebral.

9.2.1 Transformació Hemorràgica

La transformació hemorràgica (TH) forma part de l'evolució natural de l'ictus isquèmic. Abans de la introducció de la teràpia trombolítica, fins un 43% dels pacients (segons la

sèrie) presentaven sagnats a la zona infartada (Toni et al, 1996), éssent més freqüent als infarts cardiembòlics >10cc (Hornig et al, 1993). No obstant, el 89% de les hemorràgies eren petequials, sense cap repercusió clínica. Amb la introducció dels trombolítics, la freqüència i gravetat dels sagnats s'incrementà considerablement, constituint la complicació principal del tractament amb t-PA. Les hemorràgies es classifiquen segons els criteris ECASS (Larrue et al, 1997):

- **I**nfart **H**emorràgic tipus 1 (HI-1): hemorràgia petequial
- **I**nfart **H**emorràgic tipus 2 (HI-2): hemorràgia petequial confluent
- **H**ematoma **P**arenquimatós tipus 1 (PH-1): hematoma < 30% de la zona infartada amb un escàs efecte de massa
- **H**ematoma **P**arenquimatós tipus 2 (PH-2): hematoma >30% de la zona infartada amb un efecte de massa important
- **H**ematoma **P**arenquimatós remot (PH-r): dessigna qualsevol sagnat intracraneal allunyat de la zona infartada

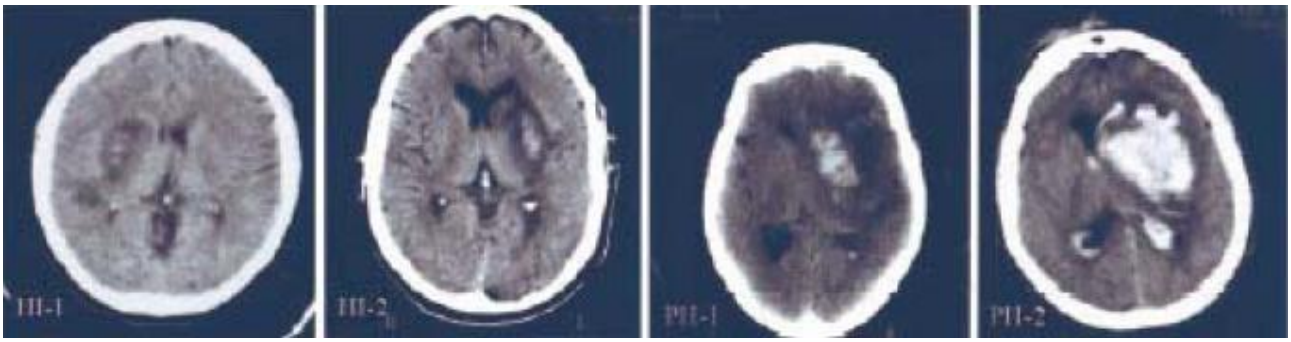


Figura 31. Classificació ECASS de la Transformació Hemorràgica. Modificada de Montaner et al, 2006.

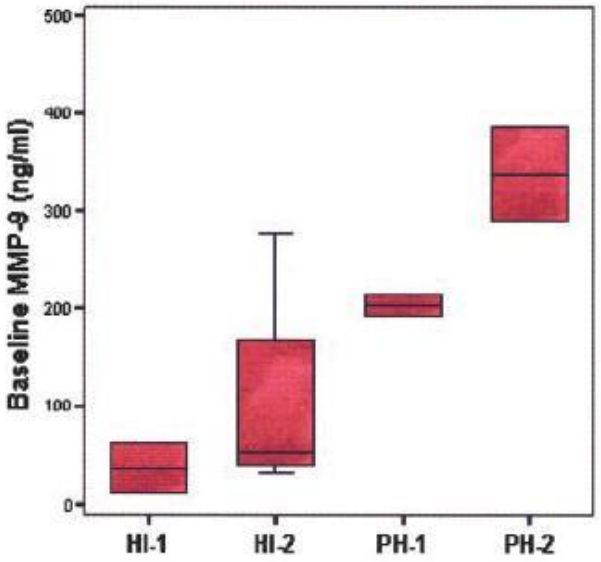
Aquests 3 últims subtipus són les hemorràgies més greus i sovint empitjoren la simptomatologia **(Figura 31)**. Les hemorràgies simptomàtiques varien en definició i freqüència entre els diferents assaigs clínics, des del 1.7% (SITS-MOST) fins el 19.8% (ECASS I). Prevaleixen els criteris de l'ECASS II, considerant-se simptomàtiques aquelles

que provoquen un deteriorament neurològic mesurat com un increment de 4 o més punts a l'escala NIHSS.

Sembla evident que les hemorràgies PH-2 van associades a un empitjorament precoç, mortalitat i pronòstic funcional (Fiorelli et al, 1999; Berger et al, 2001). Encara es debat si les PH-1 i HI-2 també tenen efectes deleteris. En ocasions, no és fàcil determinar si l'empitjorament és causat pel sagnat, el creixement de l'infart, l'edema intracerebral o altres causes no neurològiques com les infeccions. En qualsevol cas, les TH simptomàtiques són devastadoras, va provocar la mort al 45% dels afectats de l'estudi NINDS i al 83% del PROACT-II (Khatri et al, 2007).

Existeixen diferentes factors de risc associats al risc de patir transformacions hemorràgiques. L'edat (Larrue et al, 2001), plaquetopenia i hiperglucèmia (Tanne et al, 2002), hipodensitat al CT (Toni et al, 1996; Larrue et al, 2001; Tanne et al, 2002). Tots aquests són factors d'exclusió relativa per a l'administració de t-PA. Altres predictors són les lesions isquèmiques extenses, l'etiologia cardioembòlica, la hiperglucèmia i el tractament trombolític (Paciaroni et al, 2008).

L'albuminúria (ratio albúmina/creatinina > 30 mg/g), en mostres d'orina recollides durant les 3 primeres hores (Rodríguez-Yáñez et al, 2006) i els nivells plasmàtics elevats de MMP-9 (Montaner et al, 2003; Castellanos et al, 2003; Castellanos et al, 2007), fibronectina cel·lular (Castellanos et al, 2004; Castellanos et al, 2007), S100B (Foerch et al, 2004), proteïna C activada (Mendióroz et al, 2009), SSAO (Hernández-Guillamon et al, 2010) i malondialdehid (Domínguez et al, 2010) són predictores del sagnat **(figura 32)**. Recenment s'han descrit els nivells de creatinina quinasa, encara pendent de confirmar (LXII reunió de la SEN, 2010).



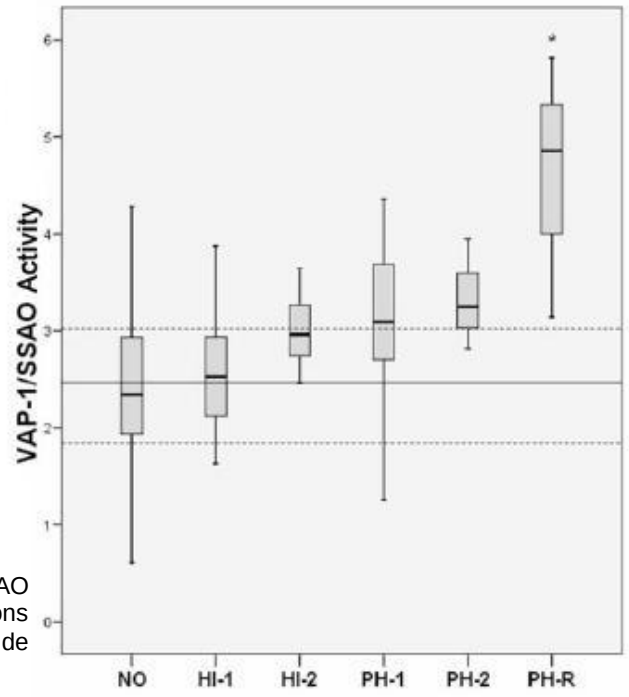


Figura 32. Els nivells basals de MMP-9 i l'activitat VAP-1/SSAO prediuen la presència i severitat de les transformacions hemorràgiques després del tractament amb t-PA. Extreta de Montaner et al, 2003 i Hernández-Guillamón et al, 2010.

9.2.2 Reoclusió arterial

La reoclusió arterial a l'ictus es defineix com una nova oclusió d'una artèria prèviament recanalitzada, per analogia a un concepte angiogràfic de la trombolisis a l'infart agut de miocardi. Un 5-14% dels pacients d'infart de miocardi pateixen una reoclusió durant les primeres hores o dies des de l'event, que comporta un increment de la mortalitat.

La primera aproximació a la reoclusió de l'ictus és el ***D**eterioration **F**ollowing **I**mprovement* (DFI) de l'assaig NINDS. El DFI es va definir com un increment en 2 o més punts de l'escala NIHSS després d'una millora inicial, havent-se descartat la transformació hemorràgica, hipotensió arterial o edema cerebral. La freqüencia del DFI al grup tractat i placebo va ésser similar, el que es va interpretar com un impacte escàs de la reoclusió a l'ictus isquèmic. En un estudi incloent 60 pacientes amb una oclusió aguda de la ACM tractats amb t-PA endovenós, el percentatge de reoclusions va ser del 34% (Alexandrov and Grotta, 2002). En un estudi de 29 pacients tractats consecutivament

amb trombolisi endovenosa i intraarterial, es va demostrar la presència de reoclusió en un 22% dels pacients que havien recanalitzat (Heo et al, 2003).

Estudis previs en infart de miocardi han demostrat que la reoclusió es més freqüent en artèries amb una estenosi residual i en situacions de major activació plaquetar. Els predictors clínics de reoclusió arterial van ser descrits al nostre centre: NIHSS≥17 i oclusió en tandem de l'artèria cerebral mitja i la caròtida ipsilateral (Rubiera et al, 2006).

9.3 Limitacions del tractament amb t-PA

El tractament amb t-PA té diverses limitacions: d'una banda, l'estreta finestra terapèutica i els estrictes criteris d'inclusió només permeten tractar un 2-14% dels pacients (Litchman et al, 2009). D'altra banda, els índex de recanalització del vas són del 60% a les 6 hores després del tractament (Molina et al, 2004), un 7-15% patirà una reoclusió (Rubiera et al, 2006) i un 2-4% patirà una transformació hemorràgica simptomàtica (Wahlgren et al, 2007).

Aquests complicacions es relacionen amb els efectes adversos del tractament: dins el parènquima, el t-PA té efectes neurotòxics per estimulació dels receptors de glutamat (Kaur et al, 2004); d'altra banda, el t-PA estimula la degranulació de MMP-9 (Cuadrado & Ortega et al, 2008) el que afavoreix la formació d'edema i hemorràgies; per últim, estimula la producció de ROS amb efectes neurotòxics i inflamatoris (Kaur et al, 2004) **(figura 33)**.

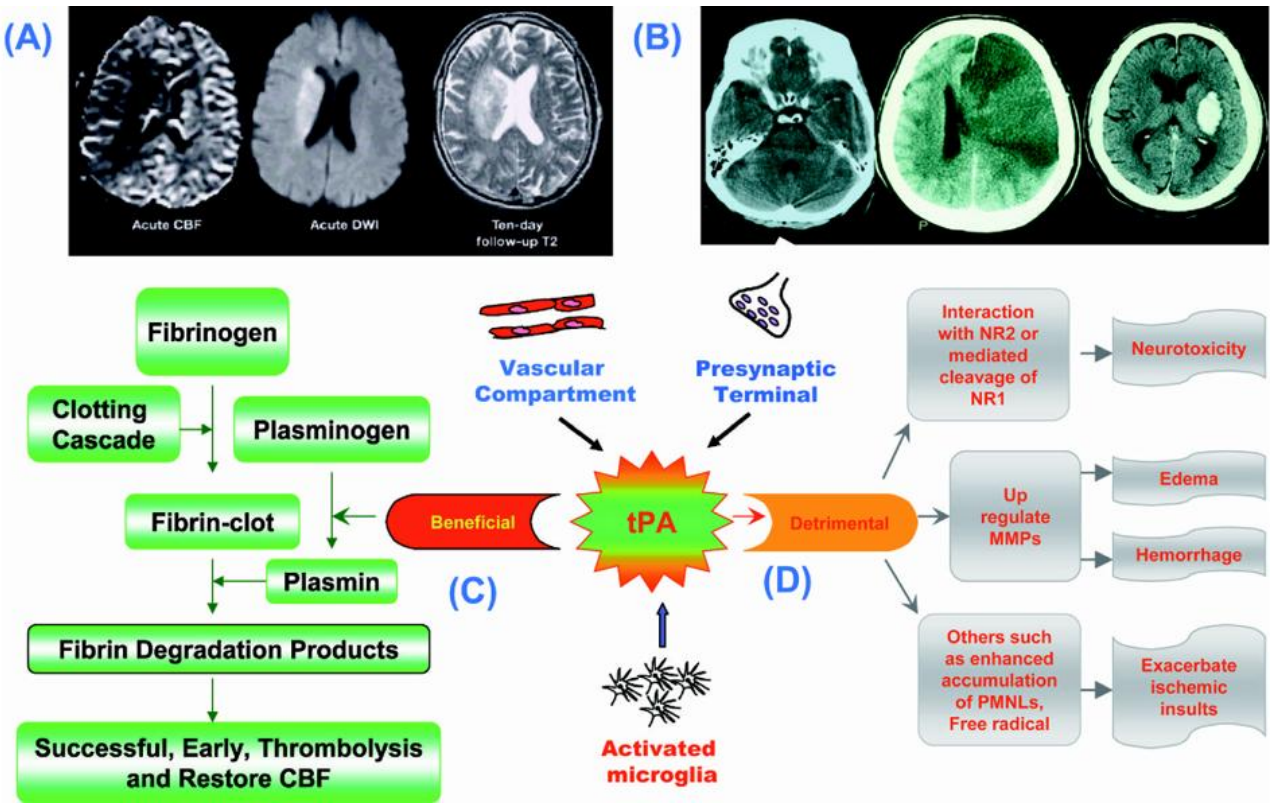


Figura 33. Efectes beneficiosos (C) i deleteris (D) del t-PA, que poden limitar el tamany de la lesió (A) o provocar una transformació hemorràgica (B). Extreta de Kaur et al, 2004.

Tot i disposar de múltiples marcadors de cadascun d'aquests fenòmens, no disposem de models predictius que permetin guiar les decisions clíniques durant la fase aguda o desenvolupar tractaments coadjuvants al tPA.

10. La Farmacogenètica

La farmacogenètica estudia la influència de la genètica a la resposta a diferents tractaments. Des del punt de vista clínic, podem emprar-la per trobar la dosi ideal de tractament o triar el millor fàrmac d'entre diverses alternatives. Des del punt de vista científic, les variants associades ens poden indicar noves vies implicades a la resposta al tractament i permetre el desenvolupament de combinacions coadjuvants més efectives i segures.

Els estudis farmacogenètics tenen a una clara limitació en la capacitat per reclutar mostres en comparació amb els estudis poblacionals. No obstant, la seva homogeneïtat permet obtenir resultats significatius amb tamanys mostrals menors si les variables resposta estan prou ben definides. Els diferents estudis GWAS publicats els darrers anys van demostrar la seva aplicació per ajustar la dosi de warfarina (Cooper et al, 2008; Takeuchi et al, 2009), acenocumarol (Teichert et al, 2009) i clopidogrel (Shuldiner et al, 2009), i definir efectes adversos de les estatines (Link et al, 2008). Per contra, els estudis amb diferents fàrmacs que utilitzaven com a variable objectiu alguna escala clínica, més subjectives i generals, no han trobat gairebé resultats positius (Daly, 2010).

Típicament, els polimorfismes associats amb la resposta a fàrmacs pertanyen a la familia de detoxificadors del Citocrom P450, perquè regulen la quantitat de fàrmac circulant. Altres enzims detoxificadors, com les glutation S-transferases també podrien participar en aquest procés.

Les glutation S-transferases (GST) és una familia de proteïnes dimèriques multifuncionals que catalitzen la conjugació entre glutations reduïts (GSH) electròfils i neutròfils. La seva funció primària és la detoxificació de compostos electròfils que s'uneixen a l'ADN, una

reacció metabòlica de fase II (Pickett y Lu, 1989). Les GSTs també catalitzen la reducció del peròxid d'hidrògen orgànic, jugant un paper clau a la protecció de l'organisme davant el stress oxidatiu (Mannervik, 1985).

Els enzims GST poden trobar-se en forma solubles o de membrana. Les GST solubles s'han classificat en cinc famílies: GSTA, GSTM, GSTP, GSTT i microsomals (Rushmore y Pickett, 1993; Mannervik, 2003), cadascuna amb diferents perfils d'afinitat per cadascun dels substrats i expressió pels diversos teixits (Mannervik, 2003). Les GSTs circulen com homodímers o heterodímers de dues subunitats de la mateixa classe.

10.1 Farmacogenètica de l'ictus

Al camp de l'ictus, no existeixen gaires estudis de farmacogenètica, la gran majoria han estat realitzats pel nostre laboratori mitjançant seqüenciació directa de gens candidats. Les variants I/D (rs1799752) de l'enzim conversor de l'angiotensina (ECA) (Fernández-Cadenas et al, 2006), 4G/5G (rs1799768) del PAI-1 (Fernández-Cadenas et al, 2007) i Thr325Ile (rs1926447) del TAFI (Fernández-Cadenas et al, 2007) han estat descrits com predictors de la recanalització arterial.

D'altra banda, el polimorfise Val34Leu (rs5985) del FXIII s'ha relacionat amb una major incidència de transformacions hemorràgiques simptomàtiques (González-Conejero et al, 2006).

10.1.1 Predictors d'eficàcia

Els polimorfismes I/D del gen de l'ECA, el polimorfisme Thr325Ile del TAFI, i la combinació Ile/Ile +4G dels gens del TAFI y del PAI-1, van predir la eficacia del tractament amb t-PA, valorada pels índex de recanalització de l'artèria cerebral mitja.

En el cas del polimorfisme I/D, els pacients homocigots DD van presentar les millors taxes de recanalització als 3 temps analitzats (1 hora: II-I/D 31.6% vs DD 69.2%; 6 hores: II-I/D 51% vs DD 91.3% i 24 hores: II-I/D 72.3% vs DD 100% després de l'administració del t-PA). Aquest polimorfisme ha estat ampliament estudiat degut a la seva associació amb els nivells i l'activitat de l'ECA (l'alel D s'associa a uns nivells més elevats de l'enzim). La presència dels homocigots DD eren un predictor independent de recanalització arterial **(figura 34)**.

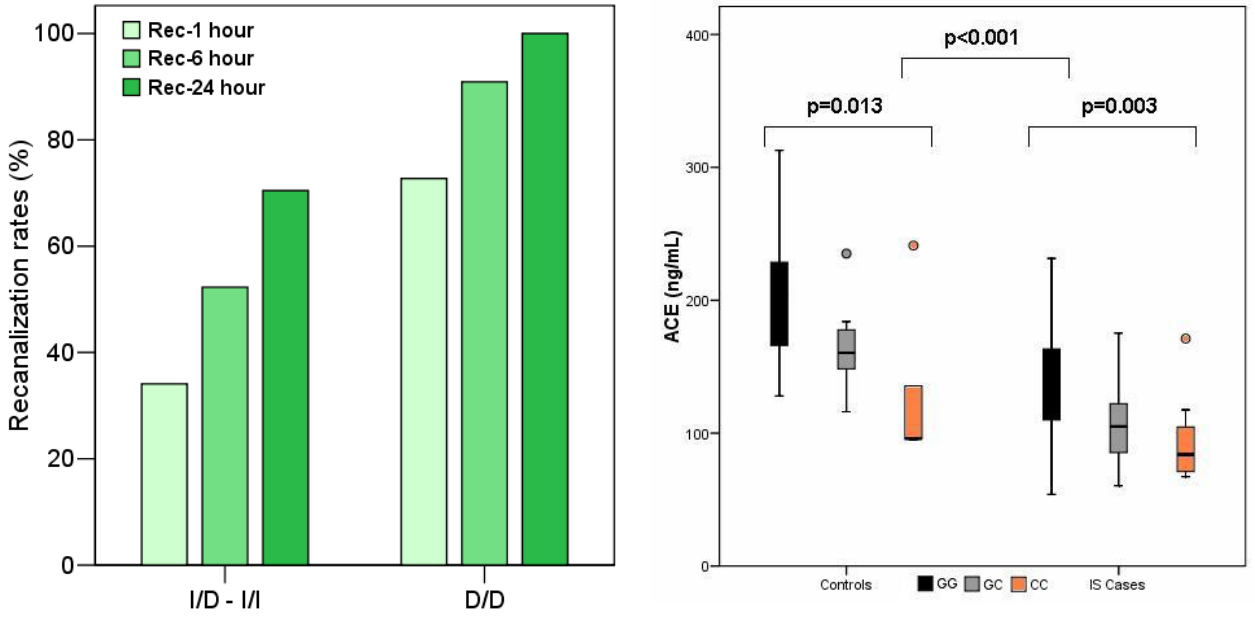


Figura 34. Índex de recanalització 1h, 6h i 24h després del tractament amb t-PA i activitat de l'enzim segons el genotip I/D o rs4341 (en LD amb I/D). Modificada de Fernández-Cadenas et al, 2006 I Domingues-Montanari et al, 2010.

El polimorfisme Thr325Ile s'ha associat a l'activitat d'aquest inhibidor (Zorio et al, 2003). Les concentracions de TAFI són un predictor de la seguretat del tractament amb t-PA (Ribo et al, 2004) i els homocigots Ile-Ile presentaven uns índex de recanalització arterial inferiors. El PAI-1 s'havia descrit com predictor de l'eficàcia del tractament amb t-PA en malalts d'ictus isquèmic (Ribo et al, 2004) i el polimorfisme 4G/5G és un modulador de la concentració d'aquest inhibidor de la fibrinòlisis (Dawson et al 1991; Eriksson et al 1995).

En combinar els genotips, els homocigots Ile-Ile que eren portadors de l'alel 4G del PAI-1 tenien una menor recanalització de l'artèria cerebral mitja tant 1h com 6h post-tPA (1h: IleIle+4G 7.1% vs Thr+5G 45.9%; 6h: IleIle+4G 25% vs Thr+5G 65.3%) **(figura 35)**.

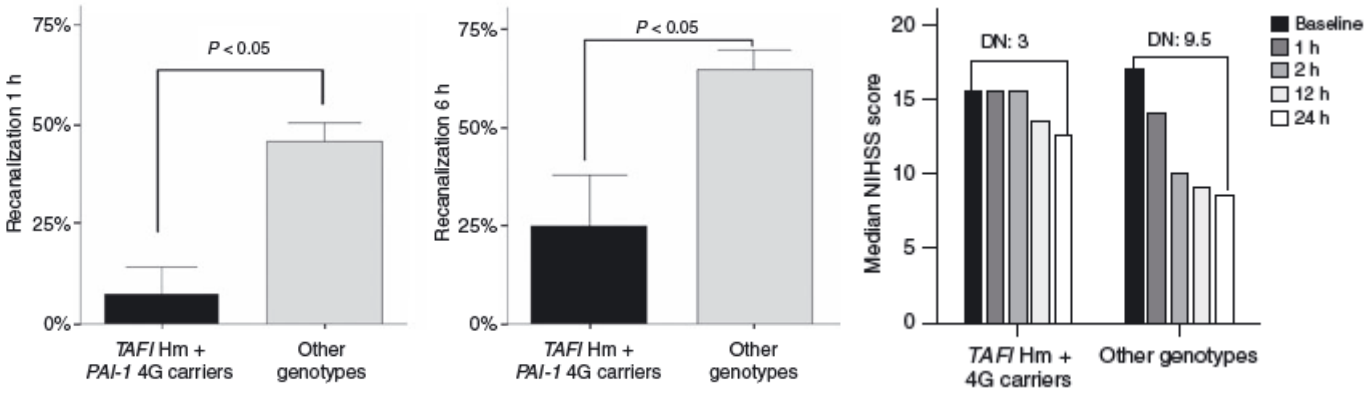


Figura 35. Índex de recanalització i puntuació a l'escala NIHSS segons els genotips Thr325Ile del TAFI i 4G/5G del PAI-1. Modificada de Fernández-Cadenas et al, 2007.

El mecanisme d'actuació del PAI-1 i del TAFI és sinèrgic, ja que ambdós son inhibidors de la fibrinòlisi en diferents vies: inhibint l'activitat del t-PA i disminuint l'activació del plasminògen sobre la malla de fibrina. El TAFI actuaria en primer lloc ja que el complex trombina / trombomodulina o la trombina l'haurien activat abans de l'administració del t-PA. Més tard, quan es produeix la infusió de t-PA, el PAI-1 inhibiria la seva activitat amb una unió 1:1 com s'ha explicat anteriorment.

10.1.2 Predictors de seguretat

El polimorfisme Val34Leu del factor XIII i la combinació factor XIII amb concentració de fibrinògen són predictors de la seguretat (mortalitat o aparició de transformacions hemorràgiques) del tractament de l'ictus isquèmic amb t-PA. Per contra, ni la variant -1562 C>T de la MMP9 ni els alels de l'apolipoproteïna E.

Els portadors del genotip Val/Val del polimorfisme Val34Leu del factor XIII presentaven

una incidència menor de transformacions hemorràgiques simptomàtiques (González-Conejero et al, 2006). A més, aquests pacients tenien una menor taxa de mortalitat, éssent un factor protector independent (González-Conejero et al, 2006).

Si es buscaven combinacions amb els nivells de fibrinògen, pacients ValVal amb fibrinògen < 3.6 g/L presentaven una mortalitat del 8.8% mentre la resta un 35.7%. Aquest subgrup també presentava una puntuació més baixa a l'escala NIHSS a tots els temps analitzats excepte el basal (1h, 2h, 12h, 24h, 48h i alta) i a l'escala modifcada de Rankin al tercer mes (González-Conejero et al, 2006).

Aquests resultats es troben en sintonía amb el rol hemorràgic que s'ha observat per a l'alel Leu (Catto et al, 1998; Catto et al, 1999). El mecanisme d'actuació seria una alteració a l'estructura de la malla de fibrina éssent més fina per l'alel 34Leu, fruit d'un increment a l'activitat del FXIII (Ariëns et al, 2000) **(figura 36)**.

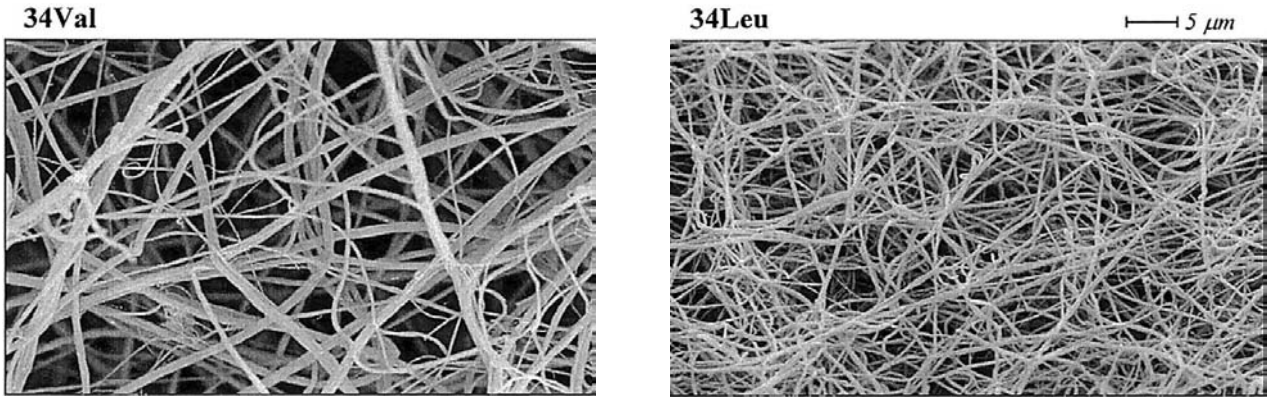


Figura 36. Imatges de microscopia electrònica del canvi estructural a la malla de fibrina entre l'alel 34Val i l'alel 34Ile. Extreta de Ariëns et al, 2000.

D'altra banda, el polimorfisme -1562 C>T del gen *MMP9* que "*in vitro"* condicionava els nivells de MMP-9 no es va associar ni a les concentracions de la proteasa ni a l'aparició de transformacions hemorràgiques (Montaner et al, 2003). Tampoc els alels de

l’apolipoproteïna E predien el sagnat després del tractament amb t-PA (Fernández Cadenas et al, 2006).

En resum, calen millors models predictius d'eficàcia i seguretat del t-PA, amb punts de tall clars que permetin prendre decisions clíniques i millorar el tractament dels malalts d'ictus isquèmic.

Donada la provada influència genètica a la resposta al t-PA, considerem que un estudi farmacogenètic de gens candidats suficientment extens ens permetrà identificar noves variants associades a l'evolució després del tractament trombolític. Els nostres resultats podrien aplicar-se ràpidament a la pràctica clínica: des del punt de vista diagnòstic utilizant kits ràpids de detecció de polimorfismes mitjançant ELISA o *Fast Real Time PCR*, que permeten determinar en 30-45 minuts el genotip d'un polimorfisme. Des d'un punt de vista terapèutic, podrien identificar-se noves dianes farmacològiques relacionades amb les variants associades.

B. Objectius

1) Identificar polimorfismes associats amb la eficacia del tractament fibrinolític i, per tant, relacionats amb la recanalització i la reoclusió arterial després de l'administració de t-PA i integrar-los amb les dades clíniques en un model predictiu.

2) Identificar polimorfismes associats amb la seguretat del tractament fibrinolític i, per tant, relacionats amb aparició de complicacions hemorràgiques i mortalitat intrahospitalària després de la administració de t-PA i integrar-los amb les dades clíniques en un model predictiu.

3) Realitzar el diagnòstic genètic de les malalties de CADASIL i CARASIL a la nostra població i identificar les noves mutacions que se'n trobin.

ARTICLE 1

Int J Neurosci. 2010; 120(4):245-51.

International Journal of Neuroscience, Early Online, 245–251, 2010

ISSN: 0020-7454 print / 1543-5245 online
DOI: 10.3109/00207451003597169

informa
healthcare

PAI-1 4G/5G Polymorphism is Associated with Brain Vessel Reocclusion After Successful Fibrinolytic Therapy in Ischemic Stroke Patients

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ABSTRACT

Background: Despite t-PA proven benefits related to vessel reopening, up to 13% of stroke patients suffer re-occlusions after t-PA. We aimed to analyze whether a functional polymorphism in a fibrinolysis inhibitor gene [plasminogen activator inhibitor-1 (PAI-1)] might be associated with reocclusion rates after stroke thrombolytic therapy. **Methods:** 165 patients with ischemic stroke who received t-PA < 3 h were studied. Reocclusion and recanalization was diagnosed by transcranial Doppler. PAI-1 4G/5G polymorphism determination was performed by sequencing. PAI-1 mRNA was studied by real-time PCR analysis. National institutes of health stroke scale (NIHSS) was serially measured since patients arrival to assess the neurological outcome, and modified ranking scale (mRS) at 3rd month was used to evaluate functional outcome following stroke. **Results:** PAI-1 4G/4G patients had higher reocclusion rates (4G/4G = 12.5% versus other genotypes = 2.7%, $p = 0.025$). . In a logistic regression, the 4G/4G genotype was the only factor associated with reocclusion (OR = 15.16 95%, CI = 1.4–163.4, $p = 0.025$). 4G/4G genotype was also associated with poor functional outcome at 3rd month (4G/4G = 4 versus others genotypes = 3, $p = 0.017$) and with mRNA levels at 12 h post stroke symptoms onset (4G/4G patients = 2.01% versus other genotypes = 0.68%, $p = 0.034$). **Conclusions:** PAI-1 4G/4G genotype is associated with reocclusion rates and poor functional outcome among stroke patients treated with t-PA.

KEYWORDS: genetic association study, plasmin, polymorphism, stroke, thrombolysis, t-PA

Pharmacogenetics has emerged as a new strong tool to individualize therapies depending on the genetic background of a patient with the purpose of pre-identifying subgroups of patients who better respond to similar treatments or to choose the most adequate dose or type of drug for an individual patient.

Therefore, pharmacogenetics might be applied to stroke thrombolysis since a huge interindividual variability to intravenous tissue plasminogen activator (t-PA) for the treatment of ischemic stroke exists (The NINDS group, 1995). Although t-PA is the only available drug for acute stroke treatment, clinical response is poor in a subgroup of patients because of lack of reopening of the occluded brain vessel (Alexandrov et al., 2000; Molina et al., 2001), or because of side effects, such as symptomatic hemorrhagic transformations, that occur in a small proportion of cases (Molina et al., 2001). Several polymorphisms and specific proteins levels have been associated with the efficacy and safety of t-PA suggesting that genetic background might influence ischemic stroke patients response to fibrinolytic therapy (Fernandez-Cadenas et al., 2006; Gonzalez-Conejero et al., 2006; Montaner et al., 2003; Montaner et al., 2006; Rosell et al., 2005; and Wahlgren et al., 2007).

In the treatment of acute myocardial infarction, reocclusion occurs in up to 30% of the cases in the first year after the event and decreases the benefit of fibrinolysis increasing 2 to 3 fold the risk of heart failure or mortality (Ohman, 1990). Recently, reocclusion of successfully recanalized brain vessels has also been recognized to be an important factor of the neurological evolution of t-PA–treated stroke patients (Rubiera et al., 2005). In fact, in the ischemic stroke field, reocclussion rates have

Received 03 July 2009

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been documented in up to 13% of patients treated with t-PA (Rubiera et al., 2005). At present, only baseline national institutes of health stroke scale (NIHSS) and tandem occlusion that is the result of the presence of an ipsilateral extracranial internal carotid occlusion have been associated with reocclusion rates (Rubiera et al., 2005), but no study has analyzed the association of genetic polymorphisms with reocclusion rates.

Several fibrinolysis inhibitor pathways might be potential candidates to be associated with reocclusion rates among ischemic stroke patients treated with t-PA. In fact we have recently described the influence of two fibrinolysis inhibitor genes (TAFI and PAI-1) on t-PA–induced recanalization (Fernandez-Cadenas et al., 2007). Plasminogen activator inhibitor 1 (PAI-1) is the main inhibitor of t-PA and blocks the activation of plasminogen by binding t-PA and forming an inactive complex that is intracellularly metabolized. A deletion/insertion polymorphism of a single guanine in the promoter region of PAI-1 has been associated with plasma level of this molecule, since the 5G variant creates a new additional binding site for an inhibitor resulting in an attenuated response to transcription factors (Dawson et al., 1993; Eriksson, Kallin, van 't Hooft, Bavenholm, & Hamsten, 1995).

In this study we aimed to explore whether the presence of the PAI-1 functional polymorphism might be implicated in reocclusion phenomena occurring in some ischemic stroke patients after t-PA fibrinolytic therapy administration.

METHODS

Study Population

Patients with an acute stroke admitted at the emergency department of a university hospital were prospectively studied. Our target group consisted of patients who had an acute ischemic stroke and admitted within the first 3 h after symptoms onset. Consecutive patients with a nonlacunar stroke involving the vascular territory of the middle cerebral artery (MCA) were evaluated. Those patients who had a documented MCA occlusion on transcranial Doppler (TCD) and received t-PA in a standard 0.9-mg/kg dose (10% bolus, 90% continuous infusion during 1 h) were included in the study.

Clinical and Transcranial Doppler Protocol

A detailed history of vascular risk factors was obtained from each patient. To identify potential mechanism of cerebral infarction, a set of diagnostic tests was performed that included electrocardiogram, chest radiography, carotid ultrasonography, complete blood count and leukocyte differential, and blood biochemistry in all patients; when indicated, some patients also underwent special coagulation tests, transthoracic ecocardiography, and Holter monitoring. With this information, and the neuroimaging data, previously defined etiologic subgroups were determined (Adams et al., 1993).

Clinical examination was performed on admission and at 1 and 2 h post–t-PA administration and again at 12, 24, and 48 h after symptoms onset and at discharge. Stroke severity as well as improvement, stability, or neurological worsening was assessed by using the NIHSS (Brott et al., 1992). Modified ranking scale (mRS) at 3rd month was also employed to assess functional outcome (mRS score >2 was considered dependency). On admission, all patients received a computed tomography (CT) within the first 3 h of stroke onset. No patient with a hypodensity involving greater than 33% of the MCA territory received t-PA in this study.

A standard TCD examination was performed by experimented neurologists in the emergency room on admission, before t-PA administration, using I-channel 2-MHz equipment (TCD 100M, Spencer Technologies, Seattle, WA). A standard set of diagnostic criteria was applied to diagnose arterial occlusion. Proximal MCA occlusion was defined as the absence of flow or the presence of minimal flow (TIBI 0 or 1) signal throughout the MCA at an insonation depth between 45 to 65 mm, accompanied by flow diversion in the ipsilateral anterior cerebral artery and posterior cerebral artery, according to the thrombolysis in brain ischemia (TIBI) grading system (Demchuk et al., 2001). Distal MCA occlusion was defined as blunted or dampened signals (TIBI 2 or 3) in the symptomatic artery with 30% lesser flow than the contralateral MCA, and flow diversion signs in ipsilateral neighboring arteries. After the site of MCA occlusion was identified, continuous monitoring of the residual flow signals was performed with a Marc 500 head frame (Spencer Technologies, Seattle, WA) or DWL metal head frame to maintain tight transducer fixation and a constant angle of insonation. Continuous TCD monitoring of recanalization was conducted during t-PA administration and an hour later (at 2 h). Changes on TCD in each patient were determined by a rater using direct visual control of monitoring display. A new TCD recording was performed after 6 h from treatment and 24 h after symptoms onset to assess arterial recanalization and reocclusion after an initial recanalization.

Recanalization on TCD was diagnosed when blunted or dampened signals appeared in a previously demonstrated absent or minimal flow (partial recanalization) and if the end-diastolic flow velocity improved to normal or elevated values (normal or stenotic signals) (complete recanalization) (Burgin et al., 2000).

Reocclusion was defined as a worsening of more than 1 grade in the TIBI flow grading system after a previously documented recanalization. Reocclusion was measured during t-PA administration and 1 h later through continuous monitoring and again at 6 h post–t-PA infusion and at 24 h after symptoms onset.

Intravenous heparin was not administered during the study period. This study was approved by the ethics committee of the hospital and all patients or relatives gave informed consent.

Determination of PAI-1 4G/5G Polymorphism

DNA was extracted from whole blood by standard methods. A 243 base pair in the promoter region of PAI-1 where 4G/5G insertion/deletion polymorphism (rs1799768) is located was amplified by polymerase chain reaction (PCR). The analysis of the polymorphism was performed by direct sequencing with standard methods.

mRNA Detection and Real-Time Analysis

RNA was extracted from 32 patients in which blood samples were obtained at baseline (before t-PA administration), 2 h post–t-PA administration, and at 12 and 24 h post symptoms onset. EDTA tubes were centrifuged at 3500 rpm for 15 min to obtain the white blood cell fraction. RiboPure^{TM}-Blood kit from Ambion (Ambion, Woodward st. Austin, TX) was used to extract total RNA following manufacturer's instructions. The PAI-1 mRNA levels were measured by real-time PCR, using TaqMan fluorogenic probes and a 7500 Real-Time PCR System (Applied Biosystems, Foster city, CA). A probe located in exons 4–5 (Hs01126603_m1) was used in all samples. Cyclophilin A (PPIA) expression (Hs0099999904_m1) was used to normalize the results. Real-time PCR was performed using a standard TaqMan® PCR kit protocol. All reactions were run in triplicate and analyzed using the Applied Biosystems SDS 7500 system software (Applied Biosystems, Foster city, CA). The results are expressed in % depending on a healthy calibrator sample used in the experiments.

Statistical Analysis

SPSS statistical package version 12.0 was used for the analysis of the data. Statistical significance for intergroup differences was assessed by the χ^2 or Fisher's exact test for categorical variables. When indicated, the Anova or *t*-test was used. Mann-Whitney U and Krustal-Wallis tests were used to analyze the association of polymorphisms with NIHSS and mRS at 3rd month. To prevent false positives, the unique end point of the study was reocclusion measured through a continuous monitoring during 2 h after t-PA bolus and 6 h after t-PA administration and 24 h after symptoms onset. Thus, none multivariable test correction is needed. Mortality and neurological evolution were secondary end points if it was a statistical association of PAI-1 genotype and reocclusion rates.

A logistic regression analysis was performed to determine factors that could be considered independent predictors of MCA reocclusion. A p value less than 0.05 was considered statistically significant.

RESULTS

We included, in the study, 165 patients (51.7% women) with an acute ischemic stroke involving the MCA territory. Mean age was 70.78 years (ranging from 26 to 91 years). A total of 49.3% of the patients were hypertensive, 31.2% were dyslipemic, and 21.4% had a history of diabetes mellitus. Median NIHSS score of the series on admission was 17 (ranging from 3 to 28). Ipsilateral extracranial internal carotid occlusion (tandem occlusion) was detected in 38.5% of the patients. PAI-1 genotype was in Hardy-Weinberg equilibrium. Successful recanalization occurred in 38.3% of the patients ($n = 54$) at 1 h and 76% of the patients ($n = 125$) at 24 h after t-PA bolus administration. PAI-1 genotype was not associated with recanalization; however, the 4G/4G presents lower levels of recanalization in later time points. (Patients 4G/4G = 38% versus patients with other genotypes = 38%, at 1 h post-t-PA, $p = 0.94$; Patients 4G/4G = 64% versus patients with other genotypes = 79.2%, at 1 h post–t-PA, $p = 0.114$).

Reocclusion was observed in 9 cases after a successful recanalization, 5.4% of all the cases. The main baseline characteristics of the patients regarding the presence of reocclusion following t-PA infusion are shown in Table 1. Among them, only the presence of the genotype 4G/4G of PAI-1 was associated with reocclusion rates (Patients 4G/4G = 12.5% versus patients with other genotypes = 2.7%, $p = 0.025$). Using a Bonferroni correction for multivariable test, the association of 4G/4G with reocclusion was also found statistically significant ($p = 0.025$).

No association was found between 4G/4G genotype and different risk factors and demographic variables including sex, stroke etiology, baseline NIHSS, or tandem occlusion (data not shown). Moreover, 4G/5G polymorphism was not associated with t-PA–related hemorrhagic transformation (data not shown).

Regarding neurological status (Figure 1), although NIHSS score at different time points is not statistically associated with 4G/4G genotype nor reocclusion as showed in Figure 1, it is clear that an increase in NIHSS appeared 12 h after reocclusion and also at 48 h among patients with the 4G/4G genotype.

TABLE 1. Univariate analysis of factors associated with MCA reocclusion rate after a successful recanalization

	Reocclusion		
	No ($n = 125$)	Yes ($n = 9$)	p value
Age $\geq$ 75 years (%)	43	33.3	0.83
Baseline NIHSS $\geq$ 17 (%)	54.8	28.6	0.18
Recanalization 1h post–t-PA (%)	37.9	42.9	0.79
Ipsilateral occlusion (%)	42.3	50	0.77
Women (%)	47.4	57.1	0.62
Currently smokers (%)	23.3	20	0.87
Presence of HT (%)	50.4	60	0.67
Diabetes mellitus (%)	22.2	40	0.36
AF (%)	41.4	60	0.4
Dyslipemia (%)	29.1	20	0.66
Etiology:			
Cardioembolic (%)	52.4	42.9	0.74
Aterothrombotic (%)	24.6	42.9	0.74
Undetermined (%)	21.4	14.3	0.74
4G/4G Homozygotes (%)	20.7	57.1	0.025

Functional outcome measured through mRS at third month showed a statistically significant association with PAI-1 genotypes, since 4G/4G polymorphism was associated with a worse functional outcome (4G/4G: mRS = 4 (median), the others genotypes: mRS = 3 (median); $p = 0.017$) as shown in Figure 2. Patients with reocclusions also showed a trend to be associated with worse functional outcome by 3rd month (reocclusion: mRS = 4.5 (median), no reocclusion: mRS = 3 (median); $p = 0.14$).

In a logistic regression model, 4G/4G genotype was the only independent factor that predicted reocclusion rates after t-PA administration (OR = 15.16 95%, CI 1.4–163.4, $p = 0.025$) after adjustment for stroke risk factors and reocclusion related factors (age, baseline NIHSS, efficacy of recanalization after 1 h post–t-PA and tandem occlusion).

In order to evaluate whether PAI-1 polymorphism was functional among our patients, we measured PAI-1 mRNA at several time points of the reperfusion process. mRNA level of PAI-1 in all time points was higher in patients 4G/4G versus the other patients (Figure 3). At 12 h post symptoms onset, mRNA level of PAI-1 among 4G/4G patients was statistically higher

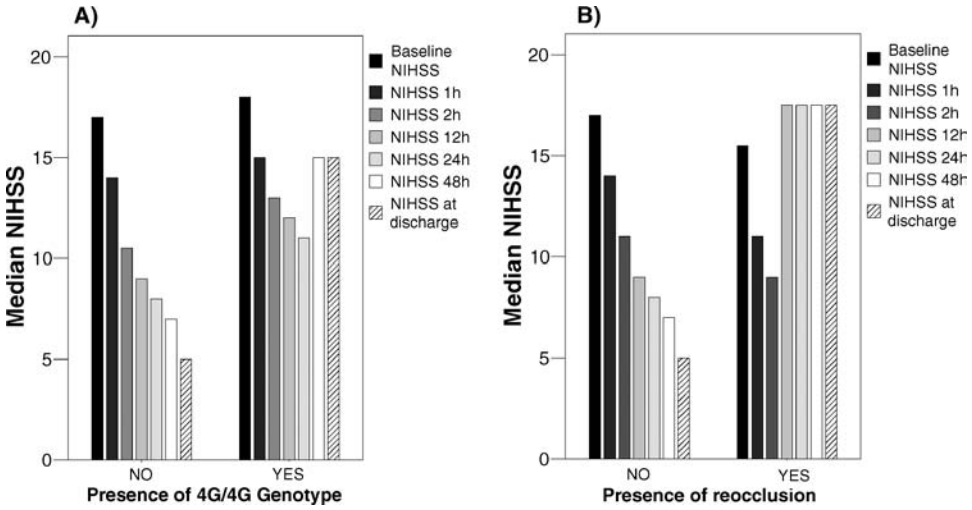

FIGURE 1. Median NIHSS scores temporal profile regarding (A) 4G/4G genotype and (B) presence of reocclusion after t-PA administration. An increase in NIHSS score may be observed for those with reocclusions and for 4G/4G patients by 12 and 24 h, respectively. NIHSS 1h: NIHSS at 1 h after t-PA administration, NIHSS 2h: NIHSS at 2 h after t-PA administration, NIHSS 12h: NIHSS at 12 h from symptoms onset, NIHSS 24h: NIHSS at 24 h from symptoms onset, NIHSS 48h: NIHSS at 48 h from symptoms onset.

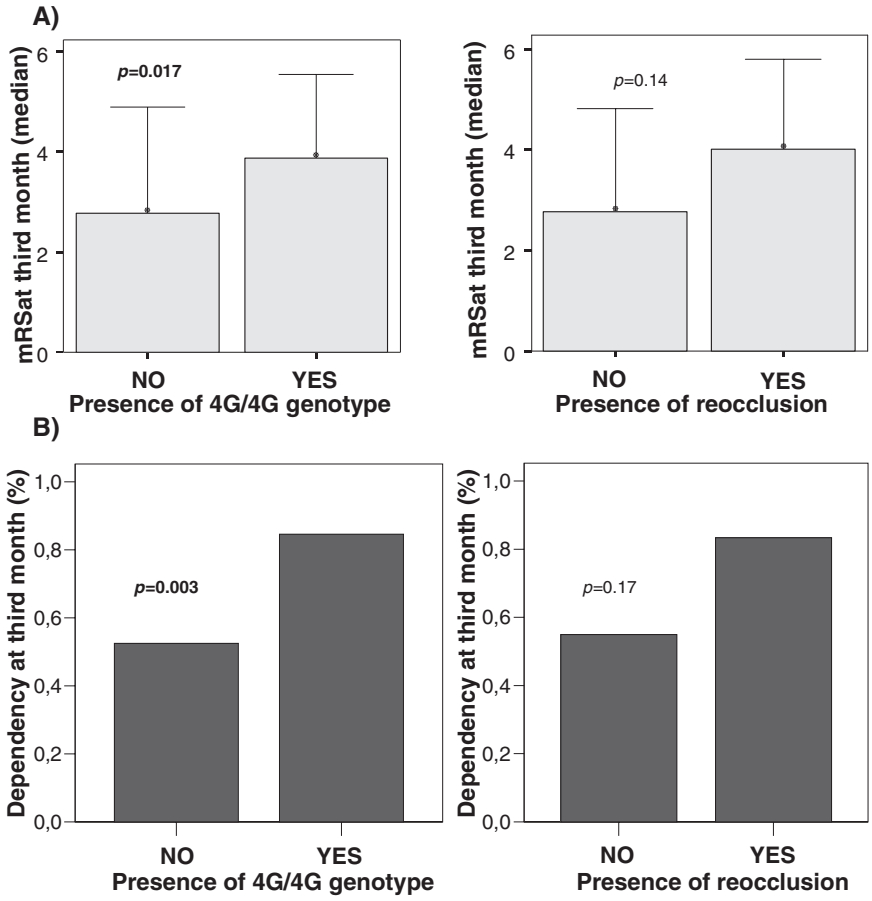

FIGURE 2. (A) Median mRS score at third month regarding the presence of 4G/4G genotype or reocclusion events. (B) Dependency at third month regarding the presence of 4G/4G genotype or reocclusion events.

than for the remaining patients (Patients 4G/4G: 2.01% versus patients with other genotypes: 0.68%; $p = 0.034$). However, no association was found among mRNA levels and reocclusion rates, mortality, or neurological worsening (data not shown).

DISCUSSION

PAI-1 is a serpin that rapidly inhibits t-PA activity through a covalent binding to t-PA. High concentration of PAI-1 has been detected in the plasma of patients during the acute phase of ischemic stroke (Lindgren, Lindoff, Norrving, stedt, & Johansson, 1996; Margaglione et al., 1994), and PAI-1 has been associated with the efficacy of t-PA treatment for ischemic stroke patients (Ribo et al., 2004). In that report, in a group of ischemic stroke patients with a proximal occlusion in the MCA, high plasma levels of PAI-1 were associated with poor rates of recanalization at 1 h and 6 h post–t-PA infusion influencing the efficacy of the fibrinolytic therapy.

In our report we have found an independent association of PAI-1 polymorphism with reocclusion rates since patients with the 4G/4G genotype presents a higher risk of reocclusion after t-PA infusion. Reocclusion after fibrinolysis in ischemic stroke patients is a recently recognized adverse event that affects neurological recovery (Rubiera et al., 2005). This is the first genetic association found with reocclusion rates after t-PA infusion pointing to a possible influence of genetic background over reocclusion occurring in ischemic stroke patients treated with t-PA.

It is well known that t-PA itself promotes thrombosis by stimulating the plasmin production, which activates platelets and transforms prothrombin into its active form. Thrombin mediates platelet activation and conversion of fibrinogen to fibrin. Moreover, activated platelets secrete native PAI-1 (Nordt et al., 1998) that is the main endogenous inhibitor of t-PA. It might be, thus, possible that increased level of PAI-1 associated with 4G/4G genotype facilitates rethrombosis or new embolic events at later time points when pharmacological activity of t-PA is

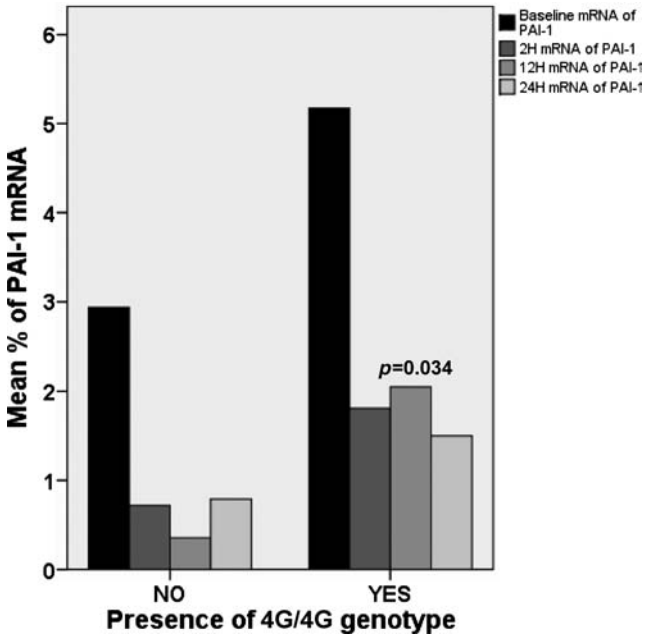

FIGURE 3. mRNA levels at different time points according to 4G/5G genotype.

decreasing. This effect on fibrinolysis activity of PAI-1 may be studied through recanalization rates using TCD. Our study showed a trend of 4G/4G genotypes to have poorer rates of recanalization at later times (6 to 24 h post–t-PA administration) but not with early recanalization (1 h) when pharmacological t-PA activity is more difficult to be inhibited by an endogenous fibrinolysis inhibitor that blocks t-PA in a 1:1 reaction (Fernandez-Cadenas et al., 2007).

We hypothesized that t-PA infusion modulates PAI-1 gene regulation and depending on the presence of 4G/5G polymorphism there is a major or minor transcription, and this variability influences reocclusion rates of the brain artery. For this reason, and in order to demonstrate functionality of the polymorphism, we studied PAI-1 mRNA, confirming an association of PAI-1 genotype with mRNA expression measured 12 h poststroke symptoms onset; interestingly at 12 h poststroke symptoms onset is when a neurological worsening occurs (increase in NIHSS scores) in patients that suffer a reocclusive event. In addition, in all time points, determined mRNA of PAI-1 was higher in patients with 4G/4G genotype than the other patients, thus, confirming the functionality of this polymorphism.

Moreover, 4G/4G related reocclusion affects functional outcome at 3rd month with larger number of dependent patients among 4G/4G carriers. In fact, mRS scores and the frequency of dependency are almost the same in patients with 4G/4G genotype and patients with reocclusion events, showing a clear connection between both 4G/4G reoclusion and poor outcome.

Regarding NIHSS evolution (Figure 1) the trends of NIHSS score are very similar among patients with reocclusion and patients with 4G/4G genotype; moreover, the dramatic increase of NIHSS of 4G/4G patients at 48 h after a clear decrease in earlier time points might be explained only by the appearance of reocclusion events or the effect of this polymorphism over recanalization rates at later time points among those patients.

Study Limitations

In another study from our group, baseline NIHSS and severe ipsilateral artery disease (tandem occlusion) were independently associated with the risk of MCA reocclusion (Rubiera et al., 2005); however, only a trend towards association for ipsilateral artery disease (tandem occlusion) and reocclusion rates was identified in the present study. This shows variability of clinical and analytical results depending on the series of individual patients; therefore, findings of this report have to be replicated by other studies with different populations to validate the association of 4G/5G polymorphism with reocclusion rates and neurological outcome, although the association of 4G/4G with mRNA levels and with the phenotype of the patients (dependency and mRS) suggest a real association of PAI-1 genotype with reocclusion events.

Moreover, the small sample size for the functional study precludes to confirm that the PAI-1 mRNA is, or is not, associated with reocclusion rates of the brain artery. In conclusion we found the first genetic association between a single PAI-1 polymorphism with reocclusion events that occurred in ischemic stroke patients treated with t-PA.

Funding Sources

This study was funded by a grant of the Spanish government (Geno-tPA project-FIS PJ060586), Mutua Madrileña (Convocatoria para la adjudicación de Ayudas a la Investigación Médica 2006) and Ramón Areces (XIV CONCURSO NACIONAL PARA LA ADJUDICACIÓN DE AYUDAS A LA INVESTIGACIÓN CIENTÍFICA Y TÉCNICA. 2006) grants and the stroke research network (RENEVAS).

We are deeply grateful to all study participants, neurologists, and nurses of the Stroke Unit from Vall d'Hebron Hospital for their contributions. We are also grateful for excellent technical support from Ortega Torres, L; Penalba, A; and García-Menéndez S. We would also like to thank the National Center for Genotyping (CeGen) for their excellent technical assistance. This study is part of the project Geno-tPA.

The Neurovascular Research Laboratory takes part in the International Stroke Genetics Consortium ISGC and in the network for Cooperative Neurovascular Research RENEVAS (Red de Investigación Cooperativa Neurovascular).

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ARTICLE 2

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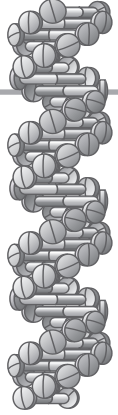

CD40 -1C>T polymorphism (rs1883832) is associated with brain vessel reocclusion after fibrinolysis in ischemic stroke

Aims: To find genetic predictors of reocclusion after successful fibrinolytic therapy during the acute phase of ischemic stroke. **Patients & methods:** This was a case–case prospective study analyzing 236 polymorphisms in a cohort of 222 patients treated with tissue plasminogen activator, from which 16 patients suffered a reocclusion event (7.2%). A predictive scale was generated using independent polymorphisms with a dominant/recessive model and tandem occlusion, weighted by their β-coefficients in logistic regression. **Results:** Using a dominant/recessive model, the rs1800801 SNP from the *MGP* gene (odds ratio [OR]*:* 15.25; 95% CI: 2.23–104.46; adjusted p = 0.006) and the rs1883832 SNP from *CD40* gene (OR: 0.077; 95% CI: 0.009–0.66; adjusted p = 0.019) were independently associated with reocclusion after logistic regression adjustment by clinical predictors. In an additive model, only the rs1883832 SNP (OR: 4.43; 95% CI: 1.62–12.15; adjusted p = 0.004) was related to reocclusion occurrence. The predictive model that was generated stratified the reocclusion risk from less than 1% to more than 70%. Reocclusions were associated with neurological worsening at 24 h (patients with reocclusion: 26.7%, versus patients without reocclusion: 4.9%; p = 0.002), as it was seen for *MGP* -7A>G (AA: 17.2% vs AG+GG: 4.5%; p = 0.027), but not for *CD40* 1C>T (CC: 4.5% vs CT+TT: 7.7%; p = 0.565). There was an association between *CD40* -1C>T genotype and *CD40* transcriptional activity in peripheral blood mononuclear cells (median expression values TT: 65.75%, CT: 70.80%, CC: 96.00%; p = 0.023). However, CD40 soluble fraction was not a useful biomarker of reocclusion status. **Conclusion:** An association was found between *MGP* -7A>G and *CD40* -1C>T polymorphisms, and reocclusion risk. The predictive scale that was generated permits the stratification of patients by their reocclusion risk with higher accuracy than clinical parameters alone.

KEYWORDS: CD40 ■ matrix γ-carboxyglutamic acid protein ■ pharmacogenetics ■ reocclusion ■ stroke ■ tissue plasminogen activator

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Pharmacogenetics has emerged as a strong new tool to individualize therapies depending on the genetic background of a patient, with the purpose of pre-identifying subgroups of patients who respond better to similar treatments, or to choose the most adequate dose or type of drug for an individual patient.

Therefore, pharmacogenetics might be applied to stroke thrombolysis, since a huge interindividual variability exists for the treatment of ischemic stroke with intravenous tissue plasminogen activator (tPA) [1]. Although tPA is the only available drug for acute stroke treatment, clinical response is poor in 9–19.5% of patients owing to reocclusion of the recanalized brain vessel [2–4] or to side effects, such as symptomatic hemorrhagic transformations, which occur in 2–4% of cases [5]. Several polymorphisms have been associated with the efficacy and safety of tPA, suggesting that genetic background might influence ischemic stroke patients' response to fibrinolytic therapy [6–8].

In the fibrinolytic treatment of acute myocardial infarction, reocclusion occurs in up to 30% of cases during the first year after the event, and decreases the benefit of fibrinolysis increasing two- to three-fold the risk of heart failure or mortality [9]. Recently, reocclusion of successfully recanalized brain vessels has also been recognized as an important factor for the neurological evolution of tPA-treated stroke patients [2]. Reocclusion rates in the Spanish population of Barcelona area have been documented in approximately 13% of patients treated with tPA [2]. At the moment, only baseline National Institutes of Health Stroke Scale (NIHSS) and tandem occlusion have been associated with reocclusion rates [2]. No study has analysed the association of SNPs with this outcome by massive genotyping techniques.

Our aim was to find useful SNP predictors of reocclusion rates and to also consider the use of their respective gene products as reocclusion biomarkers, which could help

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clinicians to identify those patients who are at higher risk and individualize their ischemic stroke treatment.

Patients & methods

Study population

Consecutive patients with an acute stroke admitted at the emergency department of the Vall d'Hebrón Universitary Hospital (Barcelona, Spain) between November 2000 and May 2007, mainly of Caucasian ethnicity and belonging to the Geno-tPA study cohort, were prospectively studied. Our target group consisted of patients who presented a nonlacunar acute ischemic stroke admitted within the first 3 h after onset of symptoms with a documented occlusion on transcranial Doppler (TCD) and received tPA in a standard 0.9-mg/kg dose. Patients without Doppler monitoring were excluded from the study.

Clinical & ultrasound protocol

On admission, all patients received a CT scan prior to tPA treatment, which was repeated after 24–36 h to determine the presence of intracranial hemorrhage. Patients also received a carotid ultrasound examination to detect severe stenosis or occlusion in the extracranial carotid artery (tandem occlusion) if present. Clinical examination was performed on admission, 1 and 2 h post-tPA administration, 12, 24 and 48 h after symptoms onset and at discharge, allowing us to classify individuals in etiologic subgroups accordingly to Trial of ORG 10172 in Acute Stroke Treatment (TOAST) criteria [10]. Stroke severity as well as neurological evolution was assessed by using the NIHSS [11], while modified Ranking Scale at the third month was employed to assess functional outcome, considering a modified Ranking Scale score of more than 2 as dependency [12].

Transcranial Doppler examination was performed by experienced neurologists as previously described [2]. Patients were examined on admission to detect arterial occlusion and received continuous TCD monitoring during tPA administration and 1 h later. New TCD recordings were performed after 6 h post-tPA bolus, after 24 h from symptoms onset and whenever clinical deterioration occurred. Changes in TCD in each patient were determined by a single rater using direct visual control of monitoring display. The presence and location of the arterial occlusion was determined by the Thrombolysis in Brain Ischemia flow grading system, as previously described [13]. Reocclusion was defined as a worsening of more than one Thrombolysis in Brain Ischemia grade after a previously documented recanalization.

Intravenous heparin was not administered during the study period. This study was approved by the Ethics Committee of the hospital, and all patients or relatives gave their informed consent.

Genetic analysis

Samples of DNA were isolated from peripheral blood samples extracted at admission and stored at -80ºC until genotyping was reformed. We attempted to genotype 236 SNPs from several candidate genes related to vascular risk factors, such as hypertension, glucose catabolism (diabetes) or atherosclerosis; other stroke-involved pathways, such as inflammation, coagulation and apoptosis, or drug metabolism, were also covered by this candidate-gene based approach. Genetic analysis was performed at the Spanish National Genotyping Centre (CeGen, Barcelona, Spain), using SNPlex™ (Applied Biosystems, Inc., CA, USA) with GeneMapper 3.5 as an allele-calling algorithm (error rates <0.5%; call rates of ~90%). Genotypes were assigned in 48-SNP batches. Finally, 212 SNPs were successfully genotyped (~90%).

mRNA detection & real-time quantitative-PCR analysis

Samples of RNA were extracted from 52 patients from whom blood samples were obtained at baseline (before tPA administration). CD40 mRNA levels were measured by real-time quantitative PCR, using the standard TaqMan® PCR kit protocol (Applied Biosystems) as instructed by the manufacturer [14]. Briefly, ethylenediaminetetraacetic acid tubes were centrifuged at 3500 rpm for 15 min to obtain the white blood cell fraction, and the total RNA was isolated with RiboPure™ Blood kit (Ambion, TX, USA) following the manufacturer's instructions. The *CD40* probe was located in exons 1–2 (Hs00386848_m1), using Cyclophilin A (*PPIA*) expression (Hs0099999904_m1) to normalize the results. All reactions were run in triplicate and analyzed using the Applied Biosystems SDS 7500 system software (Applied Biosystems). Results are expressed as a percentage relative to a healthy calibrator sample.

CD40 soluble fraction measurement

Levels of soluble CD40 were measured in 34 ethylenediaminetetraacetic acid plasma samples using a commercially available immunoassay (sCD40 ELISA product code: BMS265;

Bender Medsystems, Vienna, Austria). Plasma samples were stored at -80°C until use. Reoccluded patients were age and sex matched with two groups composed of stroke patients, the first having recanalized during the first hour after tPA treatment and the other without recanalization during the whole monitorization period. This division was performed to avoid early recanalization bias in reocclusion risk.

Statistical analysis

Sample size calculation was performed using the Ene 2.0 software. Deviation from the Hardy–Weinberg equilibrium (HWE) was assessed using a χ^2 test with one degree of freedom [15]. Statistical analysis was performed using the SPSS statistical package, version 15.0. Statistical significance for categorical variables was assessed by the χ^2 or Fisher's exact test. Genotype associations were analyzed using dominant/recessive and additive models. Odd ratios (ORs) and 95% confidence intervals (CIs) for the effect of each SNP on reocclusion risk were estimated using logistic regression models adjusted by associated clinical variables [2]. Bonferroni correction and its several variants were used for multivariable test correction, considering an adjusted p-value of less than 0.05 as statistically significant (i.e., $p < \alpha/212 = 0.000236$) [16]. A predictive model of reocclusion was performed with independently associated polymorphisms. A goodness-of-fit test by Hosmer–Lemeshow statistic was used to evaluate the model's consistency. Predictive capacity was calculated by measuring the area under the receiver operating characteristic curve (AUC). A scale was generated after giving a score to each SNP weighted by their β-coefficients on logistic regression model [17,18]. Normal distribution was determined by Kolmogorov–Smirnov, PP and QQ-plots. Continuous variables were analyzed either by Student's t-test for parametric or Mann–Whitney for nonparametric distributions. This article was written following 'Strengthening the Reporting of Genetic Association Studies' (STREGA) guidelines [19].

Results

In total, 222 tPA-treated stroke patients underwent complete Doppler monitoring and were included in the study (52.3% men). The mean age was 70.65 years (ranging from 26 to 97 years). The median NIHSS score of the series on admission was 17 (ranging from 3 to 29). Ipsilateral extracranial internal carotid occlusion (tandem occlusion) was detected in 38.5% of the patients (n = 42). Successful recanalization occurred in 38.7% of the patients after 1 h of tPA bolus administration (n = 84), in 48.1% after 2 h (n = 101), in 59.7% (n = 111) after 6 h of symptoms onset and in 74.9% after 24 h (n = 137). Reocclusion was observed in 16 cases after a successful recanalization in any time points, which was 7.2% of all the cases. The main baseline characteristics of the patients regarding the presence of reocclusion following tPA infusion are shown in Table 1.

Our sample size (n = 222) and reocclusion rates (7.2%) permit us to detect an association with $\alpha = 0.05$ and $\beta = 0.80$ for a SNP with a minor allele frequency of 0.1 only if the proportion of this minor allele in the reoccluded group is above 25.7%, giving a minimum OR of 3.68 (95% CI: 1.07–12.69) (Ene software).

Table 1. Comparison of demographic and risk factors between reocclusion and nonreocclusion groups.

Risk factors	Global	Presence of reocclusion		p-value
		Yes	*No*	
Age (years), mean ± standard deviation	70.65 ± 11.97	70.54 ± 12.19	72.00 ± 8.74	0.640
Male, % (n)	52.3 (116)	62.5 (10)	51.5 (106)	0.394
Current smoker, % (n)	18.1 (37)	25 (4)	17.6 (33)	0.498
Presence of hypertension, % (n)	54.1 (120)	68.8 (11)	53.7 (109)	0.244
Presence of dyslypidemia, % (n)	29.4 (65)	25 (4)	29.8 (61)	0.784
Presence of diabetes mellitus, % (n)	19.5 (43)	31.3 (5)	18.5 (38)	0.206
Presence of atrial fibrillation, % (n)	43.0 (95)	43.8 (7)	42.9 (88)	0.949
Presence of coronary disease, % (n)	17.3 (32)	6.7 (1)	18.1 (35)	0.477
Presence of previous stroke, % (n)	11.7 (26)	6.3 (1)	12.5 (25)	0.700
Presence of baseline NIHSS >16, % (n)	52.3 (116)	50 (8)	52.4 (108)	0.851
Presence of tandem occlusion, % (n)	38.5 (42)	66.7 (6)	36 (36)	0.085*

Values are indicated as a percentage, with the number of positive cases in brackets.
**p > 0.1.*
NIHSS: National Institutes of Health Stroke Scale.

Using a dominant/recessive model, we found eight SNPs associated with the presence of reocclusion events: rs1883832 in the *CD40* (cluster of differentiation 40) gene (CC: 2.7% vs CT+TT: 13.5%; p = 0.0036); rs3787268 in *MMP9* (GG: 2.7% vs AG+AA: 13.7%; p = 0.0039); rs3732379 in *CX3CR1* (CC: 11% vs CT+TT: 2,5%; p = 0.026); rs1042713 in *ADBR2* (GG: 12.9% vs AG+AA: 3.5%; p = 0.034); rs5370 in *EDN1* (GG: 4.2% vs GT+TT: 12.3%; p = 0.036); rs1799969 in *ICAM1* (GG: 5.6% vs AG+AA: 20%; p = 0.039); rs1800801 in *MGP* (AA: 17.2% vs AG+GG: 5.6%; p = 0.042) and rs310586 in *NOS3* (AA: 9.4% vs AG+GG: 0%; p = 0.043) (Table 2). Two variants, *CD40* (OR: 0.077; 95% CI: 0.009–0.66; adjusted p = 0.019) and *MGP* (OR: 15.25; 95% CI: 2.23–104.46; adjusted p = 0.006) were independently associated with reocclusion following logistic regression adjustment by age, sex and reocclusion risk factors (tandem occlusion and baseline NIHSS >16). No other clinical variables were found to be associated with reocclusion rates after univariate analysis.

Seven SNPs were associated in the additive model: rs1883832 in the *CD40* gene (T: 14.6% vs C: 5.5%; p = 0.003); rs4925 in *GSTO1* (C: 11.7% vs A: 3.8%; p = 0.007); rs3732379 in *CX3CR1* (C: 9.7% vs T: 2.1%; p = 0.016); rs3025010 and rs3025035 in *VEGF* (C: 15.1% vs T: 4%; p = 0.025 and T: 16.3% vs C: 6.6%; p = 0.035); rs17602729 in *AMPD1* (A: 15.9% vs G: 6.4%;p = 0.034) and rs1042713 in *ADBR2* (G: 9.8% vs A: 4%; p = 0.036). Only *CD40* was statistically independent after logistic regression (OR: 4.43; 95% CI: 1.62–12.15; adjusted p = 0.004) (Table 2). All the associated SNPs were in HWE (data not shown). None of them were significant after Bonferroni multivariable test correction (data not shown).

Reocclusions were associated with neurological worsening at 24 h (presence of reocclusion: 26.7% vs nonreocclusion: 4.9%; p = 0.002) but not with dependency at 3 months (presence of reocclusion: 84.6% vs nonreocclusion: 62.5%; p = 0.108). The same association was seen with *MGP* -7A>G, with the AA genotype showing the worst short-term outcome (AA: 17.2% vs AG+GG: 4.5%; p = 0.027), whereas *CD40* 1C>T was not associated (CC: 4.5% vs CT+TT: 7.7%; p = 0.565).

A predictive scale of reocclusion events was constructed with these two independently associated SNPs and tandem occlusion data, giving a score to the at-risk allele based on their β-coefficients on logistic regression (Table 3). The

Table 2. SNPs independently associated with reocclusion rates using an additive model or a dominant/recessive model; reocclusion rates are indicated depending on the presence of the genotype selected, along with their χ^2 p-values.

Gene	SNP	Allele	Reocclusion rate (%)		χ^2 p-value	Logistic regression	
			Yes	*No*		*OR (95% CI)*	*Adjusted p-value*[†]
Additive model							
CD40	rs1883832	T	14.6	5.5	0.003	4.43 (1.62–12.15)	0.004
GSTO1	rs4925	C	11.7	3.8	0.007	NS	NS
CX3CR1	rs3732379	C	9.7	2.1	0.016	NS	NS
VEGF	rs3025010	C	15.1	4.0	0.025	NS	NS
AMPD1	rs17602729	A	15.9	6.4	0.034	NS	NS
VEGF	rs3025035	T	16.3	6.6	0.035	NS	NS
ADBR2	rs1042713	G	9.8	4.0	0.036	NS	NS
Dominant/recessive model							
CD40	rs1883832	CC	2.7	13.5	0.0036	0.077 (0.009–0.66)	0.019
MMP9	rs3787268	GG	2.7	13.7	0.0039	NS	NS
CX3CR1	rs3732379	CC	11.0	2.5	0.026	NS	NS
ADBR2	rs1042713	GG	12.9	3.5	0.034	NS	NS
EDN1	rs5370	GG	4.2	12.3	0.036	NS	NS
ICAM1	rs1799969	GG	5.6	20.0	0.039	NS	NS
MGP	rs1800801	AA	17.2	5.6	0.042	15.25 (2.23–104.46)	0.006
NOS3	rs310586	AA	9.4	0.0	0.043	NS	NS

Logistic regression was adjusted for age, sex, tandem occlusion and NIH Stroke Scale score over 16 as covariates.
[†]p-value after logistic regression adjustment.
χ^2 p-value: Crude p-value of χ^2 test; NS: Nonsignificant; OR: Odds ratio.

Table 3. Reocclusion predictive model generated by logistic regression using dominant/recessive genotype data.

Predictive variable	At-risk group	β (SE)	Odds ratio (95% CI)	p-value	Score
Tandem occlusion	Present	2.09 (1.03)	8.11 (1.12–58.48)	0.038	2
rs1883832	T-carriers	2.53 (1.13)	12.58 (1.38–114.46)	0.025	2.5
rs1800801	AA	2.40 (1.03)	11.01 (1.47–82.37)	0.019	2.5

Odds ratio and p-value are indicated for each item inside the model. The scale score is given by rounding the β-coefficient in logistic regression analysis. SE: Standard error (of β-coefficient).

scale generated discriminates between a low risk of reocclusion (probability of reocclusion <5%) and a high-risk group (probability of reocclusion >20%). Patients with the maximum score of seven (tandem occlusion, T-carriers and AA) have a reocclusion risk of over 70%, whereas a score of zero implied a negligible risk (Figure 1B). Interestingly, those patients with a tandem occlusion but with protective alleles in both *CD40* and *MGP* genes have a moderate risk of reocclusion (only 5.7%), whereas those without tandem occlusion but with both at-risk genotypes are included in the high-risk group (Figure 1A). The predictive capacity, indicated by the AUC, was 86.7% (95% CI: 78.1–95.3%) (Figure 1C), and it was fitted to the experimental data, as indicated by a Hosmer–Lemeshow p-value of 0.428.

In a subset of 52 baseline mRNA samples, we detected an association between *CD40* -1C>T genotype and *CD40* transcription in peripheral blood mononuclear cells (median TT: 65.75%; CT: 70.80%; CC: 96.00%; p = 0.023) (Figure 2A), with the T allele, the reocclusion risk allele, associated to lower CD40 expression (median T: 68.8% vs C: 92.9%; p = 0.007) (Figure 2B). We were unable to assess *CD40* mRNA levels in reoccluded patients because no mRNA samples were available from this subset. Regarding CD40 soluble fraction levels, they were barely detectable, and no differences among groups were seen (data not shown).

Discussion

In this study, we have demonstrated that genetic background may influence stroke patients' response to thrombolytic drugs, with a subgroup of them being more prone to suffering reocclusions after a successful tPA-induced recanalization. From all the 212 SNPs genotyped, *MGP* -7A>G and *CD40* -1C>T were independently associated with reocclusions in the dominant/recessive model, while only *CD40* -1C>T was associated in the additive model. Besides, *MGP* -7A>G was correlated with reoccluded patients' clinical outcomes.

The rs1800801 variant is located in the promoter region of the *MGP* gene. *MGP* transcription is negatively regulated by retinoic acid, TGF-β and activating protein-1 (AP-1), whereas it is upregulated by vitamin D3 and cyclic AMP [20,21]. The A allele of *MGP* -7A>G has been associated with a higher risk of coronary artery calcification (CAC), renal disease and pseudoxantoma elasticum [22–24].

The functional relevance of this SNP is in doubt; on the one hand, it is located at a possible transcription start site [22], but its effects on matrix Gla protein expression are unclear [20,22] and it does not influence human matrix Gla serum levels [20]. Herrmann *et al.* hypothesized that *MGP* -7A>G is not a functional variant and the amino acid change Thr83Ala, located in the GIa-binding domain 1 in the C-terminal region of matrix Gla protein, and is in linkage disequilibrium with *MGP* -7A>G (D' = 0,97), might be responsible for *MGP* changes [22]. This amino acid substitution affects protein polarity and could affect its capacity to bind to calcium, leading to calcium deposition in the arterial wall [22].

Matrix Gla protein has a negative effect in calcium deposition and may play a protective role in atherosclerosis progression [20]. It has been found in plaque deposits, its expression is increased in senescent vascular smooth muscle cell cultures and atherosclerotic plaques, and its regulation of BMP-4 inhibits vascular calcification in endothelial cultures. Furthermore, Keutel syndrome, an autosomal recessive disorder due to nonsense mutations in *MGP*, is characterized by multiple peripheral pulmonary stenosis, brachytelephalangia, inner-ear deafness and calcification of cartilages [25]. Moreover, a knockout mouse model has been generated, showing that intense arterial calcification led to bleedings and death within the first 2 months [26]. Interestingly, matrix Gla protein activity is post-transcriptionally regulated and soluble levels of the uncarboxylated inactive form (ucMGP) have been proposed as a biomarker for CAC, although they do not allow individual risk assessment [27].

In order to establish a causal relationship between *MGP* polymorphisms and reocclusion events, we speculate that *MGP* dysfunction may cause higher atherosclerotic plaque calcification,

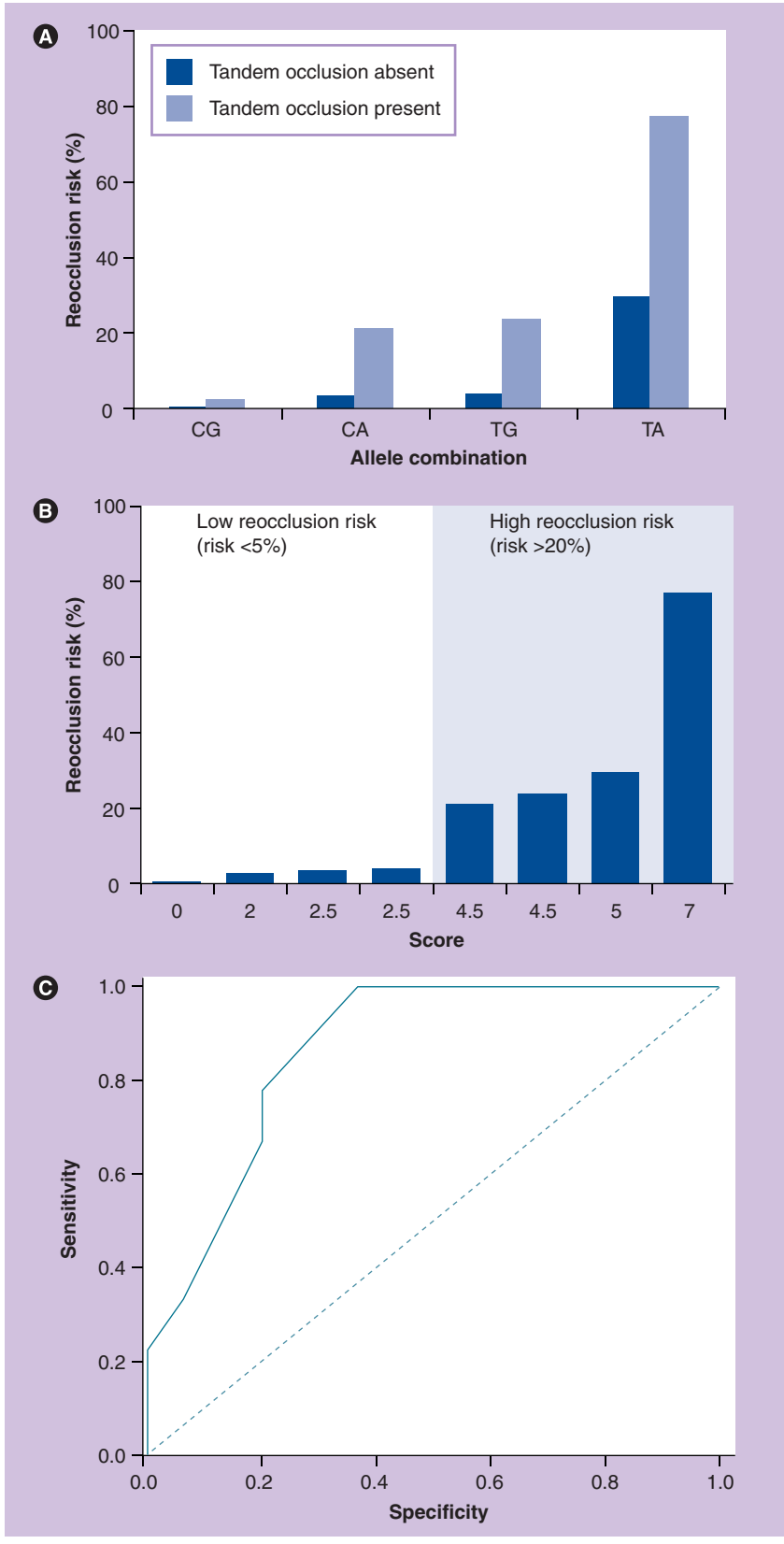

Figure 1. Reocclusion predictive model. (A) Chart plot of reocclusion risk for each allele combination (*CD40-C* or *-T*, *MGP-A* or *-G*) and tandem occlusion presence. **(B)** Chart plot of reocclusion risk stratification by the predictive scale generated. **(C)** Receiver operating characteristic curve performed for the scale, indicating its predictive capacity.

increasing atherosclerotic plaque instability and, as a consequence, an increased risk of rethrombosis after successful recanalization.

In summary, the dissimilar results observed in the *in vitro* expression studies, the low influence of this SNP in matrix Gla protein serum level and the high overlap in ucMGP level do not encourage its use as a biomarker molecule for reocclusion risk prediction. As a consequence, neither MGP mRNA nor MGP protein levels were considered for further analysis. Nevertheless, whether vitamin K inhibitors such as warfarin should be avoided or supplemented with vitamin K2, as they could increase reocclusion risk by interfering with matrix GIa protein carboxylation [28], may be considered in future specific studies.

CD40 -1C>T modifies a Kozak sequence in the translational start site of CD40, affecting translation efficiency [29]. Kozak sequences are located near the translational start site, with a consensus sequence GCC(A/G)CCAUGG, and they define translational efficiency in vertebrate genes by modulating 40S ribosomal scanning along mRNA sequence and 'AUG' start site recognition [30]. Disruption of a Kozak sequence reduces protein translation and may generate novel N-terminal isoforms owing to alternative translational start sites, mainly if there is a 'G' in the +4 position respective to the novel AUG [30]. Currently, there are several polymorphisms described in Kozak sequences, and all of them affect their protein serum levels [29]. *In vitro* studies demonstrated that the rs1883832 SNP influenced *CD40* translation process in fibroblasts, B cells and dendritic cells, while no changes in transcription were observed [29,31,32]. The T allele, which was associated with higher reocclusion levels, caused a reduction in translation levels of *CD40*.

CD40 is a cell surface trimeric receptor from the TNF-α family expressed in the immune system and in vasculature cells, where it plays a key role in antigen presentation and T-cell secondary activation [33]. Regarding tissues, it is expressed mainly in the trachea, thyroid and lymph node, but also in blood [34]. CD40 induces IL-1α, IL-1β, TNF-α, IL-6 and IL-8 production in monocytes, and nitric oxide, TNF–α and IL-12 production in macrophages [33]. It induces IL-10 and TNF–α production in T cells, unbalancing the Th1:Th2 ratio, and promotes B-cell germinal nucleus formation and immunoglobulin (Ig) isotype switching [35]. Its deficiency, due to nonsense mutations, causes hyper-IgM syndrome (HIGM), characterized by recurrent infections and high IgM levels [36].

CD40's main ligand is CD154 or CD40L, and the CD40–CD40L interaction has been suggested as the link between thrombosis and inflammation [37]. During the stroke acute phase, *CD40* transcription is upregulated in macrophages, T and B lymphocytes [38], and CD40 protein abundance is increased in monocyte cell surfaces [39]. In addition, CD40L expression in platelets increases with atherothrombotic ischemic stroke [40], and higher CD40 expression is found in symptomatic atherosclerosis plaques [41]. Monocyte and leukocyte CD40 surface markers could aggregate with CD40L platelets, as has been detected in a focal ischemia mice model [42], but lack of *CD40* -1C>T in mice impedes SNP/aggregability correlations [29].

The soluble form of CD40 has been related to cholesterol metabolism in moderate hypercholesterolemia [43] and increased levels of soluble CD40L (sCD40L) have been associated with higher platelet responsiveness to chilling, higher aggregability in percutaneous cardiovascular interventions, and numerous vascular diseases, including atherotrombotic ischemic stroke, congestive heart failure and atrial fibrillation. In a recent meta-analysis, sCD40L levels had prognostic value for coronary and cerebrovascular disease [44], but it was not useful for diagnostic purposes in a pilot clinical trial owing to the large number of infections and inflammatory diseases that also showed higher sCD40L levels at admission [45].

In the thrombosis model, CD40 from monocytes and lymphocytes aggregates with CD40L from platelets. The deleterious effects of T-cell and platelet aggregation have been demonstrated in a mouse model of transient middle cerebral artery occlusion, where they contributed to ischemic damage and neurological deficit; a process where IFN-γ would also be involved [46]. We have detected higher reocclusion rates for the carriers of the T allele, which is significantly associated with lower CD40 mRNA expression and has been related to lower CD40 production [29,31,32]. However, CD40's soluble level is not a useful biomarker of reocclusion. We hypothesize that CD40–CD40L levels may alter thrombus composition and its pattern of lyses, or maybe new CD40 isoforms are generated *in vivo* from alternative translational start sites. These aberrant isoforms might alter CD40–CD40L binding dynamics, leading to higher platelet and leukocyte adhesion, vessel reocclusion and higher neurological deficits. Nevertheless, we cannot rule out the possibility that other variants, in linkage disequilibrium with *CD40* -1C>T, are responsible of the higher reocclusion rates observed. This issue needs to be solved in future studies.

CD40 -1C>T has been intensively investigated in inflammatory diseases such as Grave's disease or multiple sclerosis, but a consistent association has only been found with Grave's disease [29,31,32]. Regarding pharmacogenetics, the only study

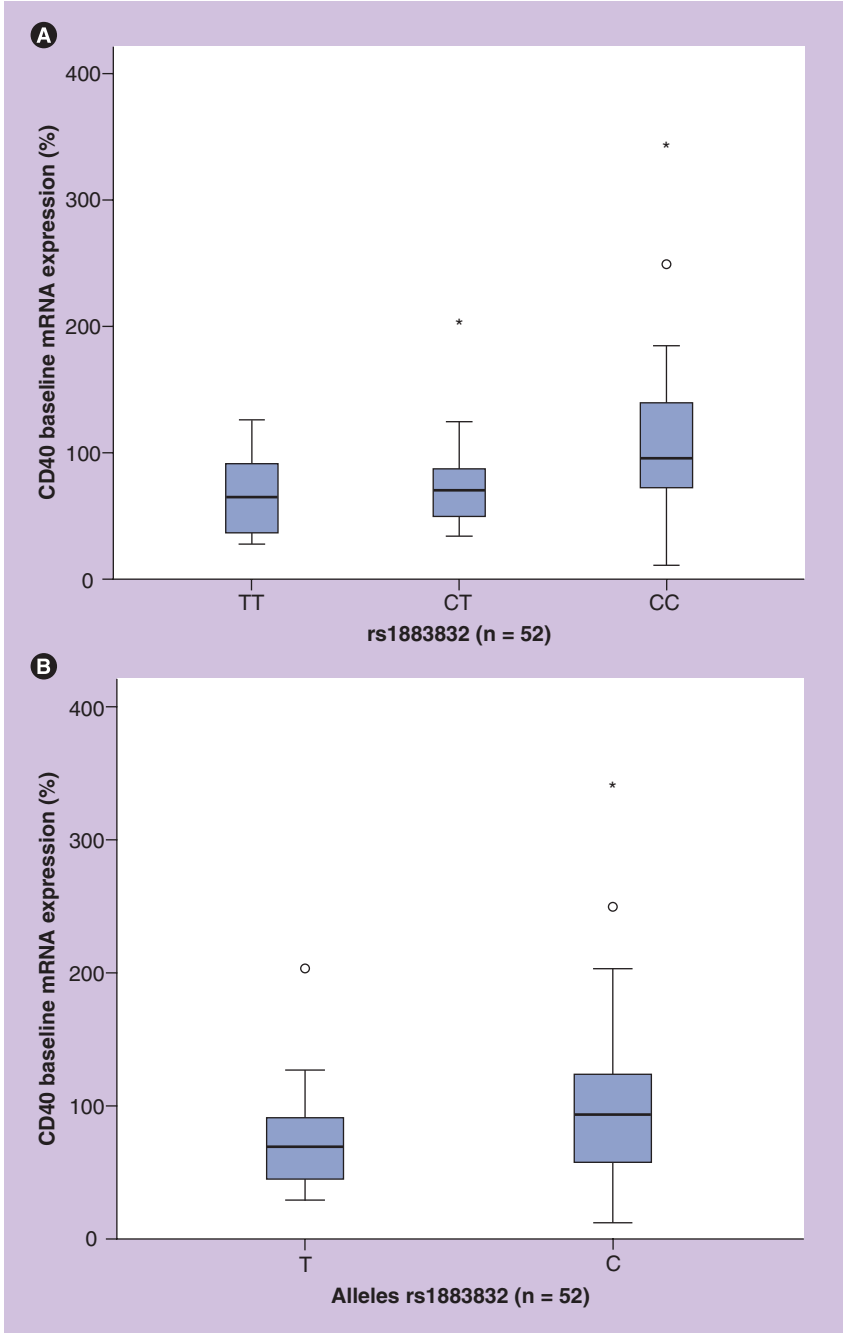

Figure 2. CD40 baseline expression in peripheral blood mononuclear cells. **(A)** Boxplot of CD40 mRNA baseline levels in peripheral blood mononuclear cells divided by genotypes. **(B)** Boxplot of CD40 mRNA baseline levels in peripheral blood mononuclear cells divided by alleles. Results are expressed as a percentage relative to a healthy calibrator control. The outliers (°) and the extreme outlier cases (*) are indicated in the boxplot graph.

performed found no effects of *CD40* -1C>T in antithyroidal withdrawal. Other unrelated *CD40* SNPs (rs1535045 and rs3765459) have been associated with atherosclerosis progression in the Diabetes Heart Study [47].

If these results are confirmed in future larger studies and replicated among other populations, we might speculate about the necessity to individualize tPA treatment depending on the genetic background of our stroke patients. Genetic risk scales such as those proposed in this article could help clinicians to anticipate which individuals will develop a reocclusion following a successful recanalization and, thus, might need new pharmacological or nonpharmacological strategies, as has been proposed for patients showing tandem occlusion [48]. A possible treatment would be to follow patients who are at risk with intensive TCD monitoring and to perform mechanical thrombectomy and/or to infuse arterial antithrombotic drugs whenever a reocclusion occurs. An alternative approach would be to give lower tPA doses in conjunction with an antiplatelet agent acting in different pathways to those of tPA, as is sometimes used in myocardial infarction therapy [49,50]. This strategy is currently being tested in clinical trials, notably with aspirin [101], and many other treatments are in preclinical or early clinical phase development [51].

Study limitations

In another study from our center, baseline NIHSS and severe ipsilateral artery disease (tandem occlusion) were independently associated with the risk of reocclusion [2]. However, only a trend towards association with ipsilateral artery disease (tandem occlusion) and reocclusion rates was identified in the present study, thus demonstrating the variability of clinical results depending on the series. Furthermore, reocclusion rates in our sample were significantly lower than those reported by other larger series [1,3,4,12], although this may be accounted for by differential reocclusion definitions.

Nevertheless, the findings of this report need to be replicated by independent studies with different populations to validate the association of rs1883832 and rs1800801 with reocclusion rates. Our results add evidence to the fact that tPA efficacy and safety are associated with patients' genetic background [5–8].

Acknowledgements

The authors are deeply grateful to all study participants, neurologists and nurses of the Stroke Unit from Vall d'Hebron Hospital (Barcelone, Spain) for their contributions. We are also grateful for excellent technical support from A Penalba, L Ortega, C Boada, JL Peiro and S García-Menéndez. We would also like to thank the National Center for Genotyping (CeGen, Barcelona, Spain) for their excellent technical assistance. This study is part of the project Geno-tPA. The Neurovascular Research Laboratory takes part in the International Stroke Genetics Consortium ISGC and the Spanish stroke research network RENEVAS (RD06/0026/0010).

Financial & competing interests disclosure

This study was funded by grants from the Spanish government (Geno-tPA project-FIS PJ060586), Mutua Madrileña (2006) and Ramón Areces (2006) Foundations. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Genetic analysis: a predictive model for vessel reocclusion

- Using a dominant/recessive model, an association between *CD40* -1 T-carrier and *MGP* AA genotypes with higher reocclusion rates after tissue plasminogen activator treatment has been found.
- This association correlated with neurological worsening for the *MGP* -7 A>G polymorphism but not for the *CD40* -1 C>T variant.
- A higher risk for reocclusion was predicted also for the *CD40* -1 T allele using an additive model.
- The predictive scale generated with the presence of tandem occlusion, *CD40*–1C>T and *MGP* -7A>G permits the stratification of patients from less than 1% to over 70% risk with higher accuracy than clinical parameters alone.

CD40 *-1C>T effects on real-time quantitative PCR & CD40 soluble fraction measurements*

- *CD40* -1 C>T is associated with CD40 mRNA expression levels in peripheral blood mononuclear cells during the acute phase of ischemic stroke.
- However, baseline CD40 plasma soluble fraction does not seem to be a useful biomarker of artery reocclusion after tissue plasminogen activator ischemic stroke treatment.

Conclusions: new treatment perspectives

- If these results are confirmed in other populations, they may prompt us to modify tissue plasminogen activator selection criteria based on a patient's genetic background.
- Vitamin K inhibitors, such as warfarin, might increase reocclusion risk due to MGP dysfunction, but this issue needs to be examined in future studies.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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ARTICLE 3

Submitted

Short title: *IL1B, VWF* and *TIMP1* variants influence t-PA efficacy

Title: *IL1B, VWF* and *TIMP1* variants are associated with fibrinolysis efficacy in Ischemic Stroke patients

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Word count: **Abstract: 232**

Article: 4,120

Tables and figures: 567

References: 2,074

Abstract

Background: Great interindividual variability exists in tissue plasminogen activator (t-PA) response in the acute phase of ischemic stroke. Therefore, we explored genetic background related with t-PA efficacy.

Methods and Results: Our prospective study attempted to genotype 250 single nucleotide polymorphisms (SNP) from 115 candidate genes by SNPlex technology in 2 cohorts accounting for 885 t-PA treated patients, from which 156 recanalized during t-PA infusion (33%). Epistasis was analyzed with Hypothesis-Free Clinical Cloning (HFCC) and predictive models were generated by logistic regression (LR) and classification tree. Functional studies included Real Time-QPCR, for Interleukin 1B (IL1B), ELISA and Searchlight protein array for Tissular Inhibitor of Metalloproteinases 1 (TIMP-1) and Matrix Metalloproteinase 9 (MMP-9) and activity measurement for von Willebrand factor and coagulation factors III (FIII), VII (FVII), VIII (FVIII) and X (FX).

After replication, rs1143627 and rs16944 in *IL1B* and rs1063856 in *vWF* influenced early recanalization, and internal validation incorporated rs207054 in *TIMP1*. The LR-based score predicted arterial recanalization 1h after t-PA bolus (area under the curve 0.668), while CRT classification tree identified similar groups of interest. Functional studies did not disclose the mechanism by which these SNPs were related to early recanalization.

Epistasis by HFCC analysis showed that combination of rs5186 (AGTR1) with rs2487151, rs2435609 or rs12703116 (ASB10) predicted early recanalization at study-wise significance ($p<2.5 \times 10^{-7}$).

Conclusions: Three SNPs were associated with t-PA efficacy in the Spanish population. We generated the first clinical-genetic predictive model for fibrinolysis effectiveness.

Keyword list: t-PA, stroke, recanalization, pharmacogenetics

Introduction

Intravenous tissue plasminogen activator (t-PA) is the only currently approved drug for acute stroke treatment. Clinical response to thrombolytics may be poor due or lack of early recanalization of the occluded vessel in 48-65% of subjects [1] safety concerns such as symptomatic hemorrhagic transformation in 1.7%-6.4% of cases [2,3] leading to 6.5-12.7% death among t-PA treated patients [2]. Recanalization time determines stroke recovery [4], especially in basilar artery occlusions, which have a devastating outcome whitout treatment and where the more aggressive intra-arterial trombolysis is common [5].

Our previous studies defined baseline PAI-1 and Thrombin-Antithrombin complex levels as biomarkers for early recanalization, as well as variants in Thrombin Activable Fibrinolysis Inhibitor (TAFI) or *CPB2* rs1926447 and Angiotensin Converting Enzyme (ACE) rs1799752 [6-9]. Besides, we proposed several biomarkers to anticipate short term neurological outcome [10-12] or long-term disability [12-13]. Recent studies have explored SNPs associated with outcome, linking COX-2 with Glasgow Outcome Score [14], GPIIIa with Barthel Index [14], and MMP-2 [15] or MBL [16] with modified Rankin Scale (mRS) score, though they need to be independently validated.

Our aim was to find new useful single nucleotide polymorphisms (SNP) predictors of early recanalization or clinic outcome in the largest cohort of t-PA treated patients with transcranial doppler (TCD) monitorization. This information could help clinicians to individualize and improve ischemic stroke treatment.

Methods

1. Study Population

Our target group was consecutive Caucasian patients suffering from an acute ischemic stroke admitted at the emergency room that had a documented occlusion on TCD and received t-PA in a standard 0.9-mg/kg dose (10% bolus, 90% continuous infusion during 1 hour) within the first 4.5 hours after symptoms onset.

Cohort A: (n=531, valid DTC=354) consisted of patients with an acute stroke admitted to the emergency department of five Spanish university hospitals between November 2000 and May 2005, which was genotyped in July 2007. Cohort B (n=354, valid DTC=143) was recruited in the same centers between May 2005 and June 2008 and genotyped in November 2008. As both cohorts were clinically similar [Table S3], they were combined for randomization methods and models generation.

2. Clinical and Transcranial Doppler (TCD) Protocol

On arrival, a detailed history of vascular risk factors and current medication was obtained from each patient. Stroke severity was assessed with the National Institutes of Health Stroke Scale (NIHSS) [17] on admission, 1 and 2 hours after t-PA administration, 12, 24 and 48 hours after symptoms onset and at discharge. For neurological outcome analysis, the difference between 24h and baseline NIHSS was calculated and categorized in improvement (≤-4 points), stability (-3 to +3) and worsening (≥+4 points) [17]. Functional outcome was assessed with mRS at 3rd month, considering mRS≥2 as dependence [18]. Finally, the analysis was repeated considering NIHSS and mRS as continuous variables to ascertain that scale categorizations did not influence the results.

The main end-point was recanalization at the end of t-PA infusion (1h after t-PA bolus) because it is associated with good outcome [1,4] and early recanalization minimizes reocclusion bias. Patients were examined on admission to detect arterial occlusion and received continuous TCD monitoring during tPA administration as described [6]. New TCD recordings were performed 2 and 6 hours after tPA bolus, 24 hours from symptoms onset and whenever clinical deterioration occurred. Changes on TCD in each patient were determined by a single experienced neurologist using direct visual control of monitoring display. Presence and location of the arterial occlusion was determined by the TIBI flow grading system [1]. Recanalization on TCD was defined as partial when blunted or dampened signals (TIBI 2-3) appeared in a previously demonstrated absent or minimal flow (TIBI 0-1) and complete recanalization if the end-diastolic flow velocity improved to elevated or normal values (TIBI 4-5; stenotic or normal) [6]. Early

recanalization was considered whenever partial or complete recanalization was detected 1 hour after t-PA bolus. This study was approved by the Ethics Committee of each recruitment center and all patients or relatives gave informed consent.

3. Genetic analysis

We attempted to genotype 250 SNPs from 115 candidate genes [Supplemental Table S1] related to angiogenesis, coagulation, drugs metabolism, inflammation, myocardial infarction (MI) or stroke risk factors, selected by manual bibliographic search in Pubmed database (e.g "stroke" and "polymorphism"; see supplemental data for details). Among candidate genes, we selected the most studied SNPs or those with a functional effect (an already known modification at transcription, translation or protein activity) or a hypothetic modification due to an aminoacid substitution.

Genotyping was carried out at the Spanish National Genotyping Centre (Barcelona, Spain), using SNPlex™ technology and 3730/3730xl DNA analyzer (Applied Biosystems, Foster City, California). Samples were assigned in groups of 90 samples plates with 48 SNPs batches using GeneMapper 3.5 as allele calling algorithm, with a mean call rate of 90%.

As quality control, two HapMap samples (NA10860 and NA10861) were included, whose genotype concordance was verified using SNPator (www.snpator.org, CeGen). Besides, SNPlex genotyping accuracy was validated in our cohort by PCR and direct sequencing [Supplemental Table S2].

Finally, 222 SNPs were successfully genotyped in cohort A, 163 SNPs in cohort B, 159 in both A and B, 140 of them accomplished the minimal Minor Allele Frequence (MAF>0.1%) [Figure 1B]. This drop was caused by repetitive freezing-thawing cycles of the probes and Genomi-Phi amplification in 2 of the 4 plates of cohort B (n=180), which reduced genotyping success rates in the latter cohort.

4. Hypothesis-Free Clinical Cloning (HFCC) analysis

HFCC combines a relatively fast computing algorithm for genome-wide epistasis detection with the flexibility to test a variety of different genetic models in multi-locus combinations. We programmed HFCC to analyze our "case-control" groups (presence/absence of early recanalization) by combining the 222 SNPs successfully genotyped of cohort A. HFCC software constructed all two-locus (digenic) variables and explored its association to recanalization occurrence by comparing nine diplotype (each 2-locus genotype combination) individually using conventional Wald's Test. As we calculated 698706 independent 2-locus strata, the study-wise corrected p-value was established at $p<2.5x10^{-7}$ after Bonferroni correction.

In order to explore the nature and strength of interactions in selected digenic patterns, we further evaluated epistasis among selected markers using Alambique software [9]. Specifically, Alambique was programmed to calculate Synergy index, AP, RERI [10], strata-specific Odds Ratios, and case-only parameter estimates [9].

5. Statistical Analysis

Sensitivity calculation was performed using the Ene 2.0 software. The sample size of 475 individuals with DTC data at 1h (cohort A+B) and the observed recanalization prevalence of 33% permit us to discriminate, with a power of 0.80, any SNP with MAF=0.1 whose minor allele rate in the recanalized group is 0.1817, giving a crude OR of 2.25 (95% CI: 1.28 – 3.93).

Statistical significance for each SNP was assessed by the Chi^2 or Fisher's exact test. Statistical analysis was performed using SPSS statistical package, version 15.0 (IBM, Chicago, US). Thereafter, we aimed to generate the best predictive model using two statistical approaches:

(1) A predictive score calculated for every subject based on logistic regression (LR) beta coefficients, using a stepwise Forward Likelihood Ratio LR (entry cut-off value 0.05), whose goodness of fit was evaluated by Hosmer-Lemeshow statistic **[13]**. Afterwards, to establish clinically relevant cut-off values, we automatically categorized this score in several risk groups

with the mathematic algorithm Chi-squared Automatic Interaction Detector (CHAID) algorithm included in SPSS software. Positive and negative predictive values (PPV and NPV) were calculated for the different cut-off values with a web-based tool (http://araw.mede.uic.edu/cgi-ebm/testcalc.pl).

(2) A classification regression tree (CRT) included in SPSS software, that creates a graphical classification model, similar to diagnosis algorithms. CRT divides the cohort in binary groups and evaluates each branch independently of the rest of the tree, whereas logistic regression gives a global score. As it is recommended that CRT minimal nodes should not be much lower than square root of total group (√n, in our case √475=21.79) **[15]**, we fixed 50 individuals as the minimum to allow node division and 20 for final nodes.

Receiver Operating Characteristics curves were plotted and predictive capacity was calculated by measuring the area under the curve (AUC). Different AUCs from the same cohort were compared using z-test **[14]** from MedCalc version 9.2.0.1 (MedCalc Software, Mariakerke, Belgium). This paper was written following STREGA guidelines **[15]**.

Results

1. Recanalization dominant-recessive association study

In cohort A, we found 20 variants associated with vessel recanalization 1h after t-PA, from which 3 were validated in cohort B: rs1063856 in *vWF* (coagulation pathway), rs1143627 and rs16944 in *IL1B* (inflammation pathway) [Table 1A].

When these variants were analyzed at later time points, a significant association was observed 2h after t-PA bolus for rs1143627 (CC: 60.5% Rec2h, T-carriers: 43% Rec2h; p=0.03) and a trend for rs1063856 (GG: 56.6% Rec2h A-carriers: 43.6% Rec2h; p=0.076) and rs16944 (AA: 56.5% Rec2h G-carriers: 43.2% Rec2h; p=0.088). Recanalization at 6 or 24h was not influenced by these genotypes (data not shown).

Regarding our previous studies, we confirmed the association of rs4341 of ACE (in complete LD with rs1799752 or I/D) with recanalization in cohort A at 1h (GG: 41.1% Rec1h C-carriers: 29.8% Rec1h; p=0.05) and a trend was seen at 24h (GG: 79.4% Rec1h C-carriers: 70.1% Rec1h; p=0.099). Unfortunately, genotyping failed in cohort B and rs4341 was excluded from further analysis.

In contrast, we did not observe an association between rs1926447 (Thr325Ile of *CBP2*) and early recanalization neither in cohort A (TT: 32.3% Rec1h C-carriers: 33.8% Rec1h, p=0.860) nor in cohort B (AA: 27.3% Rec1h T-carriers: 30.2% Rec1h, p=0.837).

2. Recanalization additive association study

Additive association study showed 8 variants associated with arterial recanalization in cohort A. Only rs2359612 was associated in both cohorts, but in the contrary sense; indicating a false positive result. Internal validation showed rs2070584 (TIMP-1) associated with artery recanalization in 787/1000 random sub-samples. In the first analysis, rs2070584 was significantly associated in cohort A (T-allele: 42.57% G-allele: 28.75%; p=0.001) but not in cohort B (T-allele: 33.3% G-allele: 27.1%; p=0.278); nevertheless, we considered TIMP-1 as a candidate for further functional studies [Table 1B].

3. Epistasis evaluation by HFCC

We found a strong association between rs5186 in *AGTR1* gene and several SNPs located between *IQCA1L* and *ASB10* [Table 3]. This region *ASB10 / IQCA1L* is not well characterized in HapMap, the best genotyped marker (rs17173189) showed differences among the four HapMap original ethnic groups. Besides, a combination between ALOX5AP variant rs4769874 and NOS2 rs1137933 also withstood Bonferroni multivariable test correction [Table 3].

4. Short-term neurological and long-term functional outcome

We analyzed SNPs influence on outcome at 24h assuming a dominant-recessive or an additive association. Dominant-recessive analysis leaded to 15 significant associations in cohort A without any consistent replication. Similar results were obtained when analyzing by alleles, with 14 significant associations in cohort A without replication [Table S4].

Internal validation of improvement (yes/no) showed marginal associations for rs10507391 in *PLA2G7* [516/1000] in the additive model and for rs1136410 in *PARP1* [573/1000] and rs5186 in *AGTR1* [582/1000] in the dominant-recessive assumption. Similar analysis for worsening (yes/no) yielded only a marginal association for rs10507391 in *PLA2G7* [536/1000] in the dominant-recessive model.

Long-term functional outcome was evaluated in presence/absence of dependence (mRS≥2). Dominant-recessive analysis leaded to 18 significant associations in cohort A without consistent replications [Table S4]. Similar results were obtained when analyzing in an additive way, with 12 significant associations in cohort A, none of them replicated [Table S4]. Internal validation with dependence (yes/no) yielded only a marginal association for rs10507391 in *PLA2G7* [562/1000] in the dominant-recessive model.

Regarding previously published results, only rs243864 was genotyped in *MMP2* without finding any association with mRS score. Other variants such as *COX-2* rs20417 or *GPIIIa* rs5918 did not influence mRS score.

5. Clinical and radiological predictors of recanalization

Univariate analysis showed an association between atherotrombotic (LAA) etiology on TOAST, absence of Atrial Fibrillation and high baseline glycemia with lower rates of recanalization, while

presence of previous stroke showed a trend [Table 3]. Regarding later time points, atherotrombotic etiology was also associated with no recanalization at 2h (LAA: 30.6% Other: 48.8%; p=0.001), 6h (LAA: 41.7% Other: 62.3%; p=0.001) and 24h (LAA: 60.6% Other: 76.7%; p=0.001) while atrial fibrillation (AF: 50.3% None: 41.5%; p=0.075) and previous stroke (Recurrence: 32.7% First event: 46.4%; p=0.068) only showed a trend at 2h.

At any time point, patients who recanalized presented lower median baseline glycemia (Rec2h: 115.6 mg/dl None: 125 mg/dl; p=0.031 / Rec6h: 116 mg/dl None: 127 mg/dl; p=0.034 / Rec24h: 116 mg/dl None: 127 mg/dl; p=0.009).

We tested independence of the SNPs identified from these clinical variables, and all of them were independent: rs1063856GG (OR: 2.847 CI95%:1.507-5.377), rs1143627CC (OR: 2.044 CI95%:1.072-3.899), rs16944TT (OR: 1.880 CI95%: 1.002-3.526) rs207584T-allele (OR: 1.504 CI95%: 1.166-1.938).

As expected, recanalization during t-PA infusion was associated with lower median NIHSS at 24h (None: 11 Rec1h: 4; p=1,73 E-8), 48h (None: 10 Rec1h: 4 ; p=4,98 E-7) and at discharge (None: 8 Rec1h: 2; p=2,41 E-5) and better functional outcome at 3rd month (None: 3 Rec1h: 2; p=7,87 E-4).

6. Recanalization models generation

We generated two predictive models for early recanalization: including genetic and clinical data or only clinical predictors.

1) Clinical-genetic model included atherothrombotic etiology on TOAST classification, rs1143627CC, rs1063856GG and rs207054 T-allelic charge.

2) Clinical model was created with atherothrombotic etiology and baseline glycemia.

Clinical-genetic model showed similar AUC than clinical model (0.668 vs 0.606; p=0.137) [Figure 1]. A more realistic approach would exclude TOAST classification, because it is not

available at emergency room department and is not useful to classify patients before t-PA infusion. Thus, clinical-genetics model would include presence of AF, rs1143627CC, rs1063856GG and rs207054 allelic charge: Score= 0.456 * AF + 0.949 * rs1143627CC + 0.904 * rs1063856GG + rs2070584 (T allele) * 0.295, whereas the clinical model included presence of AF and baseline glycemia [Figure 1]. These latter models also showed similar AUC (0.631 vs 0.574; p=0.201). CRT analysis showed similar results either using or excluding etiologic classification (data not shown).

7. Functional studies

By ELISA (n=128), TIMP-1 levels were not affected by genotypes neither among women (GG 87.11 ng/ml GT 92.40 ng/ml TT 111.68 ng/ml; p=0.272), nor in men (G 85.02 ng/ml T 76.98 ng/ml; p=0.333). Besides, TIMP-1 levels were not associated with early artery recanalization (None: 97.00 ng/ml Rec1h: 85.50 ng/ml; p=0.123) [Figure 4].

By Searchlight protein array (n=80), no effect was observed for recanalization in the whole group (None: 360.22 ng/ml Rec1h: 332.51 ng/ml; p=0.517), women genotypes (GG: 394.99 ng/ml GT: 397.58 ng/ml TT: 381.85 ng/ml; p=0.602) or in men (G: 320.45 ng/ml T: 326.64 ng/ml; p=0.808). The MMP-9/TIMP1 ratio did not show differences related to recanalization (None: 3.13 Rec1h: 3.24; p=0.978). By genotypes, a trend towards progressive ratio increase with at-risk “T” allele was seen in women (GG: 2.23 GT: 2.33 TT: 3.44; p=0.091) but not in men (G: 3.63 T: 3.39; p=0.629) [Figure 4]. As a reference, mean TIMP1 concentration in 8 healthy controls was 231.74 ng/ml with a normal range of 81.27-302.08 ng/ml. The mean ratio MMP9/TIMP1 was 2.17, with a normal range of 0.72-5.21.

As previously pointed for ischemic stroke non-treated patients [22,23], we looked for correlations between baseline TIMP-1 levels and NIHSS or mRS score. In ELISA cohort, TIMP-1 levels correlated with NIHSS score at later time points (24h: spearman coefficient 0.182, p= 0.046; 48h: spearman coefficient 0.209, p= 0.024; and discharge: spearman coefficient 0.197, p= 0.035) and functional outcome at third month measured by mRS (spearman coefficient

0.363, p= 7.38 E-5). This latter association with functional outcome was confirmed in the independent cohort analyzed by Searchlight protein array (spearman coefficient 0.316, p=0.016). In contrast, baseline MMP-9 levels did not correlate with mRS score (data not shown).

We did not detect an association of rs1063856 with vWF activity at baseline (AA: 54.57% AG: 53.38% GG: 72.92%; p=0.293) [Figure 3] nor with recanalization rates (None: 54.38% Rec1h: 59.57%; p=0.541) in 37 valid samples. No significant differences were observed for ABO or secretor genotypes too.

In contrast, an association was seen between rs1063856 and FVIII activity (AA: 115.93%, AG: 156.07%, GG: 83.42%; p=0.005) but baseline FVIII activity was not associated with 1h recanalization (None: 125.6% Rec1h: 137.64%; p=0.463) nor with short-term evolution (data not shown) but correlated with mRS functional outcome (spearman coefficient 0.263, p= 0.009) [Figure 5].

In a small cohort for which TIMP-1 levels and FVIII activity were available, we established cut-off points of TIMP-1<85 ng/ml and FVIII activity<145% for good functional outcome with 87.5% sensitivity and 63.6% specificity. However, these are preliminary results that need to be confirmed and refined in larger studies.

For *IL1B* expression, no significant differences were seen between groups for -511 or -31 genotypes (CC-TT: 106.5% CT-CT: 123.1% TT-CC: 168.2%; p=0.292) [Figure 2]. As pointed previously in an ex-vivo assay [24], we looked for an association between *IL1B* variants and FIII activity. As we found a batch effect in our results, the analysis was done in 40-samples groups. No association was seen for rs1143627 (plate 1; CC: 293.8% CT: 283.7% TT: 318.2%; p= 0.572 / plate 2; CC: 347.5% CT: 387.1% TT: 329.4%; p= 0.265) or rs16944 (plate 1; TT: 319.9% CT: 284.1% CC: 293.8%; p=0.548 / plate 2; CC: 329.1% CT: 387.1% TT: 353.7%; p=0.256) [Figure 2].

Given the negative results seen for FIII, we checked the coagulation factors FVII and FX, which form a complex with FIII in the extrinsic coagulation pathway. No association was observed for FVII with rs1143627 (CC: 129.4% CT: 124.6% TT: 115.8%; p= 0.621) or rs16944 (TT: 129.4% CT: 125.7% CC: 113.6%; p=0.401), neither for FX with rs1143627 (CC: 111.6% CT: 116.7% TT: 113.1%; p= 0.815) or rs16944 (TT: 111.6% CT: 116.1% CC: 113.8%; p=0.897) [Figure 2].

Discussion

Our genetic association study has uncovered a role for *IL1B, VWF* and less importantly *TIMP1* in early arterial recanalization following t-PA treatment. However, TIMP-1 levels give additional prognostic information. HFCC analysis identified combinations between rs5186 (*AGTR1*) and several *ASB10* variants or rs4769874 (*ALOX5AP*) and rs1137933 (*NOS2*) as predictors of early recanalization. The current predictive models must be validated and improved with new items prior its implementation in clinical routine.

No relevant associations were detected for short or long term functional outcome, measured by NIHSS and mRS scales. At present, this is the most important pharmacogenetics study that analyzes the modulation of t-PA efficacy by genetic background.

We found rs1143627 and rs16944 in *IL1B* gene associated with higher recanalization rates. IL-1β regulation is highly complex: it is regulated at transcriptional level depending on haplotype background [25], showing several inducible motifs depending on inflammatory stimulus and sequence variants [25-29]. The influence observed for these SNPs strongly vary depending on the experimental approach used: unstimulated plasma ELISA, whole blood or white blood cells “ex vivo” stimulation assay, RT-qPCR or IL-1β protein measurement from tissue biopsy, luciferase reporter expression and electronic mobility stimulation assay “in vitro”. High *IL1B* expression in peripheral blood mononuclear cells was related to worse outcome measured with Scandinavian Stroke Scale [30].

At the protein level, IL-1β also depend on protein secretion and IL-1β activity is controlled by maturation cleavage by caspase 1 or MMP-9 and degradation by MMP-3 [31]. Plasmatic IL-1β is increased during the acute phase of stroke, both in plasma [32] and CSF [33], though it remains in the picomolar range. High IL-1β has been proposed as a biomarker for cardioembolic etiology [34].

We hypothesized that rs1143627 and rs16944 would modify *IL1B* expression or FIII, FVII or FX activities (the components of tenase coagulation complex). However, in our whole blood RT-qPCR experiment, we did not see differences between genotypes or arterial recanalization with *IL1B* mRNA expression and no significant results were obtained for FIII, FVII or FX. Changes in mRNA expression may occur at different tissues (e.g. brain endothelium) or perhaps significant differences could not be detected due to the high variability during the acute phase of ischemic stroke.

We found rs1063856 in *VWF* gene associated with higher recanalization rates. VWF is synthesized by endothelial cells and megakaryocytes and forms a protein multimer from 50 to 100 monomers [35]. After activation by high shear forces, vWF binds GPIb receptor during platelet tethering, platelets become activated and vWF irreversibly binds with GP IIb/IIIa, leading to platelet aggregation and thrombus formation [35]. Alternatively, vWF directly interacts with collagen IV, releasing FVIII which in turn activates thrombin, and leading to thrombus formation by fibrinogen to fibrin conversion [35]. vWF deficiency causes von Willebrand disease and von Willebrand syndrome, both characterized by episodic mucocutaneous bleeding and excess bleedings in association with invasive procedures [35].

Plasmatic levels of vWF show great variability among individuals. Environmental factors include age, stress, exercise, pregnancy, hormonal changes and inflammatory disease [36,37]. Between 25-32% (pedigree studies) to 66-75% (twin studies) is explained by genetic factors [38], mostly by ABO blood group [39] but also Secretor [40] and a vWF promoter haplotype formed by -1793C>G, -1234C>T, -1185A>G or -1051A>G [41,42]. Blood group variants would alter vWF glycosylation and clearance [38], while the latter haplotype would influence *VWF*

mRNA expression by differential transcription factor binding [41,42]. Besides, rare mutations within VWF gene such as 1584 Y/C may increase vWF proteolysis and clearance [38].

Rs1063856 “G” allele was first linked to higher vWF levels and myocardial infarction risk in type I diabetic patients [43] and later corroborated in diabetes type II [44]; mean differences in FVIII did not reach statistical significance [43]. This association has been recently extended to healthy population, where it influenced also FVIII levels [45]. This latter approach identified also *STXBP5 (6q24), SCARA5 (8p21), STAB2 (12q23), STX2 (12q24.3), TC2N (14q32) and CLEC4M (19p13.2)* as candidate regions for vWF regulation [45], the first three also influencing FVIII levels. Additionally, Quantitative Trait Locus analysis from GAIT study linked vWF variation to five other different locus (1p36.13, 2q23.2, 5q31.1, 6p22.3, 22q11.1) [46].

We hypothesized that rs1063856, previously associated with vWF and FVIII protein levels, would influence their activity during the acute phase of ischemic stroke and t-PA mediated clot disruption. We found the highest arterial revascularization in “GG” individuals, despite “G” allele had been associated with higher protein levels both of vWF and FVIII. Our results from acute phase of ischemic stroke cases showed significant differences for FVIII activity (n=94) between genotypes, but not for vWF activity (n=37). The latter could be caused by the low sample size, since neither ABO nor secretor genotypes showed an association in that group. We can only hypothesize that rs1063856 may influence thrombus composition, which would make it easier to lysate.

Regarding therapeutic interventions, several vWF inhibitors are promising in preclinical research stages, but the approved drugs to reduce platelet adhesion (abciximab, eptifibatide and tirofiban) target GPIIb/IIIa instead of vWF [35].

We found rs2070584 associated with higher recanalization rates after t-PA administration. TIMP-1 is a ubiquitous protein whose expression is regulated by cytokines and growth factors, most notably IL-1, IL-6, IL-10, TNF-α and TGF-β [47]. It inhibits active forms of most MMPs by forming tight non-covalent 1:1 complexes, but also participates in apoptosis, angiogenesis,

neuronal plasticity or synaptic remodeling and acts as growth factor for a wide range of cells [48]. *TIMP1* is located in Xp11.23, and it has gene-specific polymorphic X-chromosome inactivation; its reactivation seems mediated by differential histone acetylation, independent of methylation or sequence variation [49].

TIMP1 polymorphisms have been inconsistently linked to abdominal aneurysms, lumbar spine disc degeneration and rheumatoid arthritis. Recently, rs2070584 “TT” and rs4898 “TT” were pointed as bad prognostic predictor in females with head and neck squamous carcinoma by regulating high TIMP-1 plasmatic levels [50]. Therefore, we hypothesized that rs2070584 might influence TIMP-1 protein levels or MMP-9/TIMP-1 ratio in the acute phase of ischemic stroke but we did not find an association neither between rs2070584 and TIMP-1 plasmatic levels nor with plasmatic MMP-9/TIMP-1 ratio in 2 independent cohort. As TIMP-1 was released by tumor endothelium [50], previous results may arise from tumor microenvironment.

In previous publications, plasmatic TIMP-1 temporal profile during ischemic stroke showed an increase between 8 and 24h after onset that was maintained in subacute phases [22]. TIMP-1 levels within the first 8 hours correlated with mRS at third month, and where lower if patients received t-PA treatment [22]. The current study extends this correlation to our 2 larger cohort of t-PA treated patients, measured either by ELISA (n=128) or Searchlight protein array (n=80).

It is hard to establish how TIMP-1 may influence stroke outcome, but it seems mediated by local effects in the brain tissue. Our group showed that, in necropsy homogenates from fatal strokes, TIMP-1 was increased in the infarct core with respect to the contralateral area, while TIMP-2 showed no differences between areas [51]. When this tissue was microdissected, no differences were observed between neuronal tissue and vessels, indicating that other components of the neurovascular unit were responsible of this increase [51].

In summary, 3 variants in *IL1B* and *VWF* genes were associated with early arterial recanalization after t-PA treatment, while rs2070584 in *TIMP1* may influence also this outcome. The mechanisms by which these SNPs modulate recanalization remain to be determined. No

variant was associated with short-term or long-term outcome when using clinically useful cut-off values.

Study limitations

This is a candidate-gene approach and we cannot rule out that other variants not included in our study would have greater influence in t-PA efficacy. Regarding functional studies, only a small number of patients were included and this may hamper our capacity to determine the causative variation of the clinical effect observed.

Sources of Funding

This study is part of the Geno-tPA project. The Neurovascular Research Laboratory takes part in the International Stroke Genetics Consortium and coordinates the Spanish Stroke Genetics Consortium (www.genestroke.com). The Neurovascular Research Laboratory receives grants from the Spanish stroke research network (RENEVAS, RD06/0026/0010) and the European Stroke Network (EUSTROKE 7FP Health F2-08-202213). This study was funded by a grant of the Spanish government (FIS PJ060586) and a grant from Ramón Areces Foundation (2006). A. del R.E. receives a PhD grant from the Vall d'Hebron Institute of Research.

Acknowledgments

We are deeply grateful to all study participants, neurologists and nurses of the Stroke Unit from Vall d'Hebron Hospital for their contributions. We are also grateful for excellent technical support from Ortega Torres, L; Penalba, A, García-Menéndez S and Félix, B. We would also like to thank the National Center for Genotyping (CeGen) for their excellent technical assistance.

Disclosures

None to declare.

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Table 1: Assuming a dominant-recessive model **(A)** or an additive model **(B)**, genetic association study for trecanalization 1 hour after t-PA bolus. Only variants with p-value<0.1 in Cohort A+B are shown, defined by their reference sequence number (rs). Minor alleles are indicated based on Hapmap-CEU population. In genotypic table **(A)**, the homozygous selected (HM1) and the other genotypes (HT-HM2) are indicated, while in additive tables **(B)** both alleles are presented. Mathematical symbols (< or >) indicate the at-risk genotypes **(A)** or alleles **(B)**. Frequencies of recanalization for each genotype group are indicated as a percentage, followed by their crude Odds Ratio (OR) and the Chi^2 p-value. *=p<0.05 in both A and B cohorts.

Table 2: Univariate analysis for other predictors of early recanalization with categorical **(A)** and continuous **(B)** variables. **(A)** For categorical variables, frequencies of recanalization (as a percentage) in presence or each absence of risk factor are indicated, followed by their Chi^2 p-value. **(B)** For continuous variables, mean or median values for recanalized and non-recanalized groups were calculated; parametric variables are indicated as mean ± Standard Deviation (S.D.) followed by t-test p-value while non-parametric as median and Interquartilic Range (IR) followed by Mann-Whitney p-values. ACE-I=Angiotensin Converting Enzyme Inhibitors, ARB-II= Angiotensin II Receptor Blockers, TOAST= Trial of ORG 10172 in Acute Stroke Treatment, OCSP= Oxfordshire Stroke Project Classication, SBP=Systolic blood pressure, DBP=diastolic blood pressure, NIHSS= National Institute of Health stroke scale, OTT=time from onset to treatment. *=p<0.05; †=p<0.1 in Cohort A+B.

Table 3: Best SNP pairs predictors as evaluated by HFCC (p-values < 10^{-5}) for early recanalization. OR10=Odd Ratio when only first SNP is present, OR01=Odd Ratio for only the second, OR11=Odd Ratio for SNP combination. Variant rs5186 belongs to AGTR1 gene, rs4769874 to ALOX5AP and rs1137933 to NOS2. The rest are located in ASB10 gene. *= p<0.05 after Bonferroni multivariable test correction.

Table 4: Predictive model of early recanalization occurrence. Parameters finally included in the logistic regression using **(A)** or excluding **(B)** TOAST data; the score is calculated using the beta coefficients. Odds Ratio (OR) and Standard Error (SE) for each item are indicated.

Table 1

A)

Gene	SNP	Genotypes HM1 / HT-HM2	Minor Allele	%Rec 1h Cohort A (n=531) HM1	HT-HM2	OR	p-value	%Rec 1h Cohort B (n=354) HM1	HT-HM2	OR	p-value	%Rec 1h Cohort A+B (n=885) HM1	HT-HM2	OR	P-value
vWF	rs1063856	GG > A-carriers	G	53.8	31.5	2.53	0.006	50.0	26.9	2.72	0.046	52.6	30.1	2.59	0.001
IL1B	rs1143627	CC > T-carriers	C	53.1	32.7	2.34	0.022	53.3	27.4	3.03	0.039	53.2	30.9	2.54	0.002
IL1B	rs16944	TT > C-carriers	T	50.0	32.0	2.12	0.038	50.0	26.2	2.81	0.049	50.0	30.2	2.31	0.005
TIMP1	rs2070584	TT < G-carriers	T	27.9	41.2	0.55	0.021	33.75	23.1	0.59	0.189	26.5	38.6	0.57	0.010
IL6	rs4722204	AA < T-carriers	A	15.8	37.0	0.32	0.010	21.4	30.7	0.62	0.471	17.3	34.9	0.39	0.012
AGTR1	rs5186	AA > C-carriers	C	38.6	27.4	1.66	0.045	32.4	25.0	1.44	0.337	36.6	26.6	1.59	0.028
CYP11B2	rs1799998	CC < T-carriers	C	24.2	38.0	0.52	0.044	21.7	31.4	0.61	0.356	23.5	35.7	0.55	0.033
F8	rs1800291	CC < G-carriers	G	32.7	39.1	0.76	0.335	25.0	46.2	0.39	0.032	30.3	41.1	0.62	0.049
MMP7	rs11568818	AA > G-carriers	G	38.4	32.4	1.30	0.324	42.1	24.0	2.30	0.036	39.5	29.8	1.54	0.050
MCP1	rs1024611	TT < C-carriers	C	28.8	41.7	0.57	0.026	28.4	29.7	0.94	0.866	28.6	37.5	0.67	0.057
NEUROD1	rs1801262	GG < A-carriers	A	32.0	36.1	0.83	0.457	12.8	35.4	0.27	0.009	27.3	35.8	0.67	0.067
ANP	rs5065	AA > G-carriers	G	37.0	24.0	1.86	0.041	30.0	29.3	1.04	0.931	34.7	25.9	1.53	0.081

B)

Gene	SNP	Alleles	Minor Allele	%Rec 1h Cohort A (n=531) Risk	No Risk	OR	p-value	%Rec 1h Cohort B (n=354) Risk	No Risk	OR	p-value	%Rec 1h Cohort A+B (n=885) Risk	No Risk	OR	p-value
TIMP1	rs2070584	G > T	T	42.4	28.75	1.82	0.001	33.3	27.1	1.34	0.278	39.5	28.3	1.65	0.001
IL1B	rs1143627	C > T	C	41.2	31.0	1.56	0.013	34.7	28.3	1.34	0.273	39.1	30.1	1.49	0.008
IL1B	rs16944	T > C	T	39.2	30.9	1.44	0.040	33.7	26.9	1.38	0.237	37.4	29.7	1.42	0.019
F8	rs1800291	G > C	G	39.3	33.1	1.31	0.250	47.2	26.7	2.46	0.012	41.6	31.1	1.58	0.020
MCP1	rs1024611	C > T	C	42.6	31.4	1.62	0.015	30.0	29.2	1.04	0.900	38.4	30.7	1.41	0.038
NEUROD1	rs1801262	A > G	A	36.9	33.1	1.18	0.347	37.4	23.6	1.94	0.013	37.0	30.3	1.35	0.040
VEGF	rs3025000	T > C	T	34.1	32.9	1.06	0.766	43.8	25.1	2.33	0.003	37.1	30.3	1.36	0.058

Table 2

A)

	% Rec 1h Cohort A (n=531)			% Rec 1h Cohort B (n=354)			% Rec 1h Cohort A+B (n=885)		
	Present	Absent	p-value	Present	Absent	p-value	Present	Absent	p-value
Vascular Risk Factors									
Gender (male)	36.9	29.5	0.144	25.3	33.8	0.266	34.0	31.2	0.518
Smoking habit	34.2	34.4	0.985	21.7	31.6	0.344	30.9	33.9	0.579
Hypertension	31.5	37.3	0.254	29.3	29.4	0.994	31.4	35.2	0.387
Diabetes mellitus	26.7	35.7	0.141	23.1	30.8	0.436	26.3	34.6	0.117
Atrial Fibrillation	39.6	30.6	0.061	34.0	27.1	0.391	38.2	29.5	0.050*
Heart disease	28.4	34.7	0.326	26.9	29.9	0.762	30.2	33.1	0.613
Dyslipidemia	28.6	36.4	0.149	38.6	26.0	0.131	31.6	33.8	0.639
Previous stroke	22.2	35.3	0.083	14.3	31.0	0.192	21.4	34.2	0.056†
Previous Treatments									
Antiplatelets	33.3	34.3	0.866	34.0	26.4	0.340	35.0	31.5	0.449
Statins	33.9	35.4	0.826	32.0	26.9	0.623	34.1	33.6	0.922
Oral antidiabetics	24.3	33.9	0.248	29.4	26.8	0.828	26.9	32.9	0.394
Diuretics	29	36.8	0.195	28.9	31.0	0.828	30.2	35.6	0.297
ARB-II	25	36.8	0.249	23.1	31.3	0.547	22.9	36.1	0.118
ACE-I	38.7	34.6	0.547	28.6	30.5	0.885	36.6	34.1	0.687
Radiological study									
Early ischemic signs	30.1	33.3	0.577	28.6	30.5	0.845	29.7	32.7	0.545
Clinical parameters									
Basilar occlusion	18.8	34.3	0.198	28.6	29.4	0.962	22.7	33.5	0.295
Atherothrombotic (TOAST)	20.7	37.5	0.005	13.3	33.0	0.034	17.9	37.2	2.03E-4*
POCI (OCSP)	18.8	33.8	0.213	20.0	30.1	0.500	20.0	33.1	0.169

B)

	Cohort A (n=531)			Cohort B (n=354)			Cohort A+B (n=885)		
	Rec 1h	No Rec 1h	p-value	Rec 1h	No Rec 1h	p-value	Rec 1h	No Rec 1h	p-value
Age (years, mean±S.D.)	71,9+11,4	71,5+13,1	0.337	72,5+10,2	72,0+12,6	0.967	71,9+11,1	71,6+12,8	0.209
SBP (mm, mean±S.D.)	147,9+27,3	152,8+26,0	0.123	152,7+27,7	153,75+26,9	0.776	149,7+27,3	152,6+26,1	0.177
DBP (mm, mean±S.D.)	79,8+14,3	84,3+17,3	0.037	83,7+13,0	82,0+14,7	0.476	80,7+13,9	83,2+16,6	0.206
Baseline NIHSS (median, IR)	17 (12-19)	17 (11-19)	0.511	18	17	0.413	36 (36,0-36,4)	36,1 (36,0-36,5)	0.195
Body temperature (ºC, median, IR)	36,2 (36,0-36,5)	36,2 (36,0-36,6)	0.875	36,0 (35,5-36,2)	36,0 (36,0-36,5)	0.030	114,5 (98,5-135)	118 (99,5-146)	0,038*
Glycemia (mg/dl, median, IR)	113 (99,7-124,2)	118 (99,25-147)	0.100	114 (97-144)	120 (99,5-163)	0.188	17 (12-20)	17 (11-20)	0.378
OTT (min, median, IR)	155 (130-183,7)	155 (133,5-195)	0.995	137 (105-195)	165 (135-190)	0.035	155 (120-195)	160 (135-195)	0.296

Table 3

SNP	Strata	OR	Wald	p-value	OR10	OR01	OR11	Mult. model	Add. model	Synergy Index	RERI	AP	Case test	p-value	Control test	p-value
rs5186-rs2487151	(1-1)	6.01	5.376	0.020	0.161	0.753	6.025	0.121	0.153	-4.627	6.111	1.014	35.45	2.62E-09*	0.089	0.765
rs5186-rs2435609	(1-1)	6.052	5.418	0.020	0.233	0.672	6.05	0.156	0.209	-4.612	6.145	1.016	33.17	8.46E-09*	0.118	0.731
rs4769874-rs1137933	(2-1)	7.537	4.075	0.044	0.425	0.354	8.493	0.15	0.239	-6.135	8.715	1.026	29.84	4.69E-08*	0.054	0.816
rs5186-rs12703116	(1-1)	6.152	5.515	0.019	0.253	0.902	6.313	0.228	0.246	-6.283	6.159	0.976	29.60	5.32E-08*	0.011	0.916
rs5186-rs2257090	(1-1)	8.539	4.811	0.028	0.247	0.847	9.877	0.209	0.236	-9.792	9.783	0.991	26.11	3.22E-07	0.1	0.752

Table 4

A)

Variables in LR score	Beta	S.E.	p-value	OR	95% CI for OR	
Atherothrombotic etiology	-1.112	0.324	0.001	0.329	0.174	0.621
rs1143627CC	0.843	0.338	0.013	2.323	1.199	4.502
rs2070584 (T-allele)	0.287	0.129	0.026	1.333	1.034	1.717
rs1063856GG	0.986	0.321	0.002	2.680	1.428	5.029

B)

Variables in LR score	Beta	S.E.	p-value	OR	95% CI for OR	
Atrial Fibrillation	0.456	0.225	0.043	1.577	1.015	2.451
rs1143627CC	0.949	0.335	0.005	2.584	1.341	4.979
rs2070584 (T-allele)	0.904	0.307	0.003	2.469	1.353	4.506
rs1063856GG	0.295	0.127	0.020	1.343	1.047	1.722

Figure 1: Predictive capacities of the different models plotted by their areas under the ROC curve using TOAST data **(A.1)** or not **(A.2)**.

Figure 2: RNA and protein determinations prior to t-PA infusion classified by *IL1B* genotypes. As rs1143627 and rs16944 were found in complete linkage disequilibrium in our population, figures are presented for only one variant. Results are indicated as a percentage of an external calibrator plasma pool. **(A)** Baseline whole blood IL1B expression. Baseline FIII **(B)**, FVII **(C)** and FX **(D)** activities classified by -511 C>T variant.

Figure 3: Baseline vWF **(A)** and FVIII activity **(B)** classified by rs1063856 genotypes. Results are indicated as a percentage of an external calibrator plasma pool.

Figure 4: Baseline TIMP-1 plasmatic levels classified by rs207584 genotypes. Due to its location in chromosome X, analysis were stratified by gender. Results are indicated as ng/ml. **A)** ELISA TIMP-1 measurements for men **(A.1)** and women **(A.2)**. **B)** TIMP-1 measurements using Searchlight protein array for men **(B.1)** and women **(B.2)**.

Figure 5: Correlation between protein levels measurements and 3rd month functional outcome measured by modified Rankin Scale score. Results are indicated as ng/ml for TIMP-1 and as a percentage of an external calibrator plasma pool for FVIII activity. **A)** TIMP-1 measurement by ELISA, **B)** TIMP-1 measurement by Searchlight protein array, **C)** FVIII activity determined by APTT measurement.

Figure 1

A.1)

A.2)

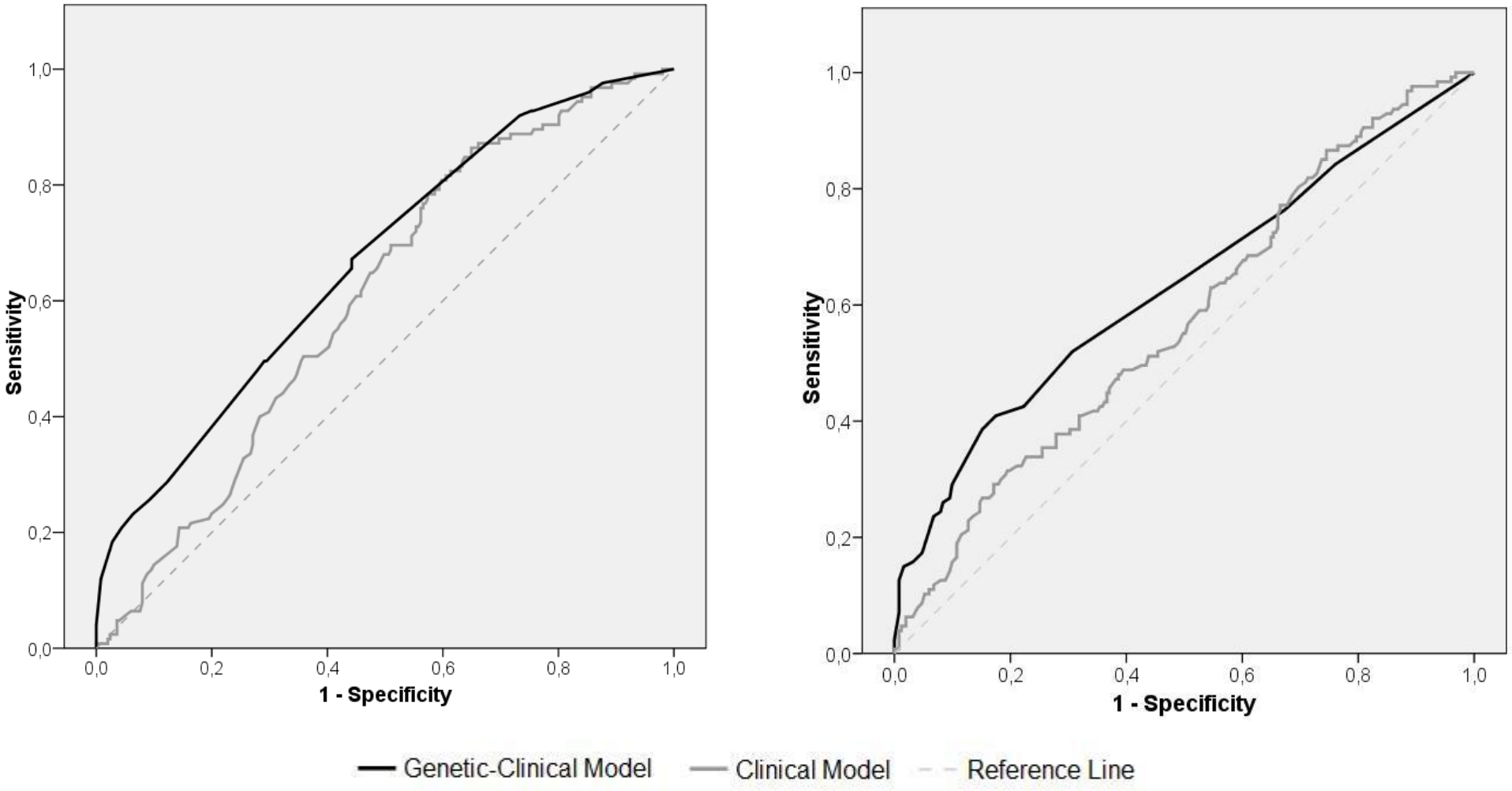


Figure 2

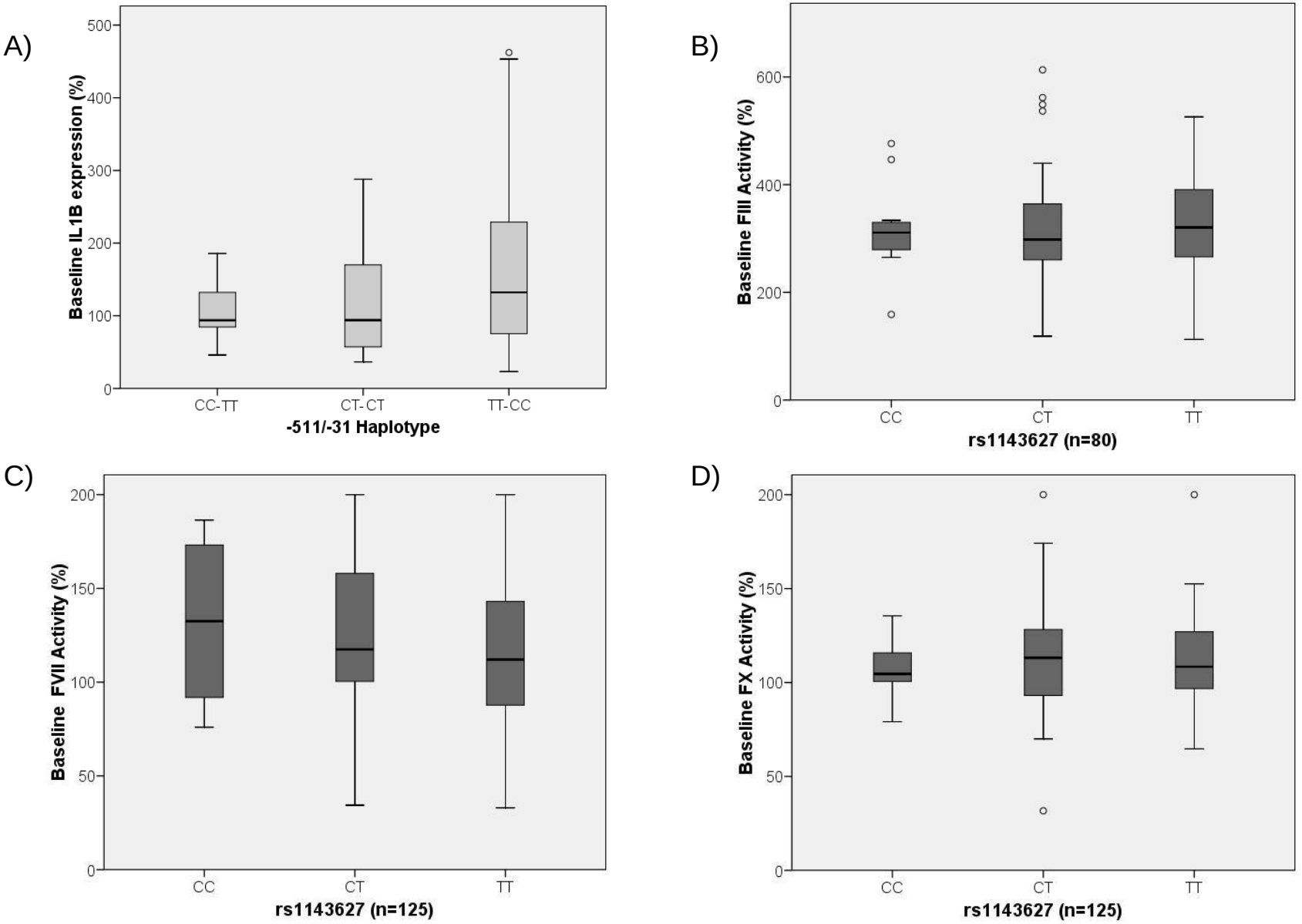

A)
Baseline IL1B expression (%)
500
400
300
200
100
0
CC-TT
CT-CT
TT-CC
-511/-31 Haplotype
B)
Baseline FIII Activity (%)
600
400
200
0
CC
CT
TT
rs1143627 (n=80)
C)
Baseline FVII Activity (%)
200
150
100
50
0
CC
CT
TT
rs1143627 (n=125)
D)
Baseline FX Activity (%)
200
150
100
50
0
CC
CT
TT
rs1143627 (n=125)

Figure 3

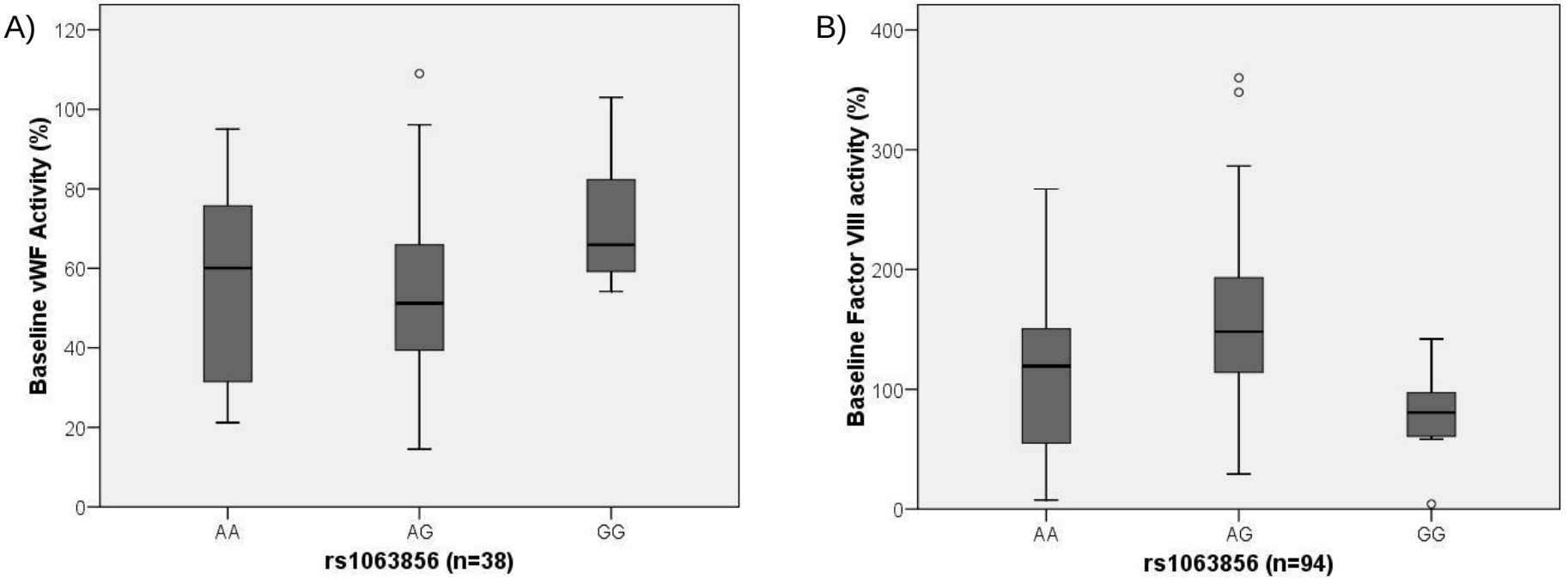
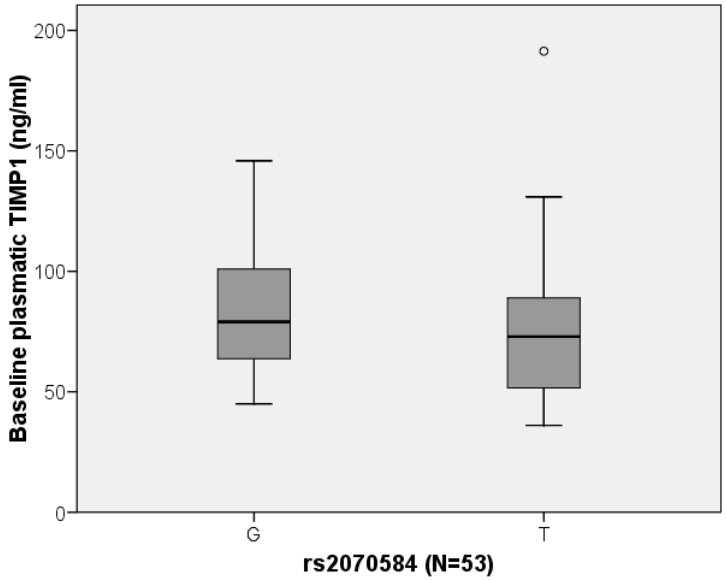
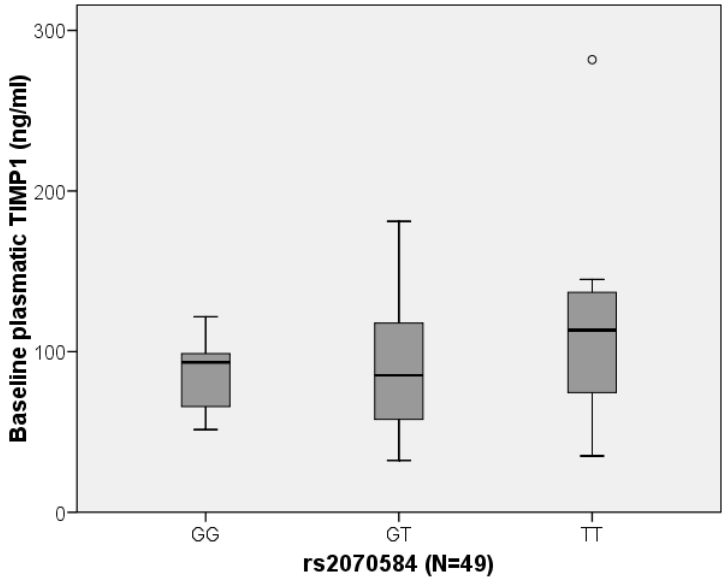

A)
Baseline vWF Activity (%)
120
100
80
60
40
20
0
AA
AG
GG
rs1063856 (n=38)
B)
Baseline Factor VIII activity (%)
400
300
200
100
0
AA
AG
GG
rs1063856 (n=94)

Figure 4

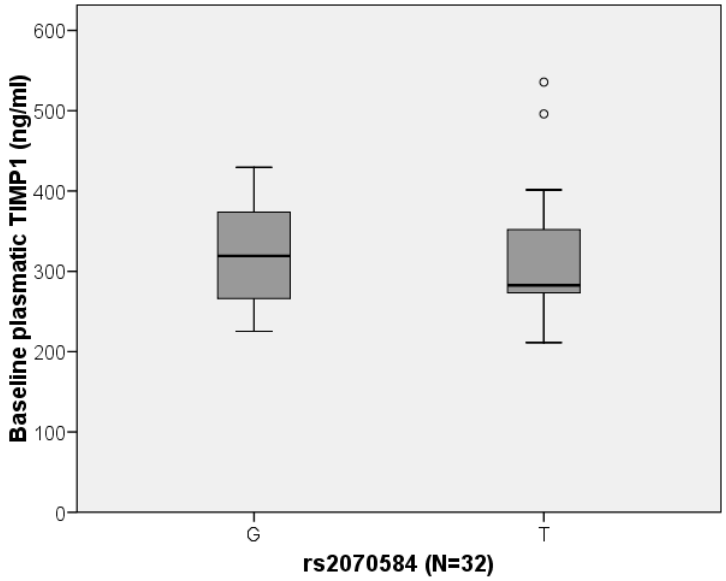
A.1)



A.2)



B.1)



B.2)

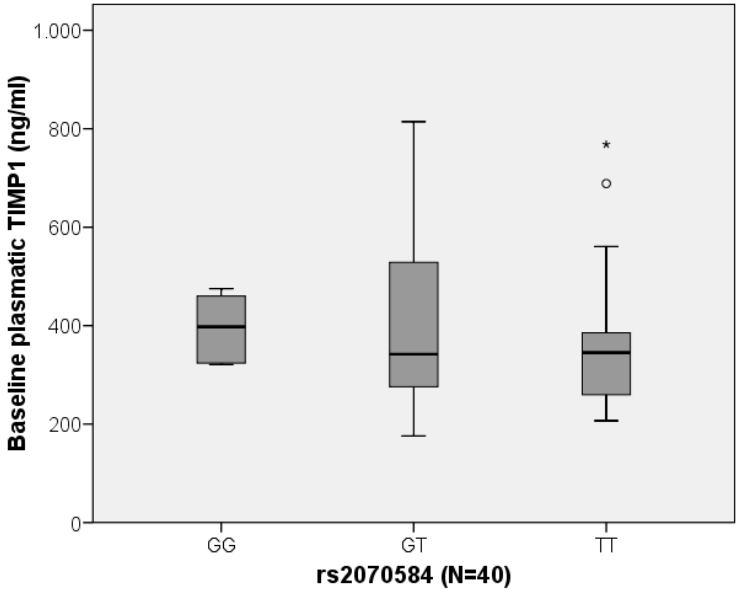
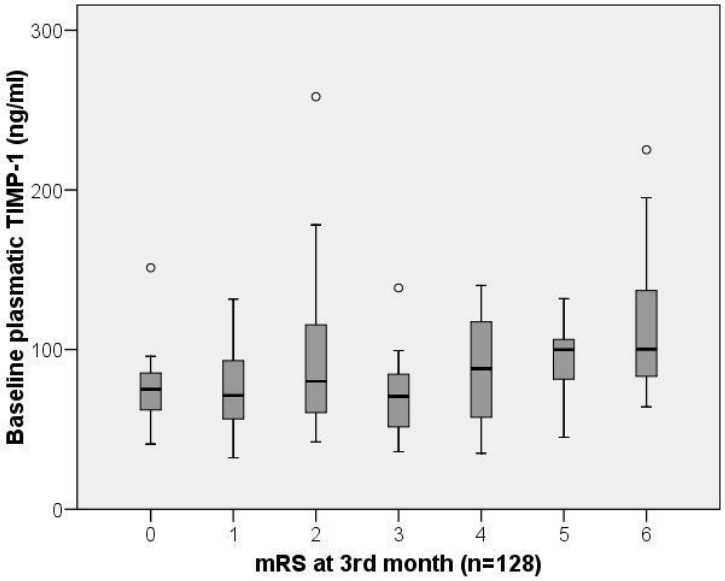
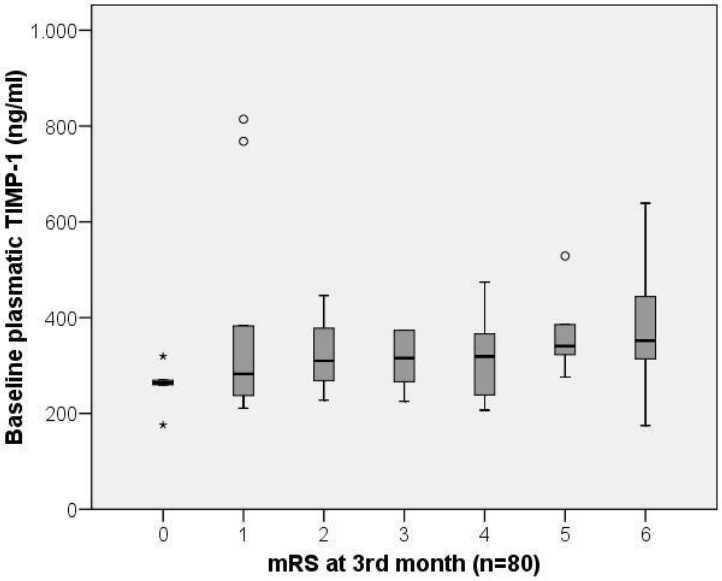


Figure 5

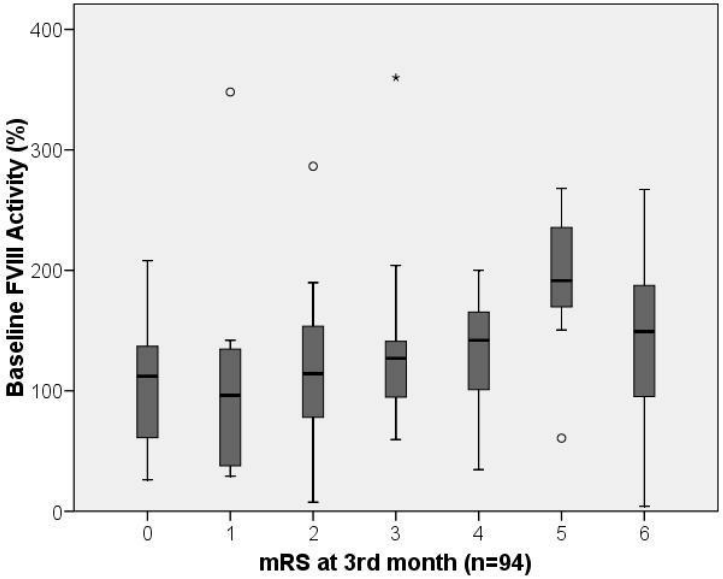
A.1)



A.2)



B)



Supplemental Data

EXPANDED METHODS

1. Clinical Study

Etiologic subgroups were determined accordingly to TOAST criteria [1]. Diagnostic set for TOAST classification included electrocardiogram, chest radiography, carotid ultrasonography, complete blood count and leukocyte differential and blood biochemistry in all patients; when indicated some patients also underwent special coagulation tests, transthoracic ecocardiography and Holter monitoring. This set was combined with neuroimaging data to define etiology as cardioembolic, atherothrombotic or other causes: arterial dissection, lacunar stroke and undetermined stroke.

Strokes were classified also according to Oxfordshire Community Stroke Project [2]: Total Anterior Cerebral Infarction (TACI), Partial Anterior Cerebral Infarction (PACI), Lacunar Cerebral Infarction (LACI) and Posterior Cerebral Infarction (POCI). A low number of lacunar stroke cases were included, since they are not expected to receive fibrynolitic therapy because of absence of defined vascular occlusion. A specific study must be conducted to extend our results to small vessel diseases.

Risk factors were defined as follows: Diabetes Mellitus: determination of fasting glucose concentration ≥ 126 mgrs/dl on venous plasma or post-glucose load ≥ to 200 mgrs/dl or current use of oral antidiabetic grugs or insuline. Coronary artery disease: any history of chest pain due to inadequate blood flow to the heart (angina pectoris) or acute myocardial infarction. Atrial fibrillation: past medical history of paroxysmal, persistent or chronic atrial fibrillation demonstrated in an EKG recording. Hypertension: history of a blood pressure reading higher or equal to 140/90 mmHg or current intake of hypotensor treatment. Hyperlipidemia: determination of total cholesterol equal or higher to 200 mgrs/dL or current use of lipid lowering drugs. Tobacco consumption: current smoking habit was recorded.

Violations of SITS-MOST criteria [3] were annotated from each center. SITS-MOST clinical exclusion criteria for t-PA treatment are age older than 80 years, previous stroke within 3 months or previous stroke with diabetes mellitus and mRS ≥ 1, INR > 1.4 or high APTT, plasma glucose<2.7mM or >22.2 mM (<50 mg/dl >400 mg/dl), baseline NIHSS >25, systolic blood pressure >185 mm Hg or diastolic blood pressure >110 mm Hg.

Only 54 basilar occlusions were included in cohorts A or B, from which 22 had DTC data and only 18 genotype and DTC data. Therefore, we were unable to perform a specific subgroup analysis.

2. Genetic analysis

The most relevant candidate genes were selected by manual searching in Pubmed using the keywords "stroke, hypertension, inflammation, drug metabolism, coagulation, diabetes mellitus or diabetes, angiogenesis, myocardial infarction, atherosclerosis, lipid metabolism" for phenotypes and the keywords "polymorphism, SNP, mutation, variant" for polymorphisms. Pathways analyzed were related to stroke risk (hypertension, diabetes mellitus, lipid metabolism and myocardial infarction), t-PA targets (coagulation) and catabolizers (drugs metabolism) and further processes linked to good (angiogenesis) and bad outcome (inflammation) after ischemia. Only articles in English or Spanish were read.

Among candidate genes, SNP selection was performed depending on previous literature (the most studied SNPs) and their functional effect, including those with an already known modification at transcription, translation or protein activity or a hypothetic modification based on an aminoacid substitution. Whenever an interesting polymorphism involved more than a single nucleotide change (e.g. Angiotensin I/D variant), a SNP in perfect linkage disequilibrium was chosen for genotyping. The 5 most interesting candidate genes (*MMP9, NOS3, IL6, LRP* and *VEGF*) were studied by TagSNPs to gain a better coverage and reduce risk of false-negative results. The SNPs list was completed with most of the previously reported stroke risk variants (e.g. PDE4D, ALOX5AP) and all the variants in gene regions linked to stroke in the first GWAS study [4]. The final list was modified to allow multiplexing in 48 probes batches. Some interesting variants, like F13 Val34Leu (rs5985) were excluded due to incompatibility with SNPlex genotyping platform.

We assume that no stratification bias has been committed, as patients were of Caucasian origin as indicated by their family names and Spain is genetically homogeneous distributed [5]. Nevertheless, it would be necessary to check a panel of intergenic unlinked markers to formally exclude this bias [6]. Further replication work is needed to expand these results to other ethnic backgrounds (African Americans, Asians …).

For SNPlex results validation, Kappa coefficient was calculated as Cohen et al [7], and stratified following the arbitrary Landis and Koch criteria [8], since there is no consensus algorithm to interpret this statistic.

Their respective CI95% and minor allele frequency (MAF) are indicated. ABO blood group determination and Secretor genotyping were carried out by PCR and direct sequencing.

All primers were designed with Primer3 [Table S2], checking in Ensembl Database (www.ensembl.org) that no nucleotide variants were present in the complementary sequence. Amplification was performed in 2720 Thermal Cycler (Applied Biosystems Inc., Foster City, USA) with an initial hot-start of 3 minutes at 94ºC, followed by 30 cycles of 30s at 94º-60ºC-72ºC and a final extension of 10 minutes at 72ºC. PCR samples were purified with ExoSap (GE Healthcare Biosciences, Waukesha, US) prior to Big Dye amplification (ABI Prism BigDye Terminator 3.0 method, Applied Biosystems Inc.; Foster City, US) and direct sequencing.

3. Real-Time qPCR analysis

Gene expression quantification was performed in 41 patients in which blood samples were obtained before t-PA administration as described [11]. Briefly, total RNA was extracted from the white blood cell fraction with RiboPure^{TM} -Blood kit (Ambion, Woodward st. Austin, USA). All reactions were run in triplicate and analyzed using the Applied Biosystems SDS 7500 system software (Applied Biosystems, Foster city, CA, USA). A probe located in exons 2-3 (Hs01535409_g1) of *IL1β* was used in all samples, with Cyclophilin A (PPIA) expression (Hs0099999904_m1) for normalization. The results are expressed as a percentage depending on an external healthy calibrator sample.

4. vWF activity determination

vWF activity was attempted to measure in 80 plasma citrate samples by ELISA (FVWF200, Axis-Shield Diagnostics Limited, Dundee, UK) in the fully automated Triturus system (Grífols, Barcelona, Spain), but only 38 measures were in the range of standard curve. Samples were stored at -80ºC before use and protein determinations were done unaware of genotype data. After first analysis, influence of ABO blood groups and Secretor genotypes was analyzed. All samples were matched by age, sex and TIBI score both at baseline (all proximal) and at the end of t-PA infusion.

5. F VII, F VIII and F X activity measurement

Activity of coagulation factors VII, VIII and X was determined by measuring APTT in 125, 94 and 125 baseline citrate plasma samples using the semi-automated coagulometer ST4 (Diagnostica Stago-Roche, Asnières, France). Factors diluent, APTT reagents, deficient serums, normal control and calibration plasma were from IZASA (Werfen Group, Barcelona, Spain). Samples were stored at -80ºC and thawed quickly in a heating bath before use. Protein determinations were done unaware of genotype data. Activities are indicated as a percentage of the normal control plasma.

6. Tissue Factor (FIII) activity determination

Tissue Factor baseline activity was measured in 81 plasma citrate samples (Assaypro, St. Charles (Missouri), US). Samples were stored at -80ºC and thawed in a heating bath before use. Measurements were done unaware of genotype data. All samples were matched by age, sex and TIBI score both at baseline (all proximal) and at the end of t-PA infusion.

7. TIMP-1 levels determination

TIMP-1 plasmatic levels were measured with Searchlight protein array (Pierce, USA) in 80 individuals and by manual ELISA (Amersham Biotrak, Buckinghamshire, UK) in 128 serum samples. Samples were stored at -80ºC before use. Measurements were done unaware of genotype data.

8. Statistical analysis

Sample size sensitivity calculation was performed using the Ene 2.0 software. Deviation from the Hardy-Weinberg equilibrium (HWE) was assessed using a Chi^2 test with 1 degree of freedom [12]. For continuous variables, Anova or Kruskal-Wallis was used depending on the normality of the distribution, as indicated by Kolmogorov-Smirnoff test and PP or QQ plots. Genotype associations were analyzed using dominant / recessive and additive models. Odd Ratios (ORs) and 95% confidence intervals (CIs) for the effect of each SNP on recanalization after tPA infusion were estimated using logistic regression models adjusted by

vascular risk factors and associated clinical variables. Bonferroni correction and its several variants were used for multivariable test correction; considering an adjusted *p*-value < 0.05 statistically significant [13].

Consistency of SNPs association was evaluated by bootstrap method with R software. The conventional method evaluates this parameter by repeating the analysis in 1000 random sub-samples of the half of the original data. In addition to this strategy, we checked for an association in 10 more random sub-samples and their corresponding non-selected counterpart. This new approach is more stringent than the traditional way.

A predictive model of early recanalization was performed with independently and consistently associated polymorphisms. Goodness of fit was evaluated by Hosmer-Lemeshow statistic and predictive capacity was calculated by measuring the area under the ROC curve (AUC). The difference between the AUCs was compared with MedCalc. For clinical use, a scale was generated after giving a score to each SNP weighted by their Beta coefficients on logistic regression adjustment [14, 15].

EXPANDED RESULTS AND DISCUSSION

1. Early recanalization model

To minimize the reduction in predictive capacity, we performed an exploratory analysis combining supraaortic Doppler data and atrial fibrillation (both available in the emergency room of our hospital) to define an “acute TOAST” as follows:

1) only presence of AF = cardioembolic

2) only presence of >50% ipsilateral carotid stenosis = atherothrombotic

3) absence or presence of both signs = undetermined

As occurred with TOAST classification, “acute atherothrombotic etiology” showed lower early recanalization rates (Atherothrombotic: 26.2% Rec1h Others: 40% Rec1h; p=0.048). For predictive purposes, when “acute atherothrombotic etiology” was included in early recanalization predictive models, ROC curves yielded similar AUC as TOAST either for genetics-combined (Acute: 0.697 vs TOAST: 0.692; p=0.927) or clinical prediction (Acute TOAST: 0.581 vs TOAST: 0.592; p=0.842) [Figure].

Agreement between “acute TOAST” and TOAST was substantial according to Landis and Koch criteria (Kappa=0.686, p=9.41E-55), but not complete. Medical knowledge and TOAST complementary tests would reclassify 20.47% of patients (52 / 254) during the following days; validation of these classification criteria escapes the scope of this paper.

2. Background from animal studies

The role of IL-1β in stroke is not totally established: traditionally, IL-1β was related to inflammatory damage, as *IL1A-IL1B* double knockout mice [16] or IL-1 pharmacological inhibition [17] showed lower MCAo infarct volumes. However, “in vitro” studies show a proangiogenic activity of IL-1β on spleen-derived endothelial progenitor cells [18]. As seen with MMPs inhibition, IL-1 may have deleterious effects in the early phase of ischemia but beneficial in the latter; further work is needed to define the exact role of IL-1 in stroke.

Besides, 7 different loci have been linked to differential vWF circulating levels in mice [19-22] by altering protein glycosylation [19], biosynthesis or *Vwf* mRNA expression [20-22]. *Vwf* knockout mice were unable to venous thrombus formation [23] and showed delayed arterial thrombosis and smaller thrombus size in experimental models [24]. Based on partial deletion mutants, it seems that GPIbα binding is the main mechanism involved in stroke pathological coagulation [25,26].

Finally, in animal models of stroke, TIMP-1 was increased in ischemic cortex at 12h, 24h and 2 days after MCAo both for permanent and 160 minutes transient occlusions [27]. Interestingly, this elevation was observed also for global forebrain ischemia [28], for 10 min MCAo (which generated brain ischemic tolerance) [29] and other neurological challenges such as Alzheimer disease, seizures and experimental autoimmune encephalitis [28, 30]. TIMP-1 upregulation is in close association with blood brain barrier (BBB) breakdown and neuroinflammatory response [28]. Accordingly, our laboratory showed that Abeta challenge to mixed rat cultures lead to TIMP-1 neuronal secretion and increased astrocyte proliferation [30]. Consequently, *Timp1* knockout mice show impaired learning and memory [31], or female-specific altered leptin signalling [32].

Regarding therapies, administration of MMP inhibitors lead to variable results, given the dual role of MMPs in ischemia [33]. Transient TIMP1/TIMP2 overexpression by adenoviral injection 3 days before MCAo reduced infarct volume and ameliorated functional outcome 14 days after ischemia [33].

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Supplemental table S1: List of SNPs attempted to genotype, classified by groups of interest. Genes, chromosomal position and genotyping results are indicated in columns.

Supplemental table S2: Agreement of genotyping results of SNPlex technology and direct sequencing to assess the quality of genetic data **(A)**. Minor allele frequencies (MAF) values are indicated because Kappa statistic depends on polymorphism frequencies. Primers used for SNPlex validation **(B)** and A2M gene sequencing **(C)**.

Supplemental table S3: Population characteristics of the 2 Geno-tPA study cohorts. Age is indicated as mean with its standard deviation. Categorical variable frequencies are indicated as a percentage with number of cases in brackets. Baseline NIHSS score is indicated as median with range in brackets. Differences between cohorts are indicated by the p-value of Chi^2 (categorical variables), t-test (age) or Kruskal-Wallis (baseline NIHSS). *=p<0.05; †=p<0.1.

Supplemental table S4: Assuming a dominant-recessive model **(A)** or an additive model **(B)**, genetic association study for short term neurological outcome **(1)** and long term functional outcome **(2)**. Only variants with p-value<0.1 in Cohort A+B are shown, defined by their reference sequence number (rs). Minor alleles are indicated based on Hapmap-CEU population. In genotypic tables **(A)**, the homozygous selected (HM1) and the other genotypes (HT-HM2) are indicated, while in additive tables **(B)** both alleles are presented. Mathematical symbols (< or >) indicate the at-risk genotypes **(A)** or alleles **(B)** for better short-term evolution **(1)** or higher dependence rates **(2)**. Frequencies of improvement (I), worsening (W) or dependency for each genotype group are indicated as a percentage, followed by their crude Odds Ratio (OR) and the Chi^2 p-value.

Supplemental figure S1: Predictive capacities of the genetic-clinical **(A)** and clinical models **(B)** using “Acute TOAST” instead of TOAST for early etiologic classification. Areas under the ROC curve are plotted separately for each model.

Supplemental figure S2: Baseline vWF activity classified either by ABO **(A)** or Secretor **(B)** genotypes. Results are indicated as a percentage of an external calibrator plasma pool.

Table S1

Biological Process	Gene Symbol	dbSNP ID	Chr	Position	Genotyping
Angiogenesis	*ABCA1*	rs28587567	9	106627854	Included
Angiogenesis	*ABCA1*	rs2230806	9	107620866	Included
Angiogenesis	*EDN1*	rs5370	6	12296254	Included
Angiogenesis	*FGF2*	rs1449683	4	123748085	Low genotyping Cohort B
Angiogenesis	*HTR2A*	rs6311	13	47471477	Included
Angiogenesis	*ICAM1*	rs5491	19	10385539	Excluded MAF<1%
Angiogenesis	*ICAM1*	rs1799969	19	10394791	Included
Angiogenesis	*ICAM1*	rs5498	19	10395682	Failed Cohort A and B
Angiogenesis	*PARP1*	rs1136410	1	226555301	Included
Angiogenesis	*TGFB1*	rs1800471	19	41858875	Failed Cohort A and B
Angiogenesis	*VCAM1*	rs1041163	1	101183824	Included
Angiogenesis	*VEGFA*	rs2010963	6	43738349	Low genotyping Cohort B
Angiogenesis	*VEGFA*	rs3025042	6	43741450	Excluded MAF<1%
Angiogenesis	*VEGFA*	rs3024994	6	43743506	Included
Angiogenesis	*VEGFA*	rs2146323	6	43745094	Failed Cohort A and B
Angiogenesis	*VEGFA*	rs3025000	6	43746168	Included
Angiogenesis	*VEGFA*	rs3025047	6	43746409	Excluded MAF<1%
Angiogenesis	*VEGFA*	rs3025010	6	43747576	Low genotyping Cohort B
Angiogenesis	*VEGFA*	rs3025030	6	43750586	Included
Angiogenesis	*VEGFA*	rs3025033	6	43751074	Included
Angiogenesis	*VEGFA*	rs3025035	6	43751358	Included
Diabetes Mellitus	*ADIPOQ*	rs2241766	3	186570891	Included
Diabetes Mellitus	*ADIPOQ*	rs1501299	3	186571122	Included
Diabetes Mellitus	*AP1M2*	rs1821282	19	10692713	Failed Cohort A and B
Diabetes Mellitus	*CAPN10*	rs3792267	2	241531173	Failed Cohort A and B
Diabetes Mellitus	*CAPN10*	rs5030952	2	241542702	Failed Cohort A and B
Diabetes Mellitus	*ENPP1*	rs1044498	6	132172367	Included
Diabetes Mellitus	*GCKR*	rs1260326	2	27730939	Included
Diabetes Mellitus	*GRM3*	rs6465084	7	86403474	Included
Diabetes Mellitus	*HNF1A*	rs1800574	12	121416863	Low genotyping Cohort B
Diabetes Mellitus	*HNF4A*	rs2144908	20	42985716	Included
Diabetes Mellitus	*IGF1R*	rs2229765	15	99478224	Included
Diabetes Mellitus	*IGFBP3*	rs2854744	7	45961074	Low genotyping Cohort B
Diabetes Mellitus	*IRS1*	rs1801278	2	227660543	Low genotyping Cohort B
Diabetes Mellitus	*PPARA*	rs1800206	22	46614273	Included
Diabetes Mellitus	*PPARG*	rs4135352	3	12458207	Excluded MAF<1%
Drug Metabolism	*CYP11B2*	rs1799998	8	143999599	Included
Drug Metabolism	*GST*	rs3211206	1	110201643	Included
Drug Metabolism	*GSTO1*	rs4925	10	106022788	Included
Drug Metabolism	*GSTP1*	rs1695	11	67352688	Included
Drug Metabolism	*MGP*	rs1800801	12	15038787	Included
Drug Metabolism	*MTHFR*	rs1801131	1	11854475	Included
Drug Metabolism	*MTHFR*	rs1801133	1	11856377	Low genotyping Cohort B
Drug Metabolism	*PTGS1*	rs3842787	9	125133506	Low genotyping Cohort B
Drug Metabolism	*PTGS2*	rs20417	1	186650320	Failed Cohort A
Drug Metabolism	*TP53*	rs1042522	17	7579471	Failed Cohort A and B
Drug Metabolism	*VKORC1*	rs2359612	16	31103795	Included

Biological Process	Gene Symbol	dbSNP ID	Chr	Position	Genotyping
Fibrinolysis & Coagulation	*ANXA5*	rs11575945	4	122617744	Included
Fibrinolysis & Coagulation	*CYP2C9*	rs1057910	10	96741052	Included
Fibrinolysis & Coagulation	*F12*	rs1801020	5	176836531	Included
Fibrinolysis & Coagulation	*F13A1*	rs7740663	6	6319003	Excluded MAF<1%
Fibrinolysis & Coagulation	*F2*	rs1799963	11	46761054	Included
Fibrinolysis & Coagulation	*F3*	rs1361600	1	95007917	Failed Cohort A and B
Fibrinolysis & Coagulation	*F5*	rs6025	1	169519048	Included
Fibrinolysis & Coagulation	*F7*	rs6046	13	113773158	Failed Cohort A and B
Fibrinolysis & Coagulation	*F8*	rs1800291	23	154158284	Included
Fibrinolysis & Coagulation	*FGA*	rs6050	4	155507589	Included
Fibrinolysis & Coagulation	*FGB*	rs1800790	4	155483707	Included
Fibrinolysis & Coagulation	*GP1BA*	rs2243093	17	4835894	Failed Cohort A and B
Fibrinolysis & Coagulation	*GP3BA*	rs5918	17	45360729	Included
Fibrinolysis & Coagulation	*PLAT*	rs2020918	8	42072437	Included
Fibrinolysis & Coagulation	*PLAU*	rs2227564	10	75673100	Low genotyping Cohort B
Fibrinolysis & Coagulation	*PROC*	rs1799810	2	128176039	Low genotyping Cohort B
Fibrinolysis & Coagulation	*PROS1*	rs6123	3	93593118	Included
Fibrinolysis & Coagulation	*RETN*	rs1862513	19	7733792	Included
Fibrinolysis & Coagulation	*SERPINC1*	rs2227606	1	173881121	Excluded MAF<1%
Fibrinolysis & Coagulation	*SERPINE1*	rs1799768	7	100769706	Included
Fibrinolysis & Coagulation	*SERPINE1*	rs7242	7	100781444	Included
Fibrinolysis & Coagulation	*TAFI*	rs1926447	13	46629943	Included
Fibrinolysis & Coagulation	*THBD*	rs1042579	20	23028723	Low genotyping Cohort B
Fibrinolysis & Coagulation	*VWF*	rs1063856	12	6153533	Included
Hypertension	*ACE*	rs1987693	17	61565764	Excluded MAF<1%
Hypertension	*ACE*	rs4341	17	61565989	Low genotyping Cohort B
Hypertension	*ADRB2*	rs1042713	5	148206439	Included
Hypertension	*ADRB2*	rs1042714	5	148206472	Low genotyping Cohort B
Hypertension	*ADRB2*	rs1800888	5	148206884	Included
Hypertension	*ADRB3*	rs4994	8	37823797	Low genotyping Cohort B
Hypertension	*AGT*	rs699	1	230845793	Failed Cohort A and low Cohort B
Hypertension	*AGTR1*	rs5186	3	148459987	Included
Hypertension	*KCNJ11*	rs5217	11	17409054	Excluded MAF<1%
Hypertension	*KCNMB1*	rs11739136	5	169810795	Included
Hypertension	*SCNN1A*	rs5742912	12	6458349	Low genotyping Cohort B
Inflammation	*CD40*	rs1883832	20	44746981	Included
Inflammation	*CRP*	rs1205	1	159682232	Included
Inflammation	*CRP*	rs1130864	1	159683090	Included
Inflammation	*CRP*	rs1800947	1	159683437	Included
Inflammation	*IFNG*	rs2430561	12	68552519	Included
Inflammation	*IL10*	rs1800872	1	206946406	Included
Inflammation	*IL10*	rs1800896	1	206946896	Included
Inflammation	*IL13*	rs1295686	5	131995842	Included
Inflammation	*IL1A*	rs1800587	2	113542959	Included
Inflammation	*IL1B*	rs1143634	2	113590389	Included
Inflammation	*IL1B*	rs1143627	2	113594386	Included
Inflammation	*IL1B*	rs16944	2	113594866	Included
Inflammation	*IL4R*	rs1805015	16	27374179	Included
Inflammation	*IL4R*	rs1801275	16	27374399	Low genotyping Cohort B
Inflammation	*IL5*	rs2069812	5	131879915	Included
Inflammation	*IL5RA*	rs2290608	3	3151758	Included

Biological Process	Gene Symbol	dbSNP ID	Chr	Position	Genotyping
Inflammation	*IL6*	rs1800797	7	22766220	Included
Inflammation	*IL6*	rs1800796	7	22766245	Included
Inflammation	*IL6*	rs1800795	7	22766644	Included
Inflammation	*IL9*	rs2069885	5	135228164	Included
Inflammation	*ITGA2*	rs1126643	5	52347368	Included
Inflammation	*MCP1*	rs1024611	17	32579787	Included
Inflammation	*MIF*	rs755622	22	24236391	Failed Cohort A and low Cohort B
Inflammation	*MMP1*	rs1799750	11	102670495	Included
Inflammation	*MMP10*	rs486055	11	102650423	Included
Inflammation	*MMP12*	rs2276109	11	102745790	Included
Inflammation	*MMP13*	rs2252070	11	102826538	Included
Inflammation	*MMP2*	rs243864	16	55512321	Included
Inflammation	*MMP21*	rs10901425	10	127462524	Failed Cohort A and B
Inflammation	*MMP3*	rs3025058	11	102715952	Included
Inflammation	*MMP7*	rs11568818	11	102401660	Included
Inflammation	*MMP8*	rs1320632	11	102596062	Included
Inflammation	*MMP9*	rs3918248	20	44071031	Included
Inflammation	*MMP9*	rs2274756	20	44076518	Included
Inflammation	*MMP9*	rs8113877	20	44635044	Included
Inflammation	*MMP9*	rs3918278	20	44635653	Included
Inflammation	*MMP9*	rs3918241	20	44635734	Included
Inflammation	*MMP9*	rs3918243	20	44636363	Excluded MAF<1%
Inflammation	*MMP9*	rs3918280	20	44636681	Excluded MAF<1%
Inflammation	*MMP9*	rs3918249	20	44638135	Included
Inflammation	*MMP9*	rs3918253	20	44639510	Low genotyping Cohort B
Inflammation	*MMP9*	rs2274755	20	44639691	Included
Inflammation	*MMP9*	rs2236416	20	44640574	Low genotyping Cohort B
Inflammation	*MMP9*	rs3918256	20	44640958	Low genotyping Cohort B
Inflammation	*MMP9*	rs3787268	20	44641730	Included
Inflammation	*MMP9*	rs2250889	20	44642405	Included
Inflammation	*NOS2A*	rs1137933	17	26105931	Included
Inflammation	*NOS3*	rs7791889	7	22960361	Failed Cohort A and B
Inflammation	*NOS3*	rs4722204	7	22960596	Included
Inflammation	*NOS3*	rs10266564	7	22961111	Low genotyping Cohort A
Inflammation	*NOS3*	rs1800779	7	150689942	Low genotyping Cohort B
Inflammation	*NOS3*	rs2070744	7	150690078	Failed Cohort A and low Cohort B
Inflammation	*NOS3*	rs2257073	7	150883286	Low genotyping Cohort B
Inflammation	*NOS3*	rs2257090	7	150883760	Low genotyping Cohort B
Inflammation	*NOS3*	rs10275136	7	150883891	Failed Cohort B
Inflammation	*NOS3*	rs12703116	7	150887941	Included
Inflammation	*NOS3*	rs2243428	7	150888657	Low genotyping Cohort A and B
Inflammation	*NOS3*	rs2288649	7	150888787	Low genotyping Cohort B
Inflammation	*NOS3*	rs2435609	7	150890072	Included
Inflammation	*NOS3*	rs2487151	7	150890240	Included
Inflammation	*NOS3*	rs2435608	7	150890805	Low genotyping Cohort B
Inflammation	*NOS3*	rs6952465	7	150891640	Included
Inflammation	*NOS3*	rs310590	7	150892346	Included
Inflammation	*NOS3*	rs310589	7	150892598	Low genotyping Cohort B
Inflammation	*NOS3*	rs310588	7	150892949	Low genotyping Cohort B
Inflammation	*NOS3*	rs310586	7	150893628	Included
Inflammation	*NOS3*	rs310585	7	150895254	Failed Cohort B
Inflammation	*NOS3*	rs310584	7	150896222	Included
Inflammation	*NOS3*	rs6977933	7	150901590	Failed Cohort B

Biological Process	Gene Symbol	dbSNP ID	Chr	Position	Genotyping
Inflammation	*SELE*	rs5355	1	169695869	Included
Inflammation	*SELE*	rs5361	1	169701059	Included
Inflammation	*SELP*	rs6133	1	169565345	Low genotyping Cohort A
Inflammation	*TIMP1*	rs11551797	23	47445939	Low genotyping Cohort B
Inflammation	*TIMP1*	rs2070584	23	47446518	Included
Inflammation	*TNF*	rs1800629	6	31543030	Low genotyping Cohort B
Inflammation	*TNFRSF1B*	rs1061622	1	12252954	Included
Lipid Metabolism	*APOB*	rs5742904	2	21229159	Excluded MAF<1%
Lipid Metabolism	*APOB*	rs1367117	2	21263899	Included
Lipid Metabolism	*APOC3*	rs2854117	11	116700141	Low genotyping Cohort B
Lipid Metabolism	*APOE*	rs405509	19	45408835	Low genotyping Cohort B
Lipid Metabolism	*APOE*	rs440446	19	45409166	Failed Cohort B
Lipid Metabolism	*APOE*	rs429358	19	45411940	Failed Cohort A and B
Lipid Metabolism	*APOE*	rs7412	19	45412078	Low genotyping Cohort B
Lipid Metabolism	*APOH*	rs1801692	17	64222163	Included
Lipid Metabolism	*CETP*	rs1800775	16	56995235	Low genotyping Cohort A and B
Lipid Metabolism	*CETP*	rs5882	16	57016091	Included
Lipid Metabolism	*FABP2*	rs179983	6	16350143	Excluded MAF<1%
Lipid Metabolism	*LDLR*	rs11669576	19	11222299	Failed Cohort A and B
Lipid Metabolism	*LEPR*	rs1137101	1	66058512	Included
Lipid Metabolism	*LIPC*	rs1800588	15	58723674	Included
Lipid Metabolism	*LPL*	rs268	8	19813528	Included
Lipid Metabolism	*LPL*	rs328	8	19819723	Included
Lipid Metabolism	*LRP1*	rs12307379	12	57518664	Included
Lipid Metabolism	*LRP1*	rs11172113	12	57527282	Included
Lipid Metabolism	*LRP1*	rs922963	12	57529292	Excluded MAF<1%
Lipid Metabolism	*LRP1*	rs17547610	12	57530340	Low genotyping Cohort B
Lipid Metabolism	*LRP1*	rs4759044	12	57530669	Included
Lipid Metabolism	*LRP1*	rs715948	12	57532981	Low genotyping Cohort B
Lipid Metabolism	*LRP1*	rs1799986	12	57535265	Failed Cohort A and B
Lipid Metabolism	*LRP1*	rs1800127	12	57539081	Low genotyping Cohort B
Lipid Metabolism	*LRP1*	rs7398375	12	57540847	Included
Lipid Metabolism	*LRP1*	rs10876966	12	57543571	Included
Lipid Metabolism	*LRP1*	rs12814239	12	57569477	Failed Cohort B
Lipid Metabolism	*LRP1*	rs2228187	12	57571248	Low genotyping Cohort B
Lipid Metabolism	*LRP1*	rs12368582	12	57582546	Low genotyping Cohort B
Lipid Metabolism	*LRP1*	rs1800168	12	57592556	Included
Lipid Metabolism	*LRP1*	rs1800159	12	57593893	Included
Lipid Metabolism	*LRP1*	rs7956957	12	57602814	Low genotyping Cohort B
Lipid Metabolism	*LRPAP1*	rs11940827	4	3533949	Failed Cohort B
Lipid Metabolism	*OLR1*	rs11053646	12	10313447	Included
Lipid Metabolism	*PPARGC1*	rs8192678	4	23815661	Included
Lipid Metabolism	*SREBF2*	rs2269657	22	42264268	Included
Myocardial Infarction	*ESR1*	rs9340799	6	152163380	Included
Myocardial Infarction	*ESR2*	rs1255998	14	64693870	Included
Myocardial Infarction	*ESR2*	rs1256065	14	64698931	Failed Cohort A and B
Myocardial Infarction	*ESR3*	rs1256030	14	64747169	Included
Myocardial Infarction	*PLA2G7*	rs1051931	6	46672942	Included
Myocardial Infarction	*PON1*	rs662	7	94937445	Included
Myocardial Infarction	*PON2*	rs7493	7	95034774	Included
Myocardial Infarction	*S100B*	rs11542310	21	48019250	Excluded MAF<1%
Myocardial Infarction	*S100B*	rs1051169	21	48022229	Included

Biological Process	Gene Symbol	dbSNP ID	Chr	Position	Genotyping
Stroke	*A2M*	rs669	12	9232267	Included
Stroke	*A2M*	rs1800433	12	9232350	Failed Cohort A and B
Stroke	*AACT*	rs4934	14	95080802	Low genotyping Cohort B
Stroke	*ADD1*	rs4961	4	2906706	Included
Stroke	*AIM1*	rs783396	6	106987369	Not attempted Cohort B
Stroke	*ALOX5AP*	rs10507391	13	31312095	Included
Stroke	*ALOX5AP*	rs4769874	13	31326440	Failed Cohort B
Stroke	*AMPD1*	rs17602729	1	115236056	Included
Stroke	*ANP*	rs5065	1	11906067	Included
Stroke	*ASTN2*	rs3761845	9	119770479	Not attempted Cohort B
Stroke	*ASTN2*	rs10817974	9	119775605	Not attempted Cohort B
Stroke	*CD14*	rs2569190	5	140012915	Included
Stroke	*CDH1*	rs16260	16	68771033	Included
Stroke	*CDK5*	rs11541602	7	150751131	Excluded MAF<1%
Stroke	*CX3CR1*	rs3732379	3	39307255	Included
Stroke	*DYX8*	rs1635712	1	35944751	Excluded MAF<1%
Stroke	*ELMO1*	rs11766187	7	36934572	Excluded MAF<1%
Stroke	*ELN*	rs17855988	7	73474824	Low genotyping Cohort B
Stroke	*FLT1*	rs7993418	13	28883060	Failed Cohort A and B
Stroke	*GJA1*	rs17653265	6	121768750	Included
Stroke	*GJA4*	rs1764391	1	35260768	Failed Cohort B
Stroke	*GNB3*	rs5443	12	6954874	Low genotyping Cohort B
Stroke	*HIF1AN*	rs2295778	10	102295835	Low genotyping Cohort B
Stroke	*HP*	rs587660	16	72094294	Excluded MAF<1%
Stroke	*HSPA6*	rs400835	1	161494731	Failed Cohort A and B
Stroke	*IMPA2*	rs7506045	18	11987271	Not attempted Cohort B
Stroke	*ITGB6*	rs10497212	2	160964699	Not attempted Cohort B
Stroke	*KCNIP4*	rs4697177	4	20760088	Not attempted Cohort B
Stroke	*KCNK17*	rs10947803	6	38989304	Not attempted Cohort B
Stroke	*KCNK17*	rs10807204	6	39273425	Not attempted Cohort B
Stroke	*LTA*	rs909253	6	31540312	Included
Stroke	*MGAT5*	rs2118844	2	135167599	Not attempted Cohort B
Stroke	*NEUROD1*	rs1801262	2	182543454	Included
Stroke	*P2RY1*	rs1065776	3	152553627	Included
Stroke	*PDE4D*	rs1396476	5	59396641	Included
Stroke	*PDE4D*	rs2910829	5	59469898	Included
Stroke	*PDE4D*	rs966221	5	59502519	Included
Stroke	*PDE4D*	rs702553	5	59736772	Included
Stroke	*PDE4D*	rs12188950	5	59783316	Not attempted Cohort B
Stroke	*PDE4D*	rs12153798	5	59787640	Not attempted Cohort B
Stroke	*PDE4D*	rs152312	5	59787815	Included
Stroke	*ROCK2*	rs9808232	2	11276571	Low genotyping Cohort A
Stroke	*SPATA13*	rs2793483	13	24759687	Not attempted Cohort B
Stroke	*SPTB*	rs229673	14	65281943	Not attempted Cohort B
Stroke	*TLR4*	rs4986790	9	120475301	Included
Stroke	*ZNF650*	rs10204475	2	170932521	Not attempted Cohort B
<Confused by rs1801177>	*Intergenic*	rs180117	17	67922657	Excluded Intergenic

Table S2

A)

	rs669	rs1801020	rs1130864	rs11575945	rs1799768	rs1800947	rs1926447	rs4341*	rs4934	rs6050
N	104	149	69	113	109	71	104	64	108	134
Kappa	0.849	0.966	0.887	0.667	0.835	0.945	0.829	0.835	0.719	0.622
K CI95%	0.754-0.943	0.920-1	0.604-0.833	0.504-0.831	0.743-0.927	0.838-1	0.733-0.925	0.709-0.961	0.604-0.833	0.494-0.749
MAF	0.30	0.18	0.35	0.12	0.47	0.05	0.30	0.42	0.43	0.22

B)

RS	Common name	Gene	Primer forward (5'-3')	Primer reverse (5'-3')	Reference
rs1130864	C1444T	*CRP*	AGCTCGTTAACTATGCTGGGGCA	CTTCTCAGCTCTTGCCTTATGAGT	[34]
rs11575945	-1C>T	*ANXA5*	GGGCACGAGTTGCAAATGGCG	GTCGCAGCATACAAAGTTGTG	[35]
rs1799768	4G/5G	*SERPINE1 (PAI1)*	TTACCATGGTAACCCCTGGT	AGCCACGTGATTGTCTAGGT	[36]
rs1800947	G1059C	*CRP*	ACTGGACTTTTACTGTCAGGGC	ATTCCCATCTATGAGTGAGAACCT	[34]
rs1801020	C46T / -4C>T	*F12*	CCAGTCCCACTATCTAGAAAAG	ATGGCTCATGGCTGTGATAG	[37]
rs1926447	Thr325Ile	*CBP2 (TAFI)*	GCTTTGTTCAGCATTGTCATAG	CAATTGTGATTGCCATAAAGTG	[38]
rs4341	LD with I/D *	*ACE*	TGGGACCACAGCGCCCGCCACTAC CTGGAGACCACTCCCATCCTTTCT	TCGCCAGCCCTCCCATGCCCATAA GATGTGGCCATCACATTGGTCAGAT	[39]
rs4934	Ala104Thr	*SERPINA3*	CAGAGTTGAGAATGGAGA	TTCTCCTGGGTCAGATTC	[40]
rs6050	Thr312Ala	*FGA*	CCTAGCAGTGCTGGAAGCTG	GGCTCCCAGGGTTTTGGT	[41]

Table S3

	Cohort A (n=531)	Cohort B (n=354)	p-value A vs B	Cohort A+B (n=885)
Age mean ± S.D.	70.68±12.0	70.25±12.0	0.616	70.51±12.0
Male gender	54.8% (289)	54.7% (181)	0.964	54.8% (470)
Current smoker	26.1% (131)	20.1% (66)	0.044*	23.7% (197)
Hypertension	58.7% (305)	59.9% (197)	0.724	59.1% (502)
Diabetes Mellitus	22.9% (120)	18.7% (62)	0.143	21.0% (184)
Atrial Fibrillation	39.3% (205)	29.6% (97)	0.004*	35.5% (302)
Dyslipidemia	33.1% (173)	32.7% (107)	0.899	33.0% (280)
Coronary disease	20.3% (103)	14.6% (48)	0.037*	18.0% (151)
TOAST				
Cardioembolism	48.9% (242)	45.5% (157)	0.421	47.6% (399)
Large-artery atherosclerosis	22.6% (110)	21.7% (75)		22.1% (185)
Other causes	28.5% (141)	32.8% (113)		30.3% (254)
Proximal occlusion	58.4% (241)	51.7% (109)	0.111	56.1% (360)
Early recanalization (1h tPA bolus)				
Hemorrhagic Transformation (HT)	22.9% (115)	20.9% (74)	0.495	22.1% (189)
Median baseline NIHSS score	16 (0-29)	15 (0-30)	0.262	16 (0-30)
In-hospital mortality	10.5% (55)	10.6% (35)	0.974	10.6% (90)

Table S4

A.1)

Gene	SNP	Genotypes	Minor	HM1		HT-HM2		Cohort A	HM1		HT-HM2		Cohort B	HM1		HT-HM2		Cohort A+B
		HM1 / HT-HM2	Allele	I	W	I	W	p-value	I	W	I	W	p-value	I	W	I	W	p-value
PLA2G7	rs1051931	GG > A-carriers	A	57.7	3.3	51.1	10.4	0.011	55.1	5.9	48.5	9.1	0.374	56.6	4.5	50.0	9.9	0.009
VEGF	rs3025030	GG < C-carriers	C	50.0	6.6	66.0	7.2	0.012	51.6	6.3	53.8	10.8	0.366	50.7	6.5	61.1	8.6	0.015
AGTR1	rs5186	AA > C-carriers	C	63.3	3.6	44.9	9.1	0.0003	52.0	8.7	51.4	5.4	0.474	58.4	5.8	47.7	7.5	0.015
LRP1	rs1800159	GG < A-carriers	A	50.7	6.9	57.6	6.3	0.361	45.2	9.0	58.4	5.4	0.050	48.4	7.8	58.0	5.9	0.032
PLAT	rs2020918	TT < C-carriers	T	43.8	6.3	55.3	6.9	0.261	47.1	17.6	52.9	5.1	0.006	45.9	12.2	54.3	6.1	0.053
MMP8	rs1320632	TT < C-carriers	C	50.9	7.4	67.9	2.6	0.017	51.7	6.3	54.7	11.3	0.311	51.2	6.9	62.6	6.1	0.056
ASB10	rs4722204	TT < A-carriers	A	51.1	10.3	56.8	4.1	0.037	48.6	7.2	55.1	6.5	0.502	50.0	9.0	56.1	5.1	0.063

A.2)

Gene	SNP	Genotypes	Minor	% Dependence Cohort A (n=531)				% Dependence Cohort B (n=354)				% Dependence Cohort A+B (n=885)			
		HM1 / HT-HM2	Allele	HM1	HT-HM2	OR	p-value	HM1	HT-HM2	OR	p-value	HM1	HT-HM2	OR	P-value
PLA2G7	rs1051931	GG < A-carriers	A	60.3	73.8	0.54	0.004	61.3	68.2	0.74	0.243	60.7	71.6	0.61	0.003
GCKR	rs1260326	TT > C-carriers	T	78.5	62.5	2.19	0.007	67.5	63.8	1.18	0.656	74.8	63.1	1.74	0.015
AGTR1	rs5186	AA < C-carriers	C	60.4	71.7	0.60	0.018	62.0	67.5	0.79	0.347	61.0	70.0	0.67	0.015
LPL	rs328	CC > G-carriers	G	69.4	58.3	1.62	0.036	65.9	56.4	1.50	0.188	67.9	57.7	1.56	0.016
PDE4D	rs966221	TT > C-carriers	T	76.3	62.5	1.93	0.021	67.6	63.9	1.18	0.667	73.5	63.1	1.62	0.032
CETP	rs5882	GG > A-carriers	G	80.0	63.9	2.26	0.024	67.9	63.2	1.23	0.629	75.6	63.6	1.78	0.036
ICAM1	rs1799969	GG > A-carriers	A	68.1	50.0	2.13	0.012	66.7	64.9	1.08	0.831	67.6	56.3	1.61	0.039
vWF	rs1063856	GG < A-carriers	G	63.5	66.9	0.86	0.629	43.8	66.8	0.39	0.011	56.0	66.8	0.63	0.050
PG IA	rs1126643	TT < C-carriers	T	54.2	68.1	0.56	0.038	61.2	64.9	0.85	0.628	57.4	66.8	0.67	0.059

B.1)

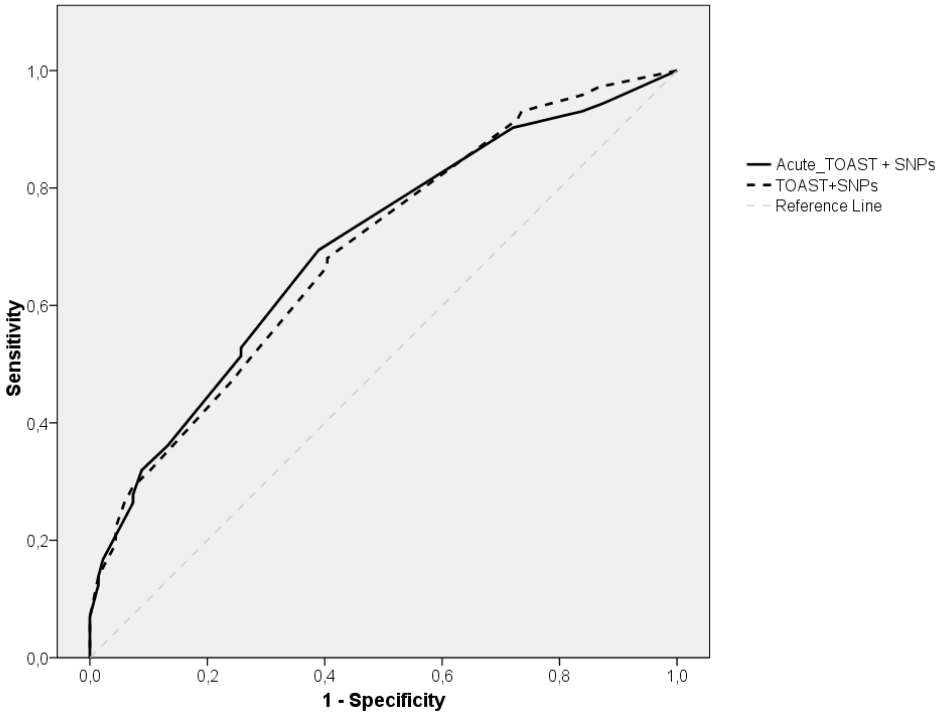
Gene	SNP	Alleles	Minor	Risk		No Risk		Cohort A	Risk		No Risk		Cohort B	Risk		No Risk		Cohort A+B
			Allele	I	W	I	W	p-value	I	W	I	W	p-value	I	W	I	W	p-value
ALOX5AP	rs10507391	A > T	A	53.5	7.6	58.2	5.6	0.345	48.0	7.7	59.4	6.3	0.026	50.9	7.7	58.7	5.9	0.018
NOS2A	rs1137933	C > T	T	49.4	6.5	55.0	6.8	0.360	46.1	4.3	53.9	7.6	0.042	47.9	5.5	54.6	7.1	0.027
PARP1	rs1136410	C > T	C	53.4	6.6	66.7	2.9	0.031	51.0	7.2	57.3	6.7	0.580	52.3	6.9	62.7	4.5	0.031
ASB10	rs4722204	A > T	A	52.4	8.2	58.0	4.1	0.047	51.8	7.6	53.4	5.4	0.575	52.1	7.9	56.0	4.6	0.040

B.2)

Gene	SNP	Alleles	Minor Allele	% Dependence Cohort A (n=531)				% Dependence Cohort B (n=354)				% Dependence Cohort A+B (n=885)			
				Risk	No Risk	OR	p-value	Risk	No Risk	OR	p-value	Risk	No Risk	OR	p-value
PLA2G7	rs1051931	A > G	A	73.2	63.8	1.55	0.016	68.8	62.7	1.31	0.209	71.5	63.3	1.45	0.008
AGTR1	rs5186	C > A	C	71.6	63.9	1.42	0.040	69.0	62.8	1.31	0.187	70.5	63.4	1.38	0.015
LPL	rs328	C > G	G	67.9	57.8	1.55	0.031	64.8	56.9	1.39	0.235	66.7	57.5	1.48	0.017
LRP1	rs11172113	T > C	C	68.5	61.5	1.36	0.043	65.9	61.9	1.19	0.353	67.5	61.6	1.29	0.030
GCKR	rs1260326	T > C	T	69.7	62.6	1.37	0.038	66.2	63.1	1.14	0.470	68.3	62.8	1.28	0.038
MMP1	rs1799750	G > X	G	68.8	62.0	1.35	0.044	65.4	63.1	1.10	0.589	67.4	62.5	1.24	0.060

Figure S1

A)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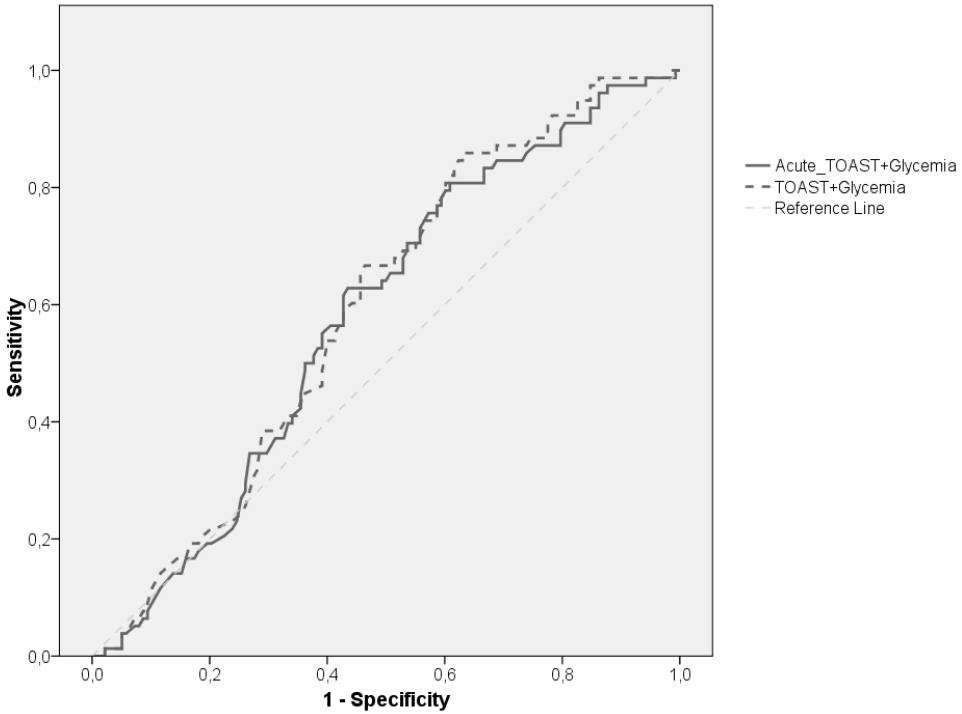
1,0
Acute_TOAST + SNPs
TOAST+SNPs
Reference Line

B)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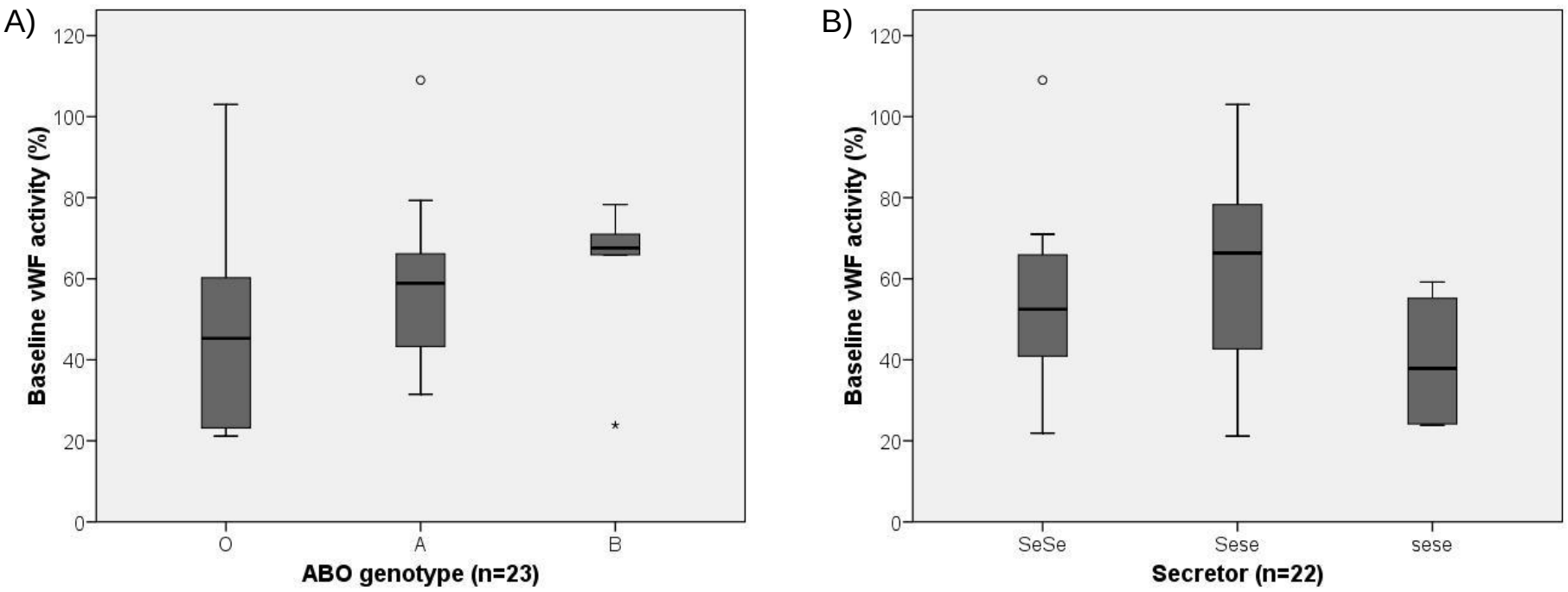
0,4
0,6
0,8
1,0
Acute_TOAST+Glycemia
TOAST+Glycemia
Reference Line

Figure S2


A)
Baseline vWF activity (%)
120
100
80
60
40
20
0
O
A
B
ABO genotype (n=23)
B)
Baseline vWF activity (%)
120
100
80
60
40
20
0
SeSe
Sese
sese
Secretor (n=22)

ARTICLE 4

Submitted

Short title: A2M, F12 and GSTP1 variants influence t-PA safety

Title: A2M, F12 and GSTP1 variants are associated with fibrinolysis safety in ischemic stroke patients

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Word count: **Abstract: 273**

Article: 3517

Tables: 356 / Figures: 217

References: 1138

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Abstract

Background: Great interindividual variability exists for tissue plasminogen activator (t-PA) treatment response in the acute phase of ischemic stroke. Therefore, we explored t-PA pharmacogenetics.

Methods: Our prospective study attempted to genotype 250 single nucleotide polymorphisms from 115 candidate genes by SNPlex in 3 cohorts accounting for 1172 t-PA treated patients, from whom 20.9% developed an hemorrhagic transformation (HT) as evaluated by systematic brain CT scan and 10.6% died. Gene-to-gene interactions were analyzed with Hypothesis-Free Clinical Cloning and predictive models were generated by logistic regression (LR) and classification tree. Functional studies included Real Time-qPCR, nephelometry and Western Blot for alpha-2-macroglobulin (A2M), ELISA for gluthatione S-transferase Pi 1 (GSTP1) and activated partial tromboplastin time measurement for coagulation factor XII (FXII).

Findings: After replication, rs669 in *A2M*, rs1801020 in *F12* and rs1695 in *GSTP1* were associated with HT, while rs1801020 was associated with in-hospital death. Val1000Ile (rs669) for HT prediction and -4C>T (rs1801020) for mortality withstood Bonferroni correction ($p<3.57$ E-4). The LR-based score predicted HT occurrence ($p=9.13$ E-15) and in-hospital mortality ($p=8.7$ E-9) and was validated in an independent cohort. The classification tree identified 4 subgroups with very low (0%), low (7.4%), high (53%) and very high (75%) HT risk, although low sample size precluded its validation. Val1000Ile modified A2M serum levels at baseline and after t-PA infusion, but not mRNA expression or protein structure; -4C>T affected FXII activity both prior and after t-PA and Ile104Val (rs1695) influenced GST-Pi serum levels at baseline and 2h after t-PA bolus.

Interpretation: Three functional polymorphisms were consistently associated with HT occurrence. Our validated LR-based score predicts t-PA safety in the Spanish population.

Funding: Spanish stroke research network, European Stroke Network, Spanish Government, Ramón Areces Foundation.

Keyword list: t-PA, stroke, hemorrhagic transformation, pharmacogenetics

Introduction

Intravenous tissue plasminogen activator (t-PA) is the only currently approved drug for acute ischemic stroke treatment. Hemorrhagic Transformation (HT) occurrence is the most critical complication of thrombolytics and it outweighs treatment benefits when given later than 4.5 hours after symptoms onset, allowing t-PA administration to only 2-14% of stroke patients. Clinical response to thrombolytics may be poor due to lack of early recanalization of the occluded vessel in 55% of subjects [3] or side effects such as symptomatic hemorrhagic transformation in 1.7%-6.4% of cases [1,2] leading to 6.5-12.7% death rates among t-PA treated stroke patients [2].

Nowadays, the clinical predictors available for HT include advanced age, high baseline National Institute of Health Stroke Scale (NIHSS) score and elevated blood pressure [4]. Radiological parameters are diffusion weighted imaging (DWI) lesion volume on Magnetic Resonance Imaging (MRI) and early computed tomography (CT) ischemic changes [4]. Plasma biomarkers are hyperglycemia [4], low platelet count [4], high levels of metalloproteinase 9 (MMP-9) and cellular fibronectin [5], high Activated Protein C [6], high S100B [7] and elevated Vascular Adhesion Protein-1 [8]. Inhibitors of proteases like MMP-9, such as alpha-2-macroglobulin (A2M) would be interesting candidates for HT prediction (see supplemental data for details on protein functions). The only predictive model available is the Hemorrhage After Trombolysis (HAT) score, combining hyperglycemia or diabetes history, baseline NIHSS and early ischemic signs [9].

Our previous studies showed several single nucleotide polymorphisms (SNPs) associated with t-PA response, but only FXIII Val34Leu predicted bleeding complications by altering fibrin density [10]. Subsequently, we searched new SNPs predictors of t-PA safety (HT occurrence or in-hospital death) among candidate pathways (e.g. *PDE4D, ALOX5AP* and *A2M* in stroke risk; *F12* in coagulation and fibrinolysis; *GSTP1, VKORC1* and *CYP11B2* in drug metabolism) in order to generate new predictive models to individualize t-PA therapy.

Methods

1. Study Population

Our target group consisted of consecutive Caucasian patients (the main ethnic group in our geographic area) suffering from an acute ischemic stroke admitted at the emergency room and that received t-PA in a standard 0.9-mg/kg dose (10% bolus, 90% continuous infusion during 1 hour), within the first 4.5 hours after symptoms onset, from which 79.4% were treated earlier than 3 hours.

Cohort A (n=531) was recruited in five Spanish university hospitals between November 2000 and May 2005, Cohort B (n=354) was recruited at the same centers between May 2005 and June 2008. Both cohorts were combined for Hypothesis Free Clinical Cloning (HFCC) analysis and models generation (cohort A+B, n=885) [Figure 1A].

These models were tested in cohort C, recruited between February 2007 and July 2009 from Hospital del Mar and Genotyping RECurrence Of Stroke (GRECOS) study (cohort C: n=287); though all clinical predictors were available only in Hospital del Mar subset (cohort C': n=98) [Figure 1A]. Finally, we extended this model to spontaneous HT in GRECOS non-tPA treated patients (cohort D: n=491) [Figure 1A]. GRECOS study excluded patients with life expectance shorter than 1 year, therefore in-hospital mortality could not be studied in cohorts C and D.

2. Clinical Protocol

HT presence was excluded before t-PA infusion by MRI or CT scan; HT was evaluated in a follow-up CT scan performed 24 hours after symptoms onset or whenever neurological worsening occurred and classified according to ECASS criteria [11]. CT scans were evaluated by neurologists and neuroradiologists experts in neurovascular diseases independently in each center, which could add variability in results obtained particularly in early ischemic signs definition [12]. Radiological and clinical evaluations were blinded mutually to genetic data. This study was approved by the Ethics Committee of each participating center; all patients or relatives gave informed written consent.

3. Genetic analysis

We attempted to genotype 250 SNPs from 115 candidate genes [Supplemental Table S1] related to angiogenesis, coagulation, drugs metabolism, inflammation, myocardial infarction or stroke risk factors, selected by manual bibliographic search in the Pubmed database (e.g "stroke" and "polymorphism"; see supplemental data for details). Among candidate genes, we selected the most studied SNPs or those with a functional effect (an already known modification at transcription, translation or protein activity) or a hypothetic modification due to an aminoacid substitution. Genotyping was carried out at the Spanish National Genotyping Centre (Barcelona, Spain), using SNPlex™ technology and 3730/3730xl DNA analyzer (Applied Biosystems, Foster City, California). The samples were assigned in 90 wells plates with 48 SNPs batches using GeneMapper 3.5 as allele calling algorithm, with a mean call rate of 90%. As quality control, two HapMap samples (NA10860 and NA10861) were included, whose genotype concordance was verified using SNPator (www.snpator.org, CeGen). Besides, SNPlex genotyping accuracy was validated in our series by PCR and direct sequencing [Supplemental Table S2]. Finally, 222 SNPs were successfully genotyped in cohort A, 163 SNPs in cohort B, 159 in both A and B, and 140 of them respected the minimal Minor Allele Frequency criteria (MAF>0.1%) [Figure 1B]. In cohort C', rs669 and rs1801020 were genotyped by PCR and direct sequencing.

4. Functional studies

Sample size of functional studies was determined depending on patients' sample availability (only from Vall d'Hebrón Hospital) and technical constraints (measurement kit size). Groups were similar in genotype frequencies and genotype-phenotype association (except for A2M full sequencing) and HT cases were age and sex-matched with non-HT cases. Alpha-2-macroglobulin (A2M) serum levels were measured by nephelometry in 140 samples both prior and 1h after t-PA bolus. A2M structure and activation was assessed by western blot (WB) in 16 samples, *A2M* transcription was analyzed by RT-qPCR. Coagulation factor XII (FXII) activity was evaluated by measuring activated partial tromboplastin time (APTT) in 110 individuals both prior and 1h after t-PA bolus. GST-Pi serum temporal profile was evaluated by ELISA in 95 samples (baseline and 1h after t-PA bolus) and in 54 samples thereafter. All results were integrated with Ingenuity Pathways (IPA) software.

5. Statistical analysis and model generation

Our sample size was 885 successfully genotyped samples (cohort A+B) with HT prevalence of 22.1% and mortality rate of 10.5%. In this conditions, we calculated the minimum OR to achieve a significant association for a SNP with MAF=0.1, α=0.05 and power of 0.80: the minimum crude OR for HT was 2.02 (95% CI: 1.26 – 3.27). and in-hospital mortality required an OR of 2.40 (95% CI 1.34 – 4.29). There were only 8 (0.009%) missing cases for HT and 10 (0.011%) for in-hospital mortality. Statistical analysis was performed using the SPSS statistical package, version 15.0 (IBM, Chicago, US). Statistical significance for each SNP was assessed by the Chi^2 or Fisher's exact test. Bonferroni correction was applied for the total of SNPs successfully genotyped in both cohorts and with a relevant MAF (140, $p<3.57$ E-4). Nevertheless, if the correction would be done for all SNPs genotyped irrespective of their MAF (159), the uncorrected p-value threshold for significance would not change importantly ($p<3.14$ E-4). Thereafter, we aimed to generate the best predictive model using two statistical approaches:

(1) A predictive score calculated for every subject based on logistic regression (LR) beta coefficients, using a stepwise Forward Likelihood Ratio LR (entry cut-off value 0.05), whose goodness of fit was evaluated by Hosmer-Lemeshow statistic [13]. Afterwards, to establish clinically relevant cut-off values, we automatically categorized this score in several risk groups with the mathematic algorithm Chi-squared Automatic Interaction Detector (CHAID) algorithm included in SPSS. Positive and negative predictive values (PPV and NPV) were calculated for the different cut-off values with a web-based tool (http://araw.mede.uic.edu/cgi-ebm/testcalc.pl).

(2) A classification regression tree (CRT) included in SPSS, that creates a graphical classification model, similar to diagnosis algorithms. CRT divides the cohort in binary groups and evaluates each branch independently from the rest of the tree, whereas logistic regression gives a global score. Moreover, CRT end-point was an ordinal scale for HT severity: No bleeding – Hemorrhagic Infarction (HI) – Parenchymal Hematoma (PH) instead of binary outcome (No HT-HT). As it is recommended that CRT minimal nodes should not be much lower than the square root of the total group (√n, in our case √885=29.75) [14], we considered 50 individuals as the minimum to allow node division and 20 for final nodes.

Receiver Operating Characteristics curves were plotted and predictive capacity was calculated by measuring the area under the curve (AUC). Different AUCs from the same cohort were compared using z-test [15] from MedCalc version 9.2.0.1 (MedCalc Software, Mariakerke, Belgium). Integrated Discrimination

Improvement (IDI) [16] was calculated as an alternative for comparison of discriminating capacity with "rcorrp.cens" function from "hmisc" R-package. This paper was written following STREGA guidelines [17].

Results

1. HT dominant-recessive association study

From the 222 SNPs successfully genotyped in cohort A (n=531), only rs669 (Val1000Ile in *A2M,* stroke-risk pathway) was statistically significant in both cohorts (Cohort A: AA 30.5% HT vs G-carriers 15% HT, p=8.8 E-5; Cohort B AA 25.7% HT vs. G-carriers 15.5% HT, p=0.02) [Table 1A.1]. The variants rs1695 and rs1801020 were associated in the cohort B, while they showed a trend in the cohort A [Table 1A.1]. Repeating analysis according to TOAST ischemic stroke subtypes [18], the strongest association of rs669 was found among cardioembolic etiology, while rs1801020 was significant both in cardioembolic and other etiologies [Supplemental Table S4A].

2. HT additive association study

In the additive model, only rs669 was coincident in cohorts A and B (Cohort A: A-alelle 25.8% HT vs. G-alelle 14.7% HT, p=0.0002; Cohort B: A-alelle 23.1% HT vs. G-allele 14.6% HT, p= 0.0136); variant rs1801020 was significantly associated in cohort B, while it remained as a trend in cohort A [Table 1B.1]. As in dominant-recessive assumption, TOAST stratification with additive model showed rs669 associated in cardioembolic etiology and rs1801020 both in cardioembolic and other etiologies [Supplemental Table S4B].

3. HT clinical and radiological predictors

Univariate analysis in Cohort A + B defined several clinical predictors of HT occurrence: cardioembolic etiology, atrial fibrillation (AF), total anterior cerebral infarction (TACI), proximal occlusion, early ischemic changes, high baseline NIHSS score or glucose levels, current loop diuretics treatment and time from onset to treatment (OTT) [Table 2]. Four of these markers resisted Bonferroni correction for clinical variables ($p<0.002$): cardioembolic etiology, AF, presence of early ischemic changes and high baseline NIHSS score

[Table 2]. Infarct volume measured in diffusion weighted image was also associated in the subgroup of samples where it was available, whereas dyslipidemia, high diastolic blood pressure (DBP) and high body temperature showed a trend ($p<0.1$) with HT occurrence [Table 2]. Both rs669 and rs1801020 were independent of all associated clinical variables, as indicated by logistic regression adjustment (rs669 OR: 1.95 [1.30-2.93] p=0.001; rs1801020 OR: 1.79 [1.07-3.02]; p=0.028), but not rs1695 (rs1695GG OR: 0.52 [0.26-1.15] p=0.111).

4. PH and in-hospital mortality studies

PH occurrence in cohort A+B (n=885) was predicted by rs669 (A=10.8% PH vs. G=7.0% PH p= 0.021) and rs1801020 showed a trend (C=10.8% T=7.0% p=0.054). No association was seen for rs1695 (A-carriers=10.3% PH vs GG=5.3% p=0.128). Regarding in-hospital mortality, rs1801020 was associated in cohorts A and B using a dominant-recessive model (Cohort A: CC 13.9% death vs. T-carriers 4.2% death, p=0.002; Cohort B: CC 14.0% death vs. T-carriers 6.1% death; p=0.03) [Table 1A.2] and additive model (Cohort A: C 12.1% death vs. T 4.5% death, p=0.006; Cohort B: C 12.9% death vs. T 6.1% death, p=0.03) [Table 1B.2]. Neither rs669 nor rs1695 were informative. Univariate analysis followed by LR was performed and rs1801020 was an independent predictor of death (rs1801020 OR 2.37 [1.26-4.45]; p=0.007). Other independent predictors were age, baseline NIHSS, previous stroke, AF and HT occurrence [Supplemental Table S5].

5. HT models generation

The predictive score from the LR model finally included baseline NIHSS score, AF, OTT, DBP, rs669 and rs1801020 in the following formula [Table 3A]: Score=0.053*baseline NIHSS score + 0.534*AF + 0.039* OTT (10 min) + 0.149 * DBP (10mm Hg) + 0.711 * rs1801020 (C allele) + 0.784 * rs669 (A allele). The coefficient for every variable was the beta value of the LR. For example, one patient with AF, DBP= 80mm Hg, baseline NIHSS= 14 and OTT= 180 min and both protective genotypes (rs1801020 TT and rs669 GG) will display a score of 14 * 0.053 + 18 * 0.039 + 0.149 * 8 + 0.534 = 3.17 (classified in G0, low risk group). The same clinical presentation but with both at-risk genotypes will have a score of 3.17 + 2 * 0.711 + 2 * 0.784 = 6.16 (G3, highest risk group). Therefore, only changes in both genotypes may reclassify a patient from the low to the highest risk group [Figure 2C].

This combined genetic and clinical model had higher predictive capacity than the previously reported HAT score [9] (0.720 vs. 0.603; p=0.0044); while the 4 clinical variables alone did not (0.654 vs. 0.603; p=0.222) [Table 3B]. As shown by AUC values, neither genetic data nor clinical data alone, but their combination, increased significantly the predictive capacity compared to the HAT score. The same relationship between models (Clinical-Genetic > 4 clinical variables > HAT score) was observed for patients treated off-label the SITS-MOST criteria and in the independent cohort C', though AUC differences were not significant [Table 3B]. Alternatively, when we calculated IDI for cohort A+B, Clinical-Genetic score improved discrimination over the other models: Clinical-Genetic vs 4 clinical variables = 5.13% (CI95% 3.41 – 6.84) and Clinical-Genetic vs HAT score = 6.04% (CI95% 3.07-9.01). This difference was further increased in cohort C': Clinical-Genetic vs 4 clinical variables = 11.37% (CI95% 3.51 – 19.22) and Clinical-Genetic vs HAT score = 14.74% (CI95% 5.30-24.18).

LR-based score was significantly increased in HT group in the Cohort A+B and in the independent cohort C, even without considering OTT and DBP, though the effect was higher in cohort C' with all clinical data available [Figure 2B]. A similar effect was observed in cohort D for non t-PA treated patients [Figure 2B].

To establish clinically useful cut-off values, LR score was stratified with CHAID algorithm in 4 groups of risk with increasing rates of HT (4.4% to 54%; p=9.13 E-15), PH (2.0% to 32.9%; p=1.49 E-10), in-hospital mortality (1.5% to 29.9%; p=8.7 E-9) and modified Rankin Scale (mRS) score at third month (p=1.81 E-10) [Figure 2C.1-3]. These groups were similar in cohort C': HT (10% to 43%; p=0.058), PH (10% to 28.6%; p=0.241), in-hospital mortality (0% to 42.9%; p=0.039) and mRS (p=0.064) [Figure 2C.4-6]. If remote PHs were excluded, the association was significant both for HT (0% to 43%; p=0.003) and PH occurrence (0% to 28.6%; p=0.025).

The lowest score cut-off value of 3.95 (G0 vs G1-G2-G3) had great sensitivity (0.98) but low specificity (0.12) for HT prediction, whereas the highest cut-off of 6.10 (G3 vs G0-G1-G2) had great specificity (0.94) but low sensitivity (0.25) [Table 3C]. As a result, PPV increased progressively from 0.24 (CI95% 0.23-0.25) obtained with the 3.95 cut-off to 0.54 (CI95% 0.43-0.65) with the 6.10 cut-off. NPV decreased from 0.96 (CI95% 0.87-0.99) obtained with the 3.95 cut-off to 0.82 (CI95% 0.80-0.83) with the 6.10 cut-off [Table 3C]. Using CRT, we identified 4 subgroups of interest [Supplemental Figure S3] but we were unable to formally validate CRT results due to sample size limitations. However, it is noticeable that the AUC was 0.734 (CI95% 0.695-0.772), slightly higher than our LR model.

6. Functional studies

To determine rs669 functionality, 15 individuals were fully sequenced for A2M without finding other variants in stronger linkage with HT [Supplemental Figure S1A.1]; no variants were detected in the thiol-ester domain in 73 patients and the Cys972Phe variant, reported to cause A2M dysfunction [19], was not found in 177 individuals. We found a significant association between rs669 "A" at-risk allele and higher A2M baseline serum levels in 140 patients (p=0.016) [Figure 3A.1], but not between A2M serum levels and HT (p=0.906). Similar results were obtained at the end of t-PA infusion for rs669 (p=0.007) [Figure 3B.1] and HT occurrence (p=0.318). Complementary analysis involving WB and mRNA did not show significant variations (see supplemental data).

Regarding rs1801020, no other variants near *F12* transcriptional or translation start site were found in stronger linkage with HT occurrence in 80 individuals [Supplemental Figure S1A.2]. We found a significant association between "C" risk allele and high baseline FXII activity in 110 individuals (p=0.002) [Figure 3A.2], but not between FXII activity and HT (p=0.519) or mortality (p=0.701). Similar results were obtained 1h after t-PA bolus for rs1801020 (p=0.008) [Figure 3B.2], bleeding (p=0.820) or mortality (p=0.369).

We found an association between rs1695 and GST-Pi plasmatic levels at baseline (p=0.032) [Figure 3A.3], but it disappeared 1h after t-PA bolus (p=0.299). No association was found in both time points with HT occurrence (p=0.551 baseline; p=0.607 at 1h). As no effect was found at the end of t-PA infusion, we checked later time points and an association was found 2h after t-PA bolus (p=0.023) [Figure 3B.3].

Discussion

The present study found rs669 and rs1801020 associated with HT occurrence after t-PA, which influenced their protein levels or activity. The best HT genetic marker was rs669; rs1801020 and rs1695 showed weaker associations but rs1801020 was an independent predictor of in-hospital death. Other clinical (baseline NIHSS score, TACI, cardioembolic etiology, AF, OTT and diuretic treatment), biochemical (glucose levels) and radiological markers (early ischemic signs or baseline DWI volume) were confirmed as HT predictors in the Spanish population.

The LR-based score generated with rs669 and rs1801020, the first integrative model of clinical and genetic data, significantly improved AUC versus HAT score [9]. Furthermore, score stratification in risk groups

predicted HT (ranging from 4.4% to 54.4%) and PH (2.9% to 32.8%) occurrence and in-hospital mortality (1.5% to 29.9%) and was validated in an independent cohort. Notably, the highest risk group (G3) doubled the global HT rates and triplicated the PH and death frequencies. This was accomplished because rs1801020, baseline NIHSS and presence of AF were independent death predictors in addition to their association with HT occurrence. The present highest PPV of 0.54 (for cut-off 6.10) might not avoid fibrinolytic treatment and the highest NPV of 0.96 (for cut-off 3.95) does not exclude HT occurrence. New items must be added before its usefulness in clinical routine: new clinical, radiological or biochemical markers or new variants coming from a GWAS or a deep-sequencing approach. Once the predictive capacity is high enough, the score could be rounded to facilitate its implementation (see supplemental data).

The CRT model generated groups of risk that showed higher rates of HT or its complete absence, but these groups were too small to be consistently replicated in Cohort C'. Nowadays, the LR-model and the risk groups generated are easier to implement in the clinical practice. This LR-based score could help to define accurately risk/benefit and individualize t-PA therapy, as current DNA extraction platforms (e.g. EZ-1 Qiagen robot; 20 minutes) combined with fast genotyping devices (e.g. MOMO-fast Real Time; 30 minutes) would permit its use during the median door-to-needle time of 60 minutes. Besides, these genes are potential therapeutic targets to increase the benefit of t-PA administration.

The A2M variant, rs669 (Val1000Ile), is not conserved during evolution [Supplemental Figure S1B] and its functionality is doubtful, but the A2M gene was fully sequenced and no other variant was more informative. High A2M levels in cerebrospinal fluid (CSF) or A2M CSF/serum ratio are classical blood brain barrier (BBB) disruption markers and iatrogenic mannitol BBB disruption caused an increase of A2M serum levels within 6h after the procedure [20]. In the present study, the "A" at-risk allele for HT was associated with higher A2M levels both baseline and after t-PA infusion, in contrast to Poller *et al.* [19]. This increase in protein levels was not due to preferential transcription, as *A2M* mRNA levels do not vary significantly in blood. Besides, no changes in disulfide bonding were observed by WB.

We have found an association between rs1801020 and FXII activity confirming previous studies [21]. The effect of rs1801020 may be exacerbated after t-PA infusion, as mean FXII activity increased after t-PA or tenecteplase infusion in myocardial infarction patients [22,23]. High FXII levels may cause brain edema, as increased FXII activity lead to angioedema type III [24], pharmacological doses of t-PA lead to bradykinin production "in vitro" [25], and bradykinin causes brain edema in both rat and mice MCAo models [26]. Accordingly, the main cause of death in our series was malignant edema (see supplemental data) and CT

evidence of edema predicted symptomatic HT in the NINDS trial [1]. FXII and kinin pathway have been involved also in orolingual edema [27], another t-PA complication that could be independently caused by Angiotensin Converting Enzyme (ACE) inhibitors [28]. We measured F12 coagulation capacity and F12 fragments (F12f) loose this function, while retaining kinin and complement activation. Maybe F12f levels or activation of these latter pathways lead to edema formation and would be statistically associated to in-hospital death. Alternatively, high FXII levels may alter monocyte and macrophages behavior [29].

GST-Pi CSF levels are markers of brain damage [30] and are increased in the infarct core [31]. GSTP-Pi serum levels correlate with both functional outcome (as indicated by mRS scale) and mortality 3 months after stroke (N. Turck, unpublished data, 2010). Interestingly, GST-Pi has a protective role against oxidative stress-induced caspase-mediated apoptosis [32], so it may influence ischemic vulnerability. In our series, GSTP1 “A-carriers” (Ile-carriers) were associated with lower GST-Pi levels and it may account for lower protective activity.

In summary, rs669 and rs1801020 are consistently associated with t-PA safety and withstand Bonferroni correction ($p<3.57$ E-4) for HT and in-hospital mortality. Both proteins are involved in fibrinolysis, kallikrein and kinin pathways (see supplemental data). Response to t-PA also seems modulated by rs1695. The rs1801020 would increase circulating FXII levels and FXII activity. The rs1695 would change GST-Pi levels and substrate specificity. The rs669 would influence A2M levels but not mRNA expression or protein structure; it could modify A2M activity, since HT patients had lower ratio or cleaved/uncleaved A2M monomer prior to t-PA infusion, an effect mirrored by rs669 genotypes.

Changes at the protein level did not correlate with HT occurrence at baseline, the association may be reached at later time points. The calculation of HT risk could allow clinicians to individualize fibrinolytic treatment, increasing the therapeutic window for low-risk patients and define more accurately t-PA risk-benefit in high-risk individuals. This study might be the first step in the development of personalized medicine and new coadjuvant treatments for t-PA.

Acknowledgments

We are deeply grateful to all patients and relatives, neurologists and nurses of the Stroke Unit from the participant Hospitals for their contributions. We are also grateful for excellent technical support from A. Penalba, L. Ortega-Torres, S. García-Menéndez and the National Center for Genotyping (CeGen). We would also like to thank A. Rosell and T. García-Berrocoso for their excellent technical advice and G. Lucas and I. Subirana for statistical counseling.

Sources of Funding

This study is part of the Geno-tPA project. The Neurovascular Research Laboratory takes part in the International Stroke Genetics Consortium and coordinates the Spanish Stroke Genetics Consortium (www.genestroke.com). The Neurovascular Research Laboratory receives grants from the Spanish stroke research network (RENEVAS, RD06/0026/0010) and the European Stroke Network (EUSTROKE 7FP Health F2-08-202213). This study was funded by a grant of the Spanish government (FIS PJ060586) and a grant from Ramón Areces Foundation (2006). A. del R.E. receives a PhD grant from the Vall d'Hebron Institute of Research.

Disclosures

None to declare.

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Table 1: Assuming a dominant-recessive model **(A)** or an additive model **(B)**, genetic association study for HT **(1)** and in-hospital mortality **(2)**. Only variants with p-value<0.1 in Cohort A+B are shown, defined by their reference sequence number (rs). Minor alleles are indicated based on Hapmap-CEU population. In genotypic tables **(A)**, the homozygous selected (HM1) and the other genotypes (HT-HM2) are indicated, while in additive tables **(B)** both alleles are presented. Mathematical symbols (< or >) indicate the at-risk genotypes **(A)** or alleles **(B)**. Frequencies of HT or death for each genotype group are indicated as a percentage, followed by their crude Odds Ratio (OR) and the Chi^2 p-value. *=p<0.05 in both A and B cohorts; †=p<0.1 in both A and B cohorts.

Table 2: Univariate analysis for other predictors of HT occurrence with categorical **(A)** and continuous **(B)** variables. **(A)** For categorical variables, frequencies of HT (as a percentage) in presence or each absence of risk factor are indicated, with number of cases in brackets and followed by their Chi^2 p-value. **(B)** For continuous variables, mean or median values for HT and non-HT groups were calculated; parametric variables are indicated as mean ± Standard Deviation (S.D.) followed by t-test p-value while non-parametric as median and Interquartilic Range (IR) followed by Mann-Whitney p-values. ACE-I=Angiotensin Converting Enzyme Inhibitors, ARB-II= Angiotensin II Receptor Blockers, TCD=Transcranial Doppler, TOAST= Trial of ORG 10172 in Acute Stroke Treatment, OCSP= Oxfordshire Stroke Project Classication, SBP=Systolic blood pressure, DBP=diastolic blood pressure, NIHSS= National Institute of Health stroke scale, OTT=time from onset to treatment. *=p<0.05 in both cohorts; †=p<0.1 in both cohorts.

Table 3: Predictive model of HT occurrence. **(A)** Parameters finally included in the logistic regression, the score is calculated using the beta coefficients. Odds Ratio (OR) and Standard Error (SE) for each item are indicated. NIHSS= National Institute of Health stroke scale, DBP=diastolic blood pressure, OTT=time from onset to treatment. **(B)** Area Under the Curve (AUC) and goodness of fit for clinical and genetics score (CG), only 4 clinical variables and HAT score with Confidence Interval at 95% (CI95%). AUC were calculated in cohort A+B, the A+B subgroup treated out of SIST-MOST criteria and the independent cohort C'. **(C)** To determine its clinical usefulness, we calculated sensitivity (SN), specificity (SP) Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of the cut-off values defined by CHAID algorithm: 3.95 (G0 vs G1-G2-G3), 5.10 (G0-G1 vs G2-G3) and 6.10 (G3 vs G0-G1-G2).

Table 1

A.1)

Gene	SNP	Genotypes HM1 / HT-HM2	Minor Allele	%HT Cohort A (n=531) HM1	HT-HM2	OR	p-value	%HT Cohort B (n=354) HM1	HT-HM2	OR	p-value	%HT Cohort A+B (n=885) HM1	HT-HM2	OR	p-value
A2M	rs669	AA > G-carriers	G	30.5	15.0	2.48	8.89E-05	25.7	15.5	1.89	0.020	28.4	15.2	2.21	7.44E-06*****
GSTP1	rs1801020	CC > T-carriers	T	25.0	18.1	1.51	0.102	24.9	12.9	2.23	0.011	25.0	15.8	1.78	0.003
F12	rs1695	GG < A-carriers	G	12.7	23.8	0.47	0.065	7.7	23.4	0.27	0.025	10.6	23.6	0.38	0.004**†**
VWF	rs1063856	GG < A-carriers	G	10.0	24.9	0.33	0.010	13.2	21.9	0.54	0.214	11.2	23.6	0.41	0.006
CYP11B2	rs1799998	TT > C-carriers	C	30.6	19.1	1.87	0.009	23.9	19.0	1.33	0.306	27.5	19.1	1.61	0.009
MCP1	rs1024611	TT < C-carriers	C	21.5	24.2	0.86	0.507	15.6	27.9	0.48	0.006	18.9	25.8	0.67	0.022
VEGFA	rs3025042	GG < T-carriers	T	22.9	37.5	0.49	0.331	20.4	60.0	0.17	0.031	21.8	46.2	0.33	0.047
VEGFA	rs3025000	CC < T-carriers	T	22.1	23.7	0.91	0.684	15.3	25.3	0.53	0.025	19.3	24.4	0.74	0.087

A.2)

Gene	SNP	Genotypes HM1 / HT-HM2	Minor Allele	Death% Cohort A (n=531) HM1	HT-HM2	OR	p-value	Death% Cohort B (n=354) HM1	HT-HM2	OR	p-value	Death% Cohort A+B (n=885) HM1	HT-HM2	OR	p-value
F12	rs1801020	CC > T-carriers	T	13.9	4.2	3.69	0.002	14.0	6.1	2.52	0.030	14.0	5.0	3.06	1.85E-04*****
LOX1	rs11053646	CC < G-carriers	G	9.8	16.1	0.57	0.158	9.3	25.0	0.31	0.007	9.6	19.3	0.44	0.006
IL1B	rs1143627	TT > C-carriers	C	14.0	7.8	1.92	0.036	13.5	8.6	1.67	0.151	13.8	8.1	1.80	0.012
PLA2G7	rs1051931	GG < A-carriers	A	6.8	15.0	0.41	0.005	9.8	11.6	0.83	0.610	8.1	13.5	0.56	0.015
IFNG	rs2430561	AA > T-carriers	A	17.0	8.9	2.10	0.026	14.7	10.0	1.54	0.261	16.0	9.4	1.83	0.016
CRP	rs1205	CC < T-carriers	T	6.7	13.0	0.48	0.029	9.0	12.7	0.68	0.281	7.8	12.9	0.57	0.020
VEGF	rs3025033	TT < C-carriers	C	10.6	11.6	0.90	0.758	8.3	19.7	0.37	0.005	9.6	14.9	0.61	0.042
ADD1	rs4961	GG > T-carriers	T	11.3	9.4	1.22	0.548	13.5	5.7	2.57	0.035	12.3	7.9	1.63	0.066

B.1)

Gene	SNP	Alleles	Minor Allele	%HT Cohort A (n=531) Risk	No Risk	OR	p-value	%HT Cohort B (n=354) Risk	No Risk	OR	p-value	%HT Cohort A+B (n=885) Risk	No Risk	OR	p-value
A2M	rs669	A > G	G	25.8	14.7	2.09	0.0002	23.1	14.6	1.75	0.014	24.7	14.6	1.91	1.00E-05*****
F12	rs1801020	C > T	T	23.9	17.3	1.43	0.074	23.0	11.5	2.30	0.004	23.5	14.6	1.80	0.001**†**
MCP1	rs1024611	T > C	C	24.2	22.1	1.06	0.529	29.1	18.2	1.84	0.003	26.3	20.4	1.39	0.015
APOB	rs1367117	A > G	A	24.6	18.5	1.45	0.049	21.6	17.2	1.32	0.208	23.3	17.9	1.39	0.021
VEGFA	rs3025042	G > T	T	37.5	23.0	2.06	0.334	60.0	20.7	5.76	0.031	46.2	22.0	3.04	0.047
IL1A	rs1800587	T > C	T	23.2	20.6	1.11	0.429	22.8	15.1	1.66	0.039	23.0	18.3	1.33	0.055
VEGFA	rs3025000	C > T	T	23.9	22.5	1.06	0.644	26.4	17.9	1.64	0.014	25.0	20.6	1.29	0.056
SERPINE1	rs7242	T > G	G	22.7	21.0	1.12	0.558	24.9	17.8	1.54	0.025	23.7	19.6	1.27	0.057
ADIPOQ	rs2241766	G > T	G	22.8	20.4	1.10	0.544	22.3	14.0	1.76	0.047	22.6	17.5	1.37	0.075

B.2)

Gene	SNP	Alleles	Minor Allele	Death% Cohort A (n=531) Risk	No Risk	OR	p-value	Death% Cohort B (n=354) Risk	No Risk	OR	p-value	Death% Cohort A+B (n=885) Risk	No Risk	OR	p-value
F12	rs1801020	C > T	T	12.1	4.5	2.58	0.006	12.9	6.1	2.27	0.030	12.4	5.2	2.56	4.84E-04*****
LOX1	rs11053646	C > G	G	15.5	10.3	1.52	0.212	26.5	10.3	3.14	0.003	19.6	10.3	2.12	0.006
F8	rs1800291	G > C	G	10.6	8.1	1.35	0.365	11.9	3.0	4.41	0.027	11.2	6.4	1.85	0.038
GJA1	rs17653265	C > T	T	16.7	13.9	1.30	0.845	33.3	10.9	4.07	0.035	26.7	12.0	2.66	0.086
VCAM1	rs1041163	C > T	C	15.0	9.4	1.55	0.038	11.3	10.7	1.06	0.859	13.4	10.0	1.39	0.098

Table 2

A)

	HT% Cohort A (n=531)			HT% Cohort B (n=354)			HT% Cohort A+B (n=885)		
	Present	Absent	p-value	Present	Absent	p-value	Present	Absent	p-value
Vascular Risk Factors									
Gender (female)	25.8% (60)	20.4% (57)	0.147	23.7% (37)	19.0% (37)	0.279	24.5% (94)	20.2% (94)	0.131
Smoking habit	17.1% (22)	24.1% (89)	0.077	23.5% (16)	20.8% (58)	0.621	19.3% (38)	23.0% (144)	0.269
Hypertension	22.3% (66)	23.7% (50)	0.712	20.4% (43)	21.7% (30)	0.760	21.6% (107)	23.0% (79)	0.633
Diabetes mellitus	23.9% (28)	22.4% (88)	0.727	21.9% (14)	20.9% (60)	0.864	24.2% (43)	21.6% (144)	0.463
Dyslipidemia	17.3% (29)	25.5% (87)	0.037	20.0% (22)	21.6% (51)	0.732	18.8% (52)	23.8% (134)	0.099
Atrial fibrillation	29.0% (58)	19.1% (59)	0.009	29.4% (30)	17.5% (43)	0.013	28.6% (85)	18.8% (102)	0.001*
Heart disease	23.8% (24)	22.1% (87)	0.718	22.2% (12)	21.0% (62)	0.842	24.3% (36)	21.5% (146)	0.447
Previous stroke	23.9% (16)	21.8% (95)	0.708	21.6% (8)	21.2% (66)	0.948	23.0% (23)	21.6% (159)	0.756
Previous Treatments									
Antiplatelets	22.0% (35)	22.6% (74)	0.878	19.3% (23)	21.8% (49)	0.595	21.9% (59)	21.8% (119)	0.975
Statins	19.7% (14)	25.0% (72)	0.350	26.5% (9)	20.5% (25)	0.455	23.1% (24)	23.5% (93)	0.920
Oral antidiabetics	21.3% (10)	23.6% (63)	0.729	31.8% (7)	20.3% (26)	0.266	26.9% (18)	22.2% (86)	0.404
Diuretics	30.6% (30)	22.4% (52)	0.115	31.1% (14)	19.0% (19)	0.108	31.9% (44)	21.3% (68)	0.015
ACE-I	27.8% (20)	22.8% (63)	0.380	22.2% (6)	22.9% (27)	0.941	26.6% (25)	23.0% (88)	0.460
ARB-II	15.4% (4)	24.6% (78)	0.289	18.8% (3)	23.3% (30)	1.000	19.0% (8)	24.2% (104)	0.455
Radiological study									
Early ischemic signs	28.6% (48)	16.2% (42)	0.002	28.7% (27)	17.0% (38)	0.018	28.8% (76)	16.6% (79)	1.00E-04*
Clinical parameters									
Proximal occlusion (baseline TCD)	28.9% (69)	20.3% (35)	0.050	24.8% (30)	17.3% (19)	0.163	27.5% (94)	19.4% (53)	0.020
Cardioembolism (TOAST)	29.8% (73)	15.5% (40)	1,24E-04	28.0% (44)	16.0% (30)	0.007	29.1% (115)	15.9% (69)	4.43E-06*
TACI (OCSP)	26.0% (76)	18.6% (36)	0.055	24.9% (54)	15.2% (20)	0.031	25.7% (126)	17.0% (54)	0.004†
End-points									
Hemorrhagic Transformation (HT)		22.9% (115)			21.1% (74)			22.1% (189)	
Hemorrhagic Infarction (HI)		13.2% (65)			12.0% (42)			12.5% (107)	
Parenchymal Hematoma (PH)		9.8% (50)			9.1% (32)			9.6% (82)	
In-hospital Mortality		10.5% (53)			10.5% (37)			10.6% (90)	

B)

	Cohort A (n=531)			Cohort B (n=354)			Cohort A+B (n=885)		
	HT	None	p-value	HT	None	p-value	HT	None	p-value
Age (years, mean±S.D.)	71.2 ± 12.3	70.5 ± 11.8	0.623	72.0 ± 10.5	70.0 ± 12.0	0.238	71.7 ± 10.9	70.5 ± 12.2	0.199
SBP (mm, mean±S.D.)	152.0 ± 26.5	153.6 ± 26.0	0.587	154.9 ± 29.7	154.1 ± 26.4	0.513	153.2 ± 28.4	153.8 ± 25.5	0.842
DBP (mm, mean±S.D.)	86.1 ± 16.9	82.6 ± 15.3	0.048	83.8 ± 15.5	82.6 ± 15.1	0.833	85.1 ± 15.8	82.4 ± 15.9	0.056
Baseline NIHSS (median, IR)	18 (13-20)	15 (9-19)	0.001	17.5 (12-20)	14 (8-19)	0.069	18 (12-20)	15 (9-19)	5.5E-05†
Body temperature (ºC, median, IR)	36.4 (36.0-36.6)	36.2 (36.0-36.6)	0.029	36.0 (35.7-36.5)	36.0 (35.9-36.4)	0.869	36.3 (36.0-36.5)	36.1 (36.0-36.5)	0.074
Glycemia (mg/dl, median, IR)	132 (109-168.5)	117 (101-143)	0.001	128 (104-161)	122 (101.5-153.5)	0.357	128 (106-163)	119 (102-148)	0.002
OTT (min, median, IR)	169 (135-192.5)	150 (120-180)	0.004	162 (120-180)	150 (120-180)	0.416	165 (130-180)	150 (120-180)	0.012

Table 3

3A)

Variables in the RL Score	Beta	SE	p-value	adj. OR	95% C.I. for adj. OR	
					Lower	Upper
Baseline NIHSS	0.053	0.017	2.06E-03	1.054	1.019	1.091
Atrial Fibrillation	0.534	0.198	6.92E-03	1.705	1.158	2.512
DBP (10mm)	0.149	0.060	1.24E-02	1.161	1.033	1.305
OTT (10min)	0.039	0.016	1.35E-02	1.039	1.008	1.072
rs1801020 (C allele)	0.711	0.199	3.62E-04	2.036	1.378	3.010
rs669 (A allele)	0.784	0.170	3.99E-06	2.190	1.569	3.056

3B)

		AUC	SE	p-value	95% C.I. for AUC		Hosmer Lemeshow
					Lower Bound	Upper Bound	
Cohort A+B (n=683)	CG score	0.720	0.028	3.20E-12	0.666	0.775	0.249
	4 Clinical variables	0.654	0.029	1.06E-06	0.598	0.711	0.286
	HAT score	0.603	0.030	1.09E-03	0.544	0.662	0.218
Off-label t-PA (A+B)' (n=300)	CG score	0.651	0.038	1.10E-04	0.576	0.726	0.500
	4 Clinical variables	0.628	0.037	1.01E-03	0.555	0.702	0.522
	HAT score	0.614	0.041	1.89E-03	0.534	0.694	0.503
Cohort C' (n=61)	CG score	0.730	0.082	1.13E-02	0.569	0.891	0.349
	4 Clinical variables	0.692	0.080	3.41E-02	0.536	0.849	0.960
	HAT score	0.688	0.080	3.80E-02	0.532	0.845	0.989

3C)

	G0	cut-off 3.95 (G0 vs G1-G2-G3)				G1	cut-off 5.10 (G0-G1 vs G2-G3)				G2	cut-off 6.10 (G3 vs G0-G1-G2)				G3
	% HT (n)	SN	SP	PPV 95%CI	NPV 95%CI	% HT (n)	SN	SP	PPV 95%CI	NPV 95%CI	% HT (n)	SN	SP	PPV 95%CI	NPV 95%CI	% HT (n)
Cohort A+B (n= 683)	4.4% (68)	0.98	0.12	0.24 (0.23-0.25)	0.96 (0.87-0.99)	13.8% (276)	0.73	0.57	0.33 (0.30-0.36)	0.88 (0.85-0.91)	27.0% (270)	0.25	0.94	0.54 (0.43-0.65)	0.82 (0.80-0.83)	54.4% (68)
Off-label t-PA (A+B)' (n=300)	5.6% (18)	0.99	0.07	0.25 (0.24-0.26)	0.94 (0.70-0.99)	18.6% (97)	0.74	0.42	0.29 (0.25-0.32)	0.83 (0.77-0.89)	21.6% (139)	0.32	0.90	0.50 (0.37-0.63)	0.81 (0.78-0.83)	50.0% (46)
Cohort C' (n=61)	10.0% (10)	0.92	0.19	0.24 (0.20-0.27)	0.90 (0.56-0.98)	8.3% (24)	0.77	0.65	0.37 (0.27-0.49)	0.91 (0.79-0.97)	35.0% (20)	0.23	0.92	0.43 (0.16-0.75)	0.81 (0.76-0.86)	42.9% (7)

Figure 1: Workflow diagram of the study. **(A)** Patients workflow: recruitment dates and sample size (n) are indicated in each cohort. QC (Quality control) indicates the final sample size in the analysis, after removing cases missing, some study variables and extreme outliers. The four steps of the current work are indicated. LR=Logistic Regression, CRT=Classification Regression Tree, RT-qPCR= Real Time quantitative PCR, WB=Western Blot, APTT=Activated Partial Thromboplastin Time **(B)** SNPs workflow: genotypes successful in each cohort are indicated, as well as those with low number of samples genotyped (low call rate) or none (failed). MAF=Minor Allele Frequency.

Figure 2: Predictive capacities of the different models plotted by their areas under the ROC curve in cohort A+B **(A.1)** and Cohort C**'** **(A.2)**. Boxplot of CG (clinical-genetics) score in HT and non-HT groups for cohort A+B **(B.1)**, Cohort C' **(B.2)**; and the incomplete CG4 (without OTT and DBP data) in Cohort C **(B.3)** and Cohort D **(B.4)**. *=p<0.05, ‡=p<E-8. **(C)** Stratification using score-based groups (G0 s≤3.95, G1 3.95<s≤5.10, G2 5.10<s≤6.10, G3 s>6.10) for presence of bleeding **(1,4)**, in-hospital mortality **(2,5)** and 3rd month mRS **(3,6)** in cohort A+B **(1,2,3)** or cohort C' **(4,5,6)**.

Figure 3: Protein determinations prior **(A)** or after t-PA infusion **(B)** classified by genotypes. **(1)** A2M plasmatic levels (g/l), classified by genotypes **(A.1 and B.1)**. **(2)** FXII activity classified by rs1801020 genotypes **(A.2 and B.2)**. Results are indicated as a percentage of an external calibrator plasma pool. **(3)** GST-Pi plasmatic levels (ng/ml) classified by genotypes **(A.3 and B.3)**. *=p<0.05; #=p<0.1 after Bonferroni correction.

Figure 1

(A)

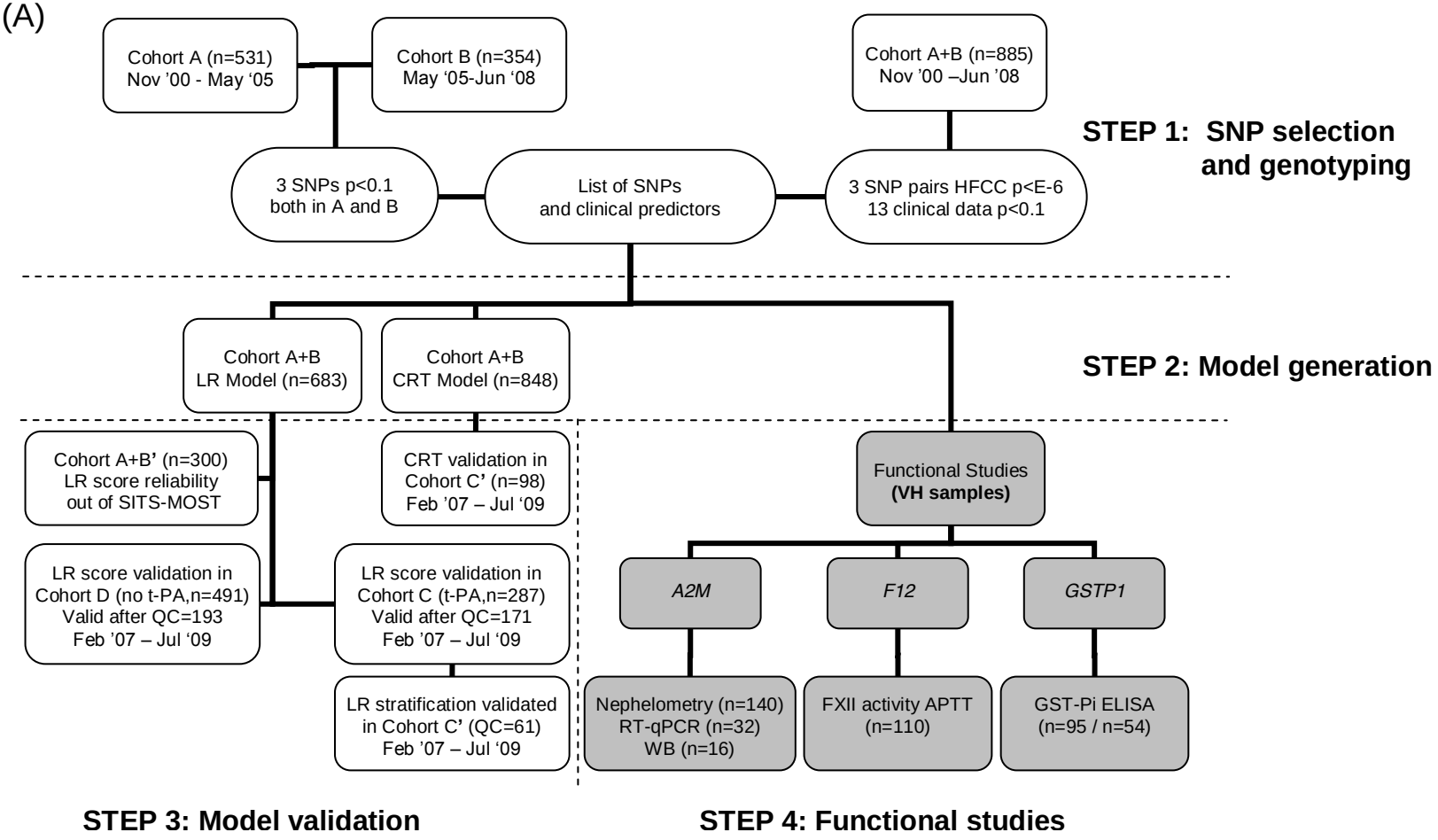

Cohort A (n=531)
Nov '00 - May '05
Cohort B (n=354)
May '05-Jun '08
Cohort A+B (n=885)
Nov '00 –Jun '08
STEP 1: SNP selection and genotyping
3 SNPs p<0.1
both in A and B
List of SNPs
and clinical predictors
3 SNP pairs HFCC p<E-6
13 clinical data p<0.1
Cohort A+B
LR Model (n=683)
Cohort A+B
CRT Model (n=848)
STEP 2: Model generation
Cohort A+B' (n=300)
LR score reliability
out of SITS-MOST
CRT validation in
Cohort C' (n=98)
Feb '07 – Jul '09
Functional Studies
(VH samples)
LR score validation in
Cohort D (no t-PA,n=491)
Valid after QC=193
Feb '07 – Jul '09
LR score validation in
Cohort C (t-PA,n=287)
Valid after QC=171
Feb '07 – Jul '09
A2M
F12
GSTP1
LR stratification validated
in Cohort C' (QC=61)
Feb '07 – Jul '09
Nephelometry (n=140)
RT-qPCR (n=32)
WB (n=16)
FXII activity APTT
(n=110)
GST-Pi ELISA
(n=95 / n=54)
STEP 3: Model validation
STEP 4: Functional studies

Figure 1

(B)

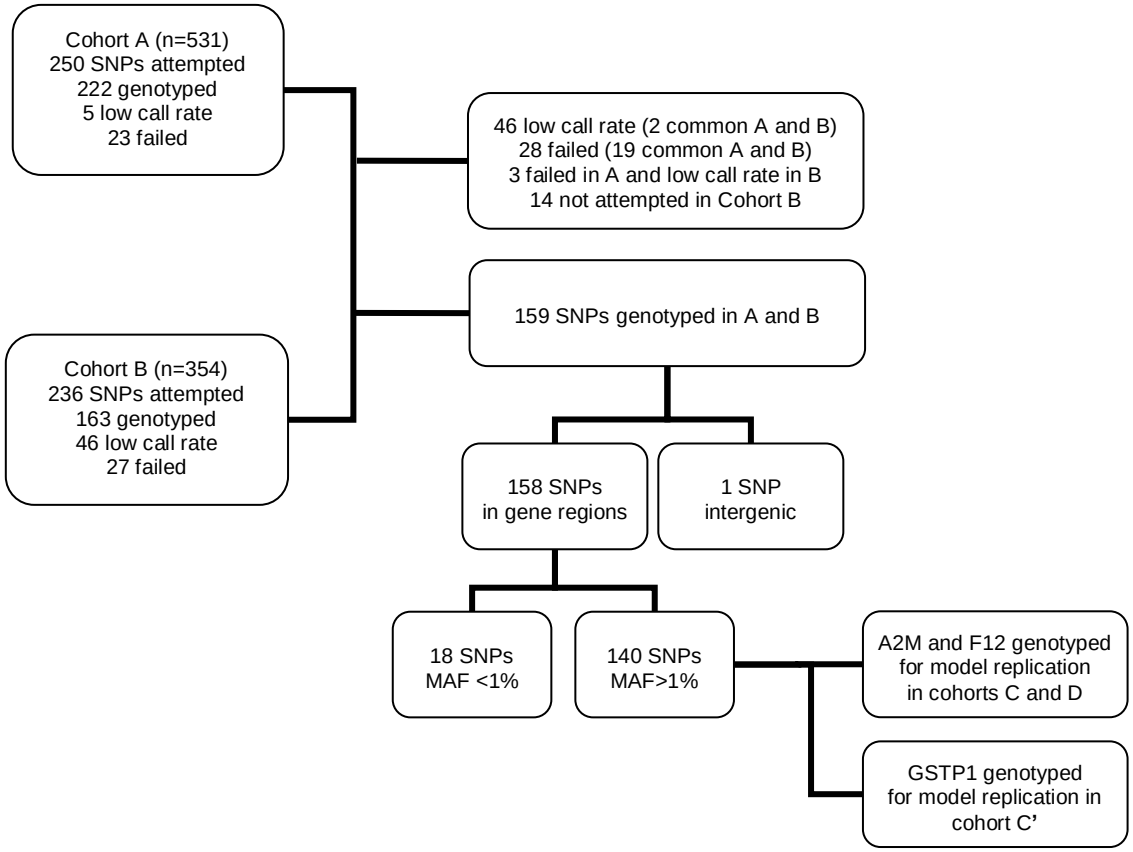

Cohort A (n=531)
250 SNPs attempted
222 genotyped
5 low call rate
23 failed
46 low call rate (2 common A and B)
28 failed (19 common A and B)
3 failed in A and low call rate in B
14 not attempted in Cohort B
159 SNPs genotyped in A and B
Cohort B (n=354)
236 SNPs attempted
163 genotyped
46 low call rate
27 failed
158 SNPs
in gene regions
1 SNP
intergenic
18 SNPs
MAF <1%
140 SNPs
MAF>1%
A2M and F12 genotyped
for model replication
in cohorts C and D
GSTP1 genotyped
for model replication in
cohort C'

Figure 2

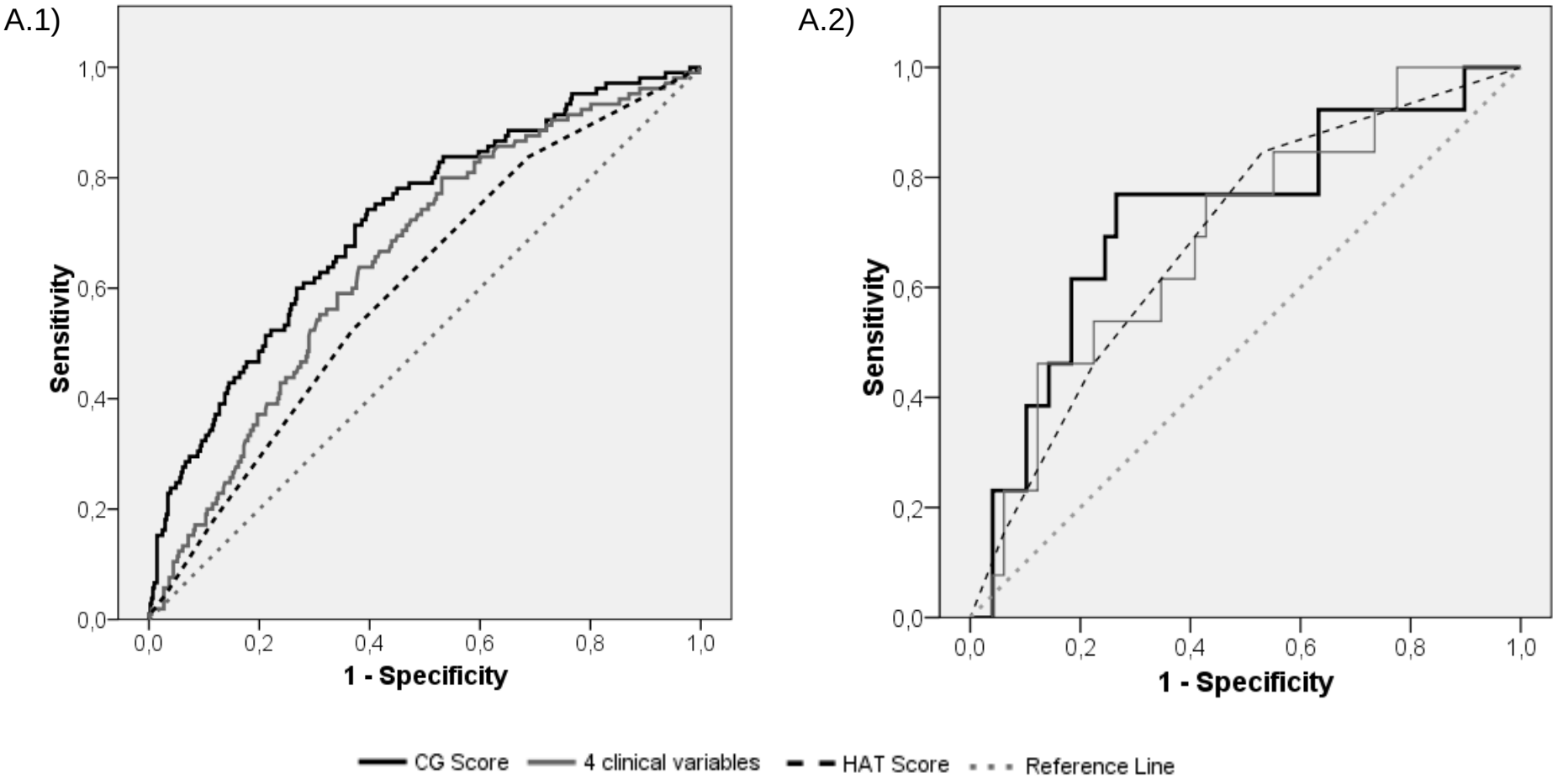
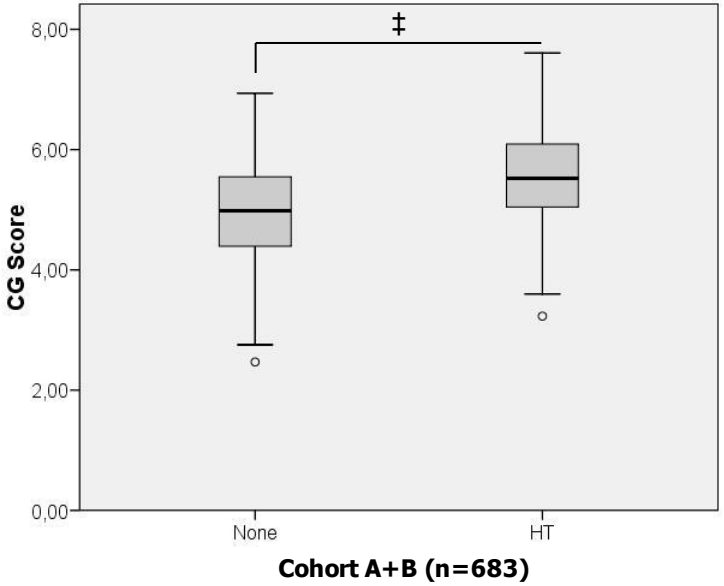
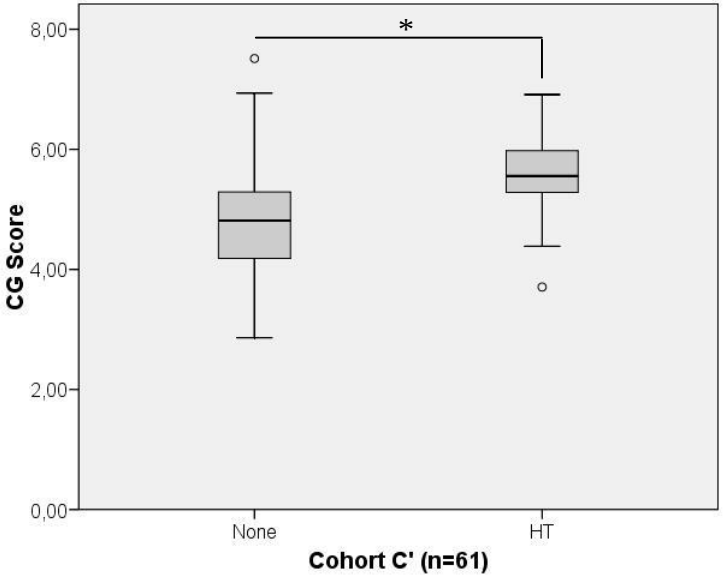

A.1)
A.2)
Sensitivity
1 - Specificity
0,0
0,2
0,4
0,6
0,8
1,0
CG Score
4 clinical variables
HAT Score
Reference Line

Figure 2

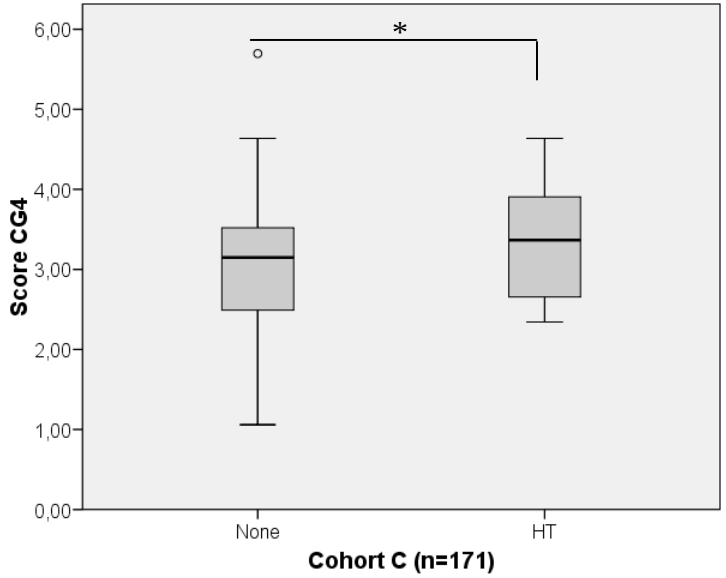
B.1)



B.2)



B.3)



B.4)

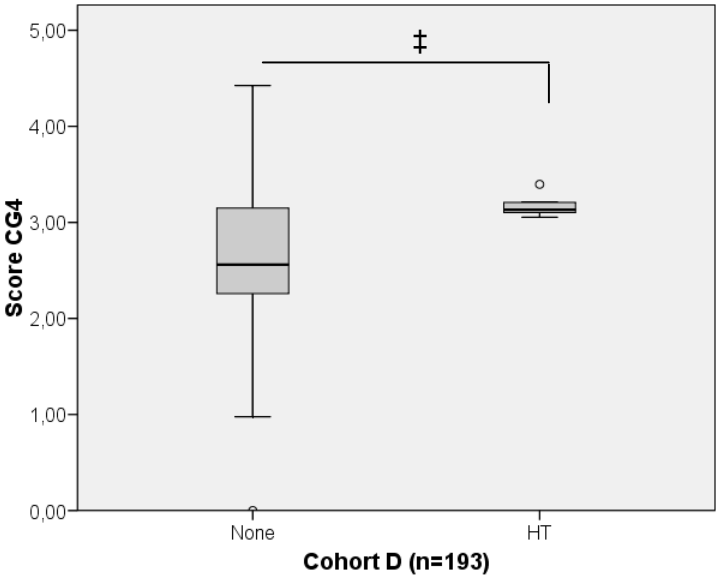
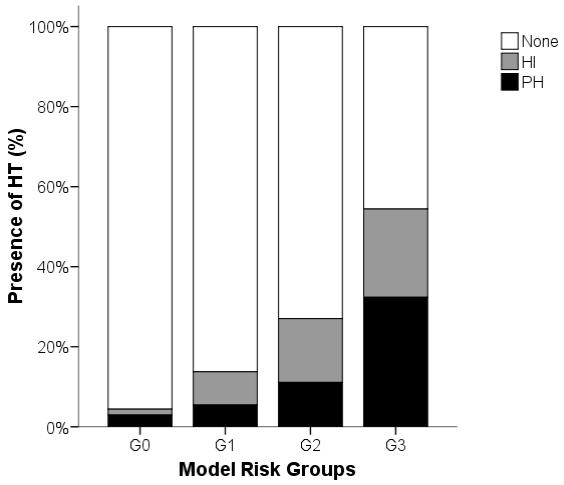
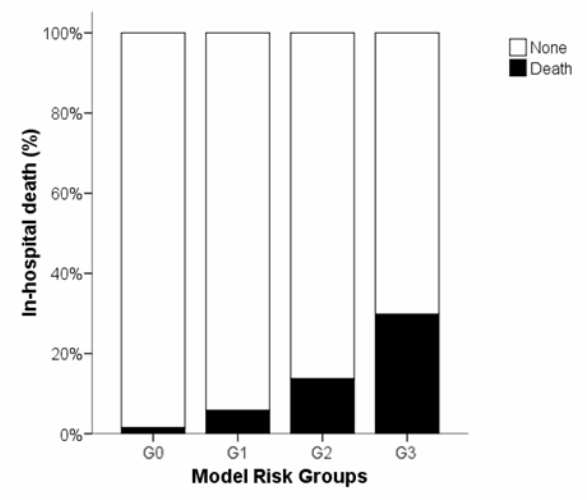


Figure 2

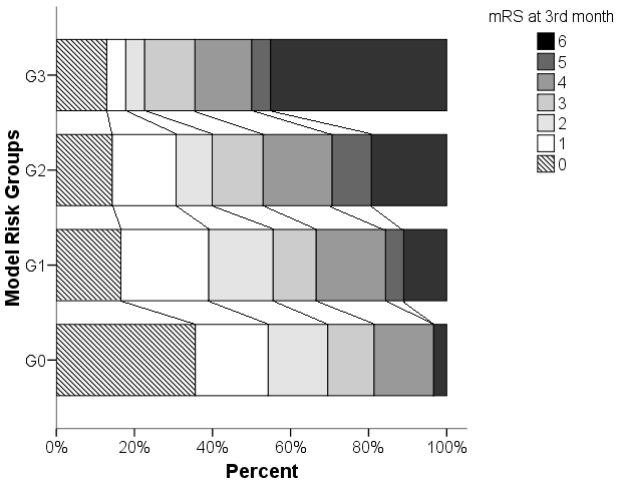
C.1)



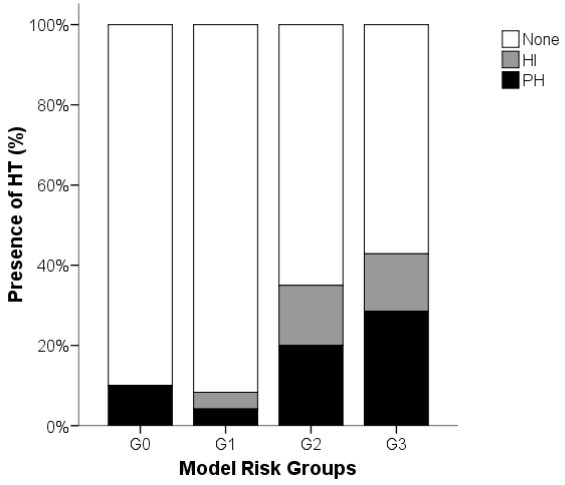
C.2)



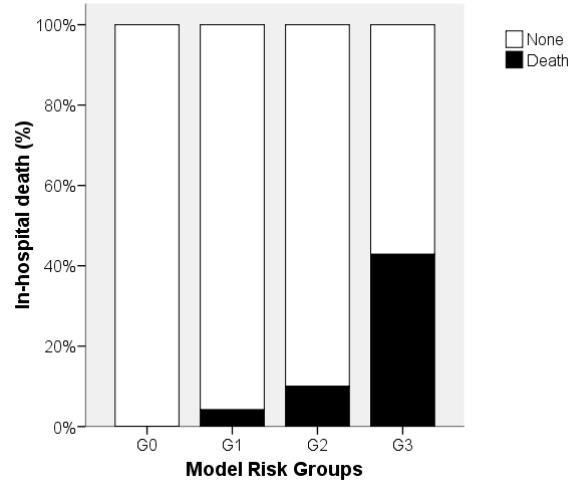
C.3)



C.4)



C.5)



C.6)

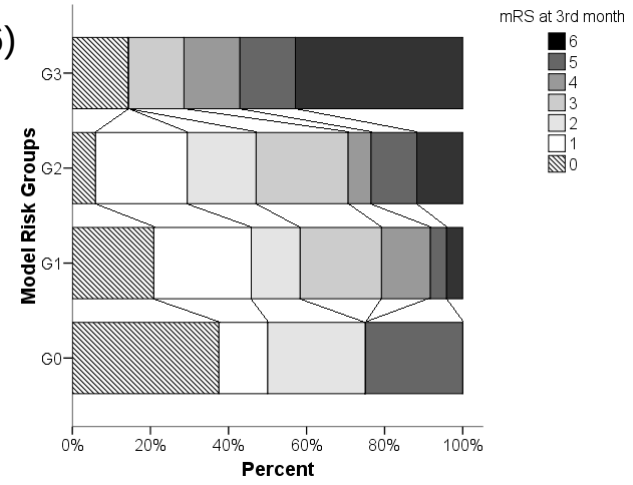
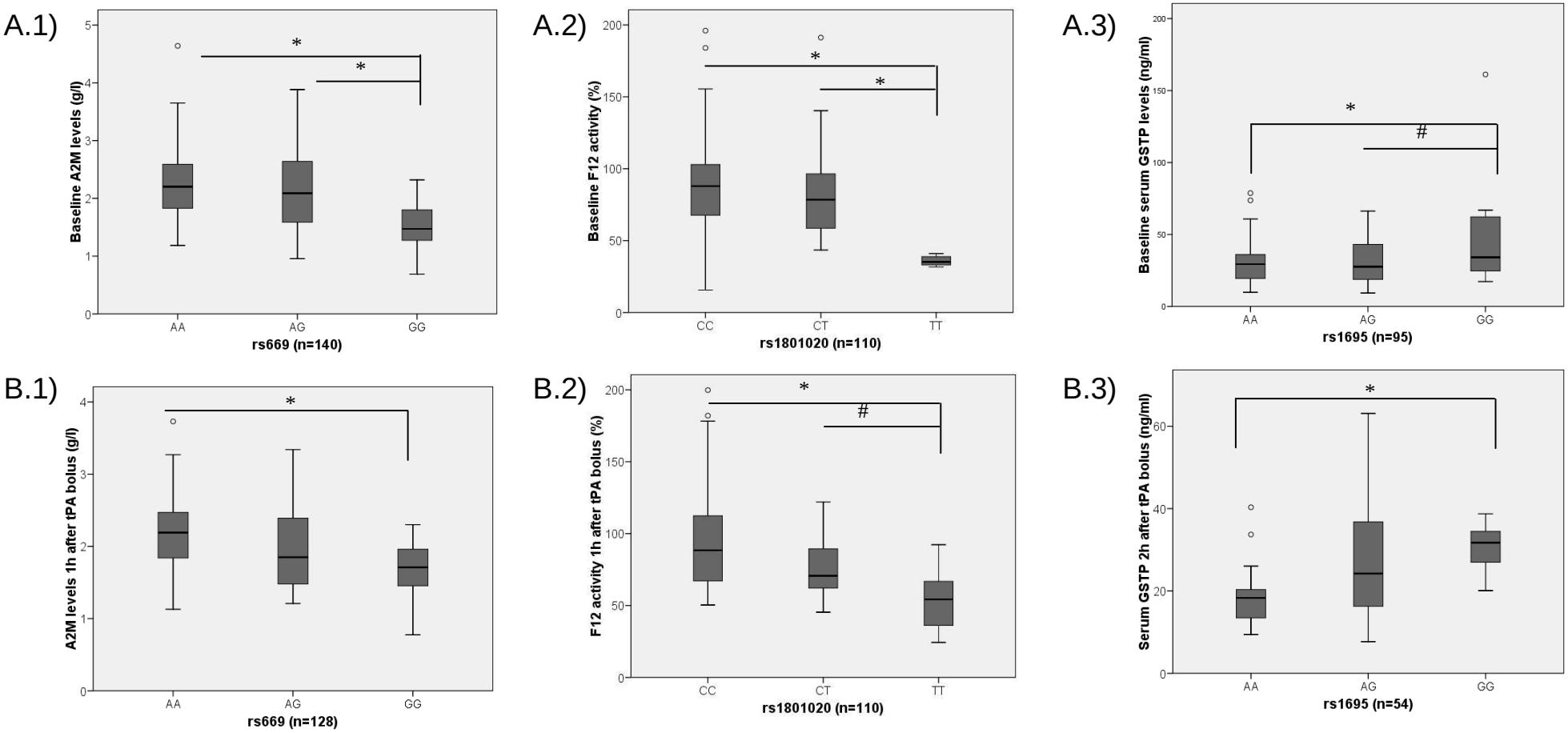


Figure 3


A.1)
Baseline A2M levels (g/l)
rs669 (n=140)
AA
AG
GG
A.2)
Baseline F12 activity (%)
rs1801020 (n=110)
CC
CT
TT
A.3)
Baseline serum GSTP levels (ng/ml)
rs1695 (n=95)
B.1)
A2M levels 1h after tPA bolus (g/l)
rs669 (n=128)
B.2)
F12 activity 1h after tPA bolus (%)
rs1801020 (n=110)
B.3)
Serum GSTP 2h after tPA bolus (ng/ml)
rs1695 (n=54)

Supplemental Data

EXPANDED METHODS

1. Clinical protocol

On arrival, a detailed history of vascular risk factors and current medication was obtained from each patient. Risk factors were defined as follows: Diabetes Mellitus: determination of fasting glucose concentration ≥ 126 mg/dl on venous plasma or post-glucose load ≥ 200 mgrs/dl or current use of oral antidiabetic drugs or insuline. Coronary artery disease: any history of chest pain due to inadequate blood flow to the heart (angina pectoris) or acute myocardial infarction. Atrial fibrillation: past medical history of paroxysmal, persistent or chronic atrial fibrillation demonstrated in an EKG recording. Hypertension: history of a blood pressure reading higher or equal to 140/90 mmHg or current intake of hypotensor treatment. Hyperlipidemia: determination of total cholesterol equal or higher to 200 mg/dL or current use of lipid lowering drugs. Tobacco consumption: current smoking habit was recorded.

Clinical examination was performed on admission, 1 and 2 hours after t-PA administration, 12, 24 and 48 hours after symptoms onset and at discharge. Stroke severity was assessed with the National Institutes of Health Stroke Scale (NIHSS) [1]. Modified Ranking Scale (mRS) at 3rd month was used to assess functional outcome (mRS score>2 was considered dependence) [2]. Etiologic subgroups were determined according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria [3]. Diagnostic set for TOAST classification included electrocardiogram, chest radiography, carotid ultrasonography, complete blood count and leukocyte differential and blood biochemistry in all patients; when indicated some patients also underwent special coagulation tests, transthoracic ecocardiography and Holter monitoring. This set was combined with neuroimaging data to define etiology as cardioembolic, atherothrombotic or other causes: arterial dissection, lacunar stroke and undetermined stroke.

Stroke was classified also according to the Oxfordshire Community Stroke Project (OCSP) criteria [4]: Total Anterior Cerebral Infarction (TACI), Partial Anterior Cerebral Infarction (PACI), Lacunar Cerebral Infarction (LACI) and Posterior Cerebral Infarction (POCI). A low number of lacunar stroke cases were included in A, B

and C cohorts, since they are not expected to receive fibrinolytic therapy because of absence of defined vascular occlusion. A specific study must be conducted to extend our results to small vessel diseases.

According to ECASS criteria, Hemorragic Infarction (HI) was defined as small petechiae along the margins of the infarct (HI-1) or more confluent petechiae within the infarcted area but without space-occupying effect (HI-2). Parenchymal Hematoma (PH) was defined as hematoma in less than 30% of the infarcted area with some slight space-occupying effect (PH-1); as dense hematoma over 30% of this area (PH-2) or further classified into remote PH if it occurred out of the ischemic area, within presumably normal brain (remote PH). Infarct volume was recorded when baseline MRI was available.

Violations of Safe Implementation of Thrombolysis in Stroke-Monitoring Study (SITS-MOST) criteria [5] were annotated from each center: age older than 80 years, previous stroke within 3 months or previous stroke with diabetes mellitus and mRS ≥ 1, INR > 1.4 or high APTT, plasma glucose<2.7mM or >22.2 mM (<50 mg/dl >400 mg/dl), baseline NIHSS >25, systolic blood pressure >185 mm Hg or diastolic blood pressure >110 mm Hg.

Finally, one neurologist from each of the participant hospitals reviewed clinical records from all patients deceased during the study in A, B and C' cohorts to establish in detail what was the cause of death. Several times, there would be different coexistent complications and one single cause could not be established. Both causes were noted for graphical representation and these cases were counted independently for percentage calculation of causes of death.

2. Genetic analysis

The most relevant candidate genes were selected by manual searching in Pubmed using the keywords "stroke, hypertension, inflammation, drug metabolism, coagulation, diabetes mellitus or diabetes, angiogenesis, myocardial infarction, atherosclerosis, lipid metabolism" for phenotypes and the keywords "polymorphism, SNP, mutation, variant" for polymorphisms. Pathways analyzed were related to stroke risk (hypertension, diabetes mellitus, lipid metabolism and myocardial infarction), t-PA targets (coagulation) and catabolizers (drugs metabolism) and further processes linked to good (angiogenesis) and bad outcome (inflammation) after ischemia. Only articles in English or Spanish were read.

Among candidate genes, SNP selection was performed depending on previous literature (the most studied SNPs) and their functional effect, including those with an already known modification at transcription, translation or protein activity or a hypothetic modification based on an aminoacid substitution. Whenever an interesting polymorphism involved more than a single nucleotide change (e.g. Angiotensin I/D variant), a SNP in perfect linkage disequilibrium was chosen for genotyping. The 5 most interesting candidate genes (*MMP9, NOS3, IL6, LRP* and *VEGF*) were studied by TagSNPs to gain a better coverage and reduce risk of false-negative results. The SNPs list was completed with most of the previously reported stroke risk variants (e.g. PDE4D, ALOX5AP) and all the variants in gene regions linked to stroke in the first GWAS study [6]. The final list was modified to allow multiplexing in 48 probes batches. Some interesting variants, like F13 Val34Leu (rs5985) were excluded due to incompatibility with SNPlex genotyping platform.

We assume that no stratification bias has been committed, as patients were of Caucasian origin as indicated by their family names and Spain is genetically homogeneous distributed [7]. Nevertheless, it would be necessary to check a panel of intergenic unlinked markers to formally exclude this bias [8]. Further replication work is needed to expand these results to other ethnic backgrounds (African Americans, Asians …).

To exclude any influence of genotyping errors, we repeated statistical analysis applying more stringent genetic quality controls (SNP call rate>90%, MAF>1%, sample call rate>95%, sample heterozigosity between 20-35%) to the Cohort A+B (high QC n=662) without changes in the significant associations observed. For SNPlex results validation, Kappa coefficient was calculated as Cohen et al [9], and stratified following the arbitrary Landis and Koch criteria [10], since there is no consensus algorithm to interpret this statistic. Their respective CI95% and minor allele frequency (MAF) are indicated.

All primers were designed with Primer3 [Table S2], checking in Ensembl Database (www.ensembl.org) that no nucleotide variants were present in the complementary sequence. Amplification was performed in 2720 Thermal Cycler (Applied Biosystems Inc., Foster City, USA) with an initial hot-start of 3 minutes at 94ºC, followed by 30 cycles of 30s at 94º-60ºC-72ºC and a final extension of 10 minutes at 72ºC. PCR samples were purified with ExoSap (GE Healthcare Biosciences, Waukesha, US) prior to Big Dye amplification (ABI Prism BigDye Terminator 3.0 method, Applied Biosystems Inc.; Foster City, US) and direct sequencing.

3. Hypothesis-Free Clinical Cloning (HFCC) analysis

HFCC combines a relatively fast computing algorithm for genome-wide epistasis detection with the flexibility to test a variety of different genetic models in multi-locus combinations. We programmed HFCC to analyze our "case-control" groups (presence/absence of HT or death) by combining the 227 SNPs successfully genotyped of cohort A. HFCC software constructed all two-locus (digenic) variables and explored its association to HT occurrence by comparing nine diplotype (each 2-locus genotype combination) individually using conventional Wald's Test. As we calculated 698,706 independent 2-locus strata, the study-wise corrected p-value was established at $p<7.16$ E-8 after Bonferroni correction.

In order to explore the nature and strength of interactions in selected digenic patterns, we further evaluated epistasis among selected markers using Alambique software [11]. Specifically, Alambique was programmed to calculate Synergy index, AP, RERI [12], strata-specific Odds Ratios, and case-only parameter estimates [11].

4. Linkage and conservation in F12 and A2M genes

Linkage blocks were plotted with Haploview [13] using the theoretical haplotype block distribution obtained from CEU population in HapMap database [14], and compared against our experimental data. A2M protein sequences were aligned between species using Homologene NCBI tool [15] and a score for pathogenicity was obtained with PolyPhen-2 [16].

The screening of the Alpha-2-Macroglobulin (A2M) gene region was carried out by polymerase chain reaction (PCR) amplification of 15 ischemic stroke samples (5 individuals for each genotype) and direct sequencing. Groups were matched by age, gender and HT occurrence.

5. A2M serum levels quantification

Alpha-2-macroglobulin (A2M) levels were measured by nephelometry in 140 available baseline and after t-PA serum samples in a BNProSpec System (N Antiserum to human A2M, 0842768009655, Dade Behring-Siemens, Marburg, Germany). Samples were stored at -80ºC and thawed before use. Protein determinations

were done unaware of genotype data; A2M levels are indicated as g/l. Using a commercial available control, the coefficient of variation (CV) intra-essay was 1.76% and inter-essay 2.21%.

6. Real-Time Quantitative PCR analysis

Gene expression quantification of *A2M* was performed in 32 available patients in whom blood samples were obtained before t-PA administration, as described [17]. A probe located in exons 1-2 (Hs00929976_m1) of *A2M* was used, with Cyclophilin A (PPIA) (Hs0099999904_m1) expression for normalization (Applied Biosystems, Austin, US). Results are expressed as a percentage depending on an external healthy calibrator sample, based on Livak method. Maximum intra-essay CV was 2.63% and inter-essay 2.65%

7. Western Blot of serum samples

Western blot was performed in 16 available samples selected by rs669 genotype (8 AA, 6 AG, 2 GG) and HT occurrence (6 HT, 10 none) as described [18]. Briefly, 1:30 serum dilutions (≈15 ug of total protein) were run in 4-6% SDS-PAGE (non-reducing conditions=N-R) or 4-8% (reducing conditions=R) adding 10% of 50 mM Dithiothreitol aqueous solution (Sigma-Aldrich Co; St. Louis, US). Membranes were incubated with 1:3,000 (cleaved monomer, R) or 1:15,000 (uncleaved monomer R, N-R) polyclonal anti-A2M (Sigma-Aldrich Co; St. Louis, US) followed by 1:15,000 donkey anti-rabbit secondary antibody (GE Healthcare, Little Chalfont Buckinghamshire, UK).

We used recombinant A2M protein (63013.5MG-F, Sigma-Aldrich Co; St. Louis, US) as a positive control for N-R, at a 0.5 ug/ul dilution. This protein was activated either by overnight incubation at 37ºC with 200mM methylamine (M0505, Sigma-Aldrich Co; St. Louis, US) in TrisHCl pH 8.1 [19] or by 1h incubation at 37ºC with trypsine (T9201, Sigma-Aldrich Co; St. Louis, US) in 0.1M $NaPO_4$ pH 8.0, followed by 5 minutes inactivation with 1 mM PMSF (93842, Sigma-Aldrich Co; St. Louis, US) [20] to use it as positive control for cleaved/uncleaved monomer ratio determination. All experiments were done in duplicate, only an example membrane is shown. Pure methylamine activated-A2M was chosen for band densitometry quantification, similar trends were obtained with trypsin or methylamine 1/5 (v/v) dilution as positive controls.

8. GSTP ELISA serum quantification

A home-made sandwich ELISA was performed to quantify GST-P in serum, including 95 samples at baseline and after t-PA infusion, and 54 available samples 2h after t-PA, 12 and 24 hours after symptoms onset. Each sample was assayed in duplicates and distributed randomly on the plates. Protein determinations were done unaware of genotype data; results are expressed as ng/ml. Intra and inter-run CV were below 10%.

9. FXII activated partial tromboplastin time (APTT) determination

FXII activity was determined by measuring APTT in 110 available baselines and after t-PA citrate plasma samples using the semi-automated coagulometer ST4 (Diagnostica Stago-Roche, Asnières, France). Factors diluents, APTT reagents, FXII-deficient serum, normal control and calibration plasma were from IZASA (Werfen Group, Barcelona, Spain). Samples were stored at -80ºC and thawed quickly in a heating bath before use. Protein determinations were done unaware of genotype data. FXII activity is indicated as a percentage of the normal control serum. CV intra-assay was 5.76% and inter-assay 5.28%.

10. Statistical Analysis

Sample size sensitivity calculation was performed using Ene 2.0 software. Although, from a clinical point of view, it would be interesting to determine polymorphisms associated with symptomatic HT or PH occurrence, we focused on HT to increase our statistical power. Nevertheless, all interesting predictors of HT have been later tested for PH (n=93) but not for symptomatic HT, due to the low number of events (n=39).

Deviation from Hardy-Weinberg equilibrium (HWE) was assessed in the whole group of subjects using a Chi^2 test with 1 degree of freedom [21]. Statistical significance for categorical variables was assessed by the Chi^2 or Fisher's exact test. For continuous variables, Anova or Kruskal-Wallis was employed depending on the normality of the distribution, as indicated by Kolmogorov-Smirnoff test and PP or QQ plots. Correlations were performed with Spearman Rho and differences between paired non-parametric samples were assessed by Friedman and Wilcoxon Signed Rank tests. Genotype associations were analyzed using dominant / recessive and additive models. Odd Ratios (ORs) and 95% confidence intervals (CIs) for the

effect of each SNP on HT risk were estimated using logistic regression (LR) models adjusted for vascular risk factors and associated clinical variables. Bonferroni correction was used for multivariable test correction; considering a 2-tailed adjusted *p*-value < 0.05 statistically significant. No imputation of missing data was performed.

To determine whether observed associations were influenced by sample selection (i.e. the order in which patients arrived to the hospital), we performed 3 different approaches:

1) Manual division of A+B cohort in 10 random half-sized groups, chi-square statistic in both groups (i.e. group1 and group1 complementary) and notation of the times a variant was associated significantly in both groups.

2) Automatic division of A+B cohort in 1000 random half-sized groups, calculation of chi-square statistic and notation of the times a variant was associated significantly. SNPs with minor homozygosity<0.08 were excluded to avoid repeated analytical failures.

3) Bootstrap calculation for the Odds Ratio and CI95% in a random sample of 885 patients with replacement from A+B cohort, using a modified version of bootstrap R-package. Only variants with p-value<0.1 in cohort A+B were included.

11. Network analysis with Ingenuity Pathways (IPA)

The IPA software (Ingenuity Systems, Redwood, US) was used to find new associations with other molecules and disease functions stored in its knowledge base. The information is scientist-curated, updated, and integrated from the published literature and other databases such as OMIM, Gene Ontology, and KEGG.

We performed a core analysis [Figure S4] to see connections between the different molecules previously related to HT occurrence (MMP9, FN1, SSAO, PLAT, PLG, S100B, ACP) and the new gene variants described here (*A2M, F12, GSTP1*). The first IPA network was used as a backbone, checking manually in Pubmed for all genes with low number of connections.

EXPANDED RESULTS AND DISCUSSION

1. Study population

The 2 cohorts recruited for model generation (cohort A, n=540 and cohort B, n=360) were very similar regarding clinical variables; differences were seen only for smoking habit, presence of atrial fibrillation (AF) and coronary artery disease [Table S3]. Thus, both cohorts were combined in the Cohort A+B (n=885) for model generation, with 22.1% HT, 9.6% PH and 10.6% in-hospital death.

Cohort C (n=287) was similar to training set with 20.7% HT and 8.1% PH, though patients had younger age, lower rates of AF and cardioembolism on TOAST classification and lower baseline NIHSS [Table S3]. Cohort D (n=491), which did not receive t-PA treatment showed greater differences: as expected this cohort had only 4.9% HT and 1.4% PH; with higher male gender and diabetes mellitus frequency, along with lower AF and cardioembolism rates and lower NIHSS score on admission [Table S3].

2. HT univariate analysis and predictive models

TOAST classification was excluded from predictive models because it is not available at emergency room department and consequently is not useful from a clinical point of view to classify patients before t-PA infusion.

Baseline DWI volume was found to predict HT occurrence in the samples were it was available (no HT: 10 cc vs. HT: 72 cc; p=2.19 E-6; n=259) but failed to enter in the model. Platelet count (no HT: 222,000 vs. HT: 229,000; p=0.595; n=506) did not predict HT in our series, contrary to previous reports [22,23].

LR predictive score could be adapted to accurately predict PH or symptomatic HT in very large prospective series to overcome statistical power limitations. To facilitate score implementation in clinical practice, our predictive score can be simplified. All beta coefficients were divided by the minimum beta coefficient (0.039) and rounded to the natural number. Besides, OTT and DBP data were stratified in groups of 10 units (min or mm). The resulting equation was: Score= baseline NIHSS + OTT (rounded 10min) + DBP (rounded 10mm)

x 3 + AF x 12 + rs1801020 (C-allelic charge) x 16 + rs669 (A-allelic charge) x 17. In our series, the score ranged from 53 to 174, and predictive capacity decreased slightly (AUC: 0.702 CI95% 0.648-0.759) respect to the exact approach. After CHAID score stratification, cut-off natural values would be G0<84, 84≤G1<110, 110≤G2<132 and G3≥132.

The most interesting groups of CRT analysis were a high risk group (NIHSS>13, rs669=AA, DBP>85, rs1801020=CC; n=66) with 53% HT (from which 24.2% were PH) and a very high risk group (NIHSS>13, rs669=AA, DBP>95, rs1801020=CC; n=20; 75% HT, 50% of them PH). Complementarily, a low risk group (NIHSS<13, without early ischemic signs, rs669 G-carriers; n=153) with only 7.4% HT (4.6% PH) and a very low risk group (NIHSS>13, rs669=AA, DBP≤85, rs1695=GG; n=20) without HT cases [Figure S3].

We were unable to formally validate CRT results, as only 4 patients were included in the high risk CART group in cohort C', from which one suffered an HI and another a PH (2/4, 50%). The low risk group was filled by 16 individuals, from which one suffered an HI and another patient a PH (2/16, 12.5%). The node before GSTP1 genotyping (NIHSS>13, rs669=AA, DBP≤85) had only 9 individuals, and "GG" group included only one patient who suffered an HI-2 (1/1, 100%).

3. In-hospital death univariate analysis and explicative model

We tested all clinical data as predictors of in-hospital mortality. Among categorical variables [Table S5A], we found hypertension, atrial fibrillation, previous stroke, anti-platelets or loop diuretics therapy, baseline proximal occlusion, SITS-MOST protocol violations, HT occurrence and TOAST classification associated with higher death rates. Among continuous variables [Table S5B] we found a link with older age, higher baseline NIHSS and hyperglycemia.

Six of them resisted Bonferroni correction ($p<0.05 / 29 = 1.72$ E-3): atrial fibrillation, loop diuretics treatment, proximal occlusion, HT occurrence, old age and baseline NIHSS score. Besides, ACE-I therapy, early ischemic signs on CT scan and basilar artery occlusion showed a trend towards high mortality.

All these data were introduced in a stepwise Forward Likelihood Ratio LR with rs1801020 to define the independent predictors of in-hospital death after t-PA treatment. This model would not be useful from a clinical point of view, since several of the items introduced are not known at baseline, but it could explain how these variables relate to fatal outcome [Table S5C].

Finally, the analysis of medical records was possible in 70 of the 90 deceased cases in cohort A+B (77.78%). Brain edema (28/70, 40.00%) and infections (26/70, 37.14%) were the major causes of death, followed by HT occurrence (12/70, 17.14%) [Fig. S5.1]. In cohort C' data was obtained for all deceased individuals (12/12, 100%). Brain edema (7/12, 58.33%) and HT occurrence (4/12, 33.33%) were the main causes in this cohort [Fig. S5.2].

4. HFCC analysis results

Without epistasis, none of the SNPs pairs resisted bonferroni correction [Table S4], crude data from gene-gene interaction analysis is available upon request. The best combinations involved rs669 for HT prediction and rs1051931 for in-hospital mortality. Notewhorthy, the rs669-rs1801020 pair (grey) used in our LR-based score was among the best predictive combinations for HT. Regarding epistasic SNP pairs; one pair (rs5361-rs2230806) was associated with HT and resisted Bonferroni correction [Table S5]. Another pair (rs11053646-rs4986790) was linked in two idependent stratus with in-hospital mortality [Table S5].

All these combinations must be tested at least in 2 independent cohorts to validate them. If the association is confirmed, they can be added as new items to the predictive score generated to increase its predictive capacity.

5. A2M complementary functional studies

No significant association between rs669 and *A2M* expression (p=0.669) nor *A2M* mRNA levels and HT occurrence (p=0.614) was found in peripheral blood mononuclear cells (PBMC) of 32 individuals [Figures S4G / S4H].

Western Blot of plasmatic samples showed no alterations in disulfide bond pattern [Figure S4A]. A2M ratio significantly increased between baseline and after t-PA infusion (p=0.001) [Figure S4D]. We hypothesize that HT occurrence may be associated with lower A2M activation, as non-significant differences were seen in the ratio between the cleaved [Figure S4C] and uncleaved monomer [Figure S4B] at baseline (None:0.68 HT:0.37; p=0.125) but not 1h after t-PA bolus (None:1.17 HT: 1.21; p=0.900). This effect was mirrored by the rs669 genotype, both at baseline (AA: 0.53 AG: 0.60 GG: 0.78; p=0.743) or after t-PA (AA: 1.13 AG: 1.28 GG: 1.10; p=0.854) [Figure S4D]. We believe it could be an association between A2M activity and HT occurrence, but we would need a sample size of 55 individuals to reach statistical significance (α=0.05, power=0.80).

6. Biological background

A2M is a general inhibitor of proteases mainly produced by hepatocytes but also fibroblasts, monocytes, neurons and glia. Upon its activation by cleavage of the bait region, A2M suffers a conformational change which sterically traps the protease and binds to lipoprotein-related protein 1, which internalizes the complex for degradation, a mechanism applied for t-PA [24], plasmin and MMP-9 [25]. Furthermore, A2M has been involved in polimorphonuclear migrations, the major source of MMP-9 to the HT [26], and whose degranulation is induced by t-PA *in vitro* [18]. Nevertheless, A2M also interacts with numerous growth factors and cytokines.

The knockout *A2M* mice show protective phenotype against sepsis and inflammation but increased sensitivity to pancreatic diseases [27]. Noteworthy, A2M is a typical acute phase protein in classic mammalian animal models (i.e. rat and mice) but not in humans, which must be kept in mind for new therapies development.

HT patients had lower ratio between cleaved and uncleaved monomer at baseline, while an increase was seen at the end of t-PA infusion, which was mirrored by rs669 genotypes. This decrease in A2M ratio could be related to lower A2M activity or early A2M-protease complex internalization, as HT patients have higher baseline levels of proteases [5]. We believe that the at-risk rs669 “A” allele may induce lower A2M activity, but this hypothesis must be demonstrated in further studies.

FXII has a dual role in homeostasis. It becomes activated due to contact with negatively charged surfaces in presence of Zn^{2+} [28] and active FXII cleaves kallikrein. At this point, FXIIa could activate FXI and thereafter FIX, initiating the intrinsic coagulation pathway, or kallilrein may activate urokinase promoting the conversion of plasminogen to plasmin, synergically with t-PA action [28]. FXII is also involved in kinin-kallikrein system and complement system activation. Of particular interest, coagulative, immune and vasoactive properties of F12 can be dissociated by generation of F12 fragment F12f, which looses its capacity to activate FXI but retains its ability to initiate both kinins and complement pathways [50].

Lower FXII activity and the TT genotype of rs1801020 have been inconsistently linked to CAD, deep venous thrombosis and recurrent miscarriages. FXII deficiency causes an increase in APTT without higher hemorrhagic risk whereas excessive FXII activity causes hereditary angioedema, particularly type III [29]. The *F12* knockout models showed prolonged APTT [28], defects in thrombus formation [30] and lower infarct size after middle cerebral artery occlusion (MCAo) without higher bleeding tendency, caused by higher cortical reperfusion [31]. Pre-treatment with FXII inhibitors of MCAo mice show variable results depending on target specificity.

The rs1801020 (-4C>T) creates a new translation initiation site, disrupts a Kozak sequence and reduces the translation efficiency of *F12* [32]. Although -58G>C and -63C>T also alter *F12* expression via HNF4 interaction [33], the main influence in FXII levels comes from rs1801020 as indicated by Quantitative Trait Locus analysis [34]. In fact, rs1801020 frequencies account for FXII levels variation between Asiatic and Caucasic populations [32]. Estrogen levels, FXII antibodies or IL6 levels influence FXII activity, which lead to only moderate correlation between FXII levels and FXII activity [35].

We hypothesize that F12f may participate in edema formation without altering coagulation capacity. Maybe generation of this degradation product of F12 may explain why coagulation tests did not show any relationship to HT or death occurrence, because a secondary product would be involved in phenotype.

Finally, *GSTP1* belongs to glutathione transferases (EC 2.5.1.18), a dimeric enzyme involved in detoxification. Expression and regulation of GST is complex and highly species-dependent. *GSTP1* is expressed in dopaminergic neurons, astrocytes, oligodendrocytes and brain capillaries [36]. The *GSTP1* knockout mice have no obvious phenotype, unless differential response to carcinogens [37].

The rs1695 (Ile104Val) is located within the substrate binding site of GST-Pi and alters substrate specificity, as demonstrated in mutagenesis studies in-vitro [38,39] activity measurements ex-vivo [40,41] and the crystal structure of 3 independent mutant protein-substrate complexes [42].

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Supplemental table S1: List of SNPs attempted to genotype, classified by groups of interest. Genes, chromosomal position and genotyping results are indicated in columns.

Supplemental table S2: Agreement of genotyping results of SNPlex technology and direct sequencing to assess the quality of genetic data **(A)**. Minor allele frequencies (MAF) values are indicated because Kappa statistic depends on polymorphism frequencies. Primers used for SNPlex validation **(B)** and A2M gene sequencing **(C)**.

Supplemental table S3: Population characteristics of the 4 Geno-tPA study cohorts. Age is indicated as mean with its standard deviation. Categorical variable frequencies are indicated as a percentage with number of cases in brackets. Baseline NIHSS score is indicated as median with range in brackets. Differences between cohorts are indicated by the p-value of Chi^2 (categorical variables), t-test (age) or Kruskal-Wallis (baseline NIHSS). *=p<0.05; †=p<0.1.

Supplemental table S4: As HT risk was influenced by TOAST stroke etiology, the polymorphisms included in the final model have been analyzed separately for cardioembolic, atherothrombotic or other etiologies. HT risk for each genotype and their corresponding Chi^2 p-value in dominant-recessive (A) or additive (B) model are indicated.

Supplemental table S5: Univariate analysis for in-hospital mortality predictors with categorical **(A)** and continuous **(B)** variables. **(A)** Frequencies of death for presence or absence of risk factor are indicated as a percentage with number of cases in brackets, followed by their Chi^2 p-value. For TOAST classification, death rates are indicated for each category. **(B)** Continuous variables are indicated, depending on normal distribution, as mean ± Standard Deviation (S.D.) or median and Interquartilic Range (IR) followed by t-test or Mann-Whitney p-values. SBP=Systolic blood pressure, DBP=diastolic blood pressure, OTT=time from onset to treatment. **(C)** Independent predictors of mortality after stepwise forward LR.

Supplemental table S6: Best SNP pairs predictors as evaluated by HFCC (p-values < 10^{-5}) for HT **(A)** in-hospital mortality **(B)** or epistatic interactions for both conditions **(C)**. OR10=Odd Ratio when only first SNP is present, OR01=Odd Ratio for only the second, OR11=Odd Ratio for SNP combination. D=in-hospital death.

Supplemental figure S1: Linkage and conservation analysis of rs669 and rs1801020 variants. Physical map of A2M **(A1)** and F12 **(A2)** genes are represented in abscises with R^2 linkage values in each square; the darker the square is, the stronger the linkage disequilibrium is. Conservation analysis of Val1000Ile **(B)**, with mutated residue indicated in white over dark grey.

Supplemental figure S2: Western Blot of blood serum samples showing A2M dimer **(A)** uncleaved monomer **(B)** and cleaved A2M **(C)**. Densitometry results are expressed relative to recombinant A2M positive control. The ratio of cleaved / uncleaved A2M was calculated and compared among rs669 genotypes **(D)**, and presence / absence of HT **(E)**. Error bar display +/- 1 Standard Error. *=p<0.05 after Bonferroni correction.

A2M baseline mRNA expression in PBMC classified by rs669 genotypes **(F)** or presence / absence of HT **(G)**, indicated as a percentage of a healthy calibrator sample.

Supplemental figure S3: Tree classification model in Cohort A+B, groups showing the most extreme HT frequencies (either high or low) are indicated with black arrows. Cut-off values or selected categories used for node separation in Chi^2 test are indicated over each node or group of patients.

Supplemental figure S4: Network analysis of HT occurrence. Genes and molecules up-regulated in HT cases or at-risk genotypes are indicated in red while those in green are down-regulated. A2M=Alpha-2-Macroglobulin, AOC3= semicarbazide sensitive amine oxidase or vascular adhesion protein 1, ERK1/2=

dimmer formed by mitogen-activated protein kinase 1 and 3, F12= coagulation factor 12, FN1=fibronectin 1, FOS= FBJ murine osteosarcoma viral oncogene homolog, GRB2= growth factor receptor-bound protein 2, GSTP1= glutathione S-transferase pi 1, H2O2= hydrogen peroxide, IL1B= interleukin 1 beta, IL6= interleukin 6, Jnk= mitogen-activated protein kinase 8, JUN= jun oncogene, KLK3= kallikrein-related peptidase 3, KLKB1= kallikrein B, plasma (Fletcher factor) 1, MAP3K5= mitogen-activated protein kinase kinase kinase 5, MMP12= matrix metallopeptidase 12 (macrophage elastase), MMP13= matrix metallopeptidase 13 (collagenase 3), MMP14= matrix metallopeptidase 14 (membrane-inserted), MMP9= matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase), PLAT= plasminogen activator, tissue (t-PA), PLG=plasminogen, TNF= tumor necrosis factor, TRAF2= TNF receptor-associated factor 2.

Supplemental figure S5: Causes of death from deceased patients of cohorts A+B (left side) or cohort C' (right side). Percentages for each category are indicated. HT=Hemorrhagic Transformation, AMI=Acute Myocardial Infarction.

Table S1

Biological Process	Gene Symbol	dbSNP ID	Chr	Position	Genotyping
Angiogenesis	*ABCA1*	rs28587567	9	106627854	Included
Angiogenesis	*ABCA1*	rs2230806	9	107620866	Included
Angiogenesis	*EDN1*	rs5370	6	12296254	Included
Angiogenesis	*FGF2*	rs1449683	4	123748085	Low genotyping Cohort B
Angiogenesis	*HTR2A*	rs6311	13	47471477	Included
Angiogenesis	*ICAM1*	rs5491	19	10385539	Excluded MAF<1%
Angiogenesis	*ICAM1*	rs1799969	19	10394791	Included
Angiogenesis	*ICAM1*	rs5498	19	10395682	Failed Cohort A and B
Angiogenesis	*PARP1*	rs1136410	1	226555301	Included
Angiogenesis	*TGFB1*	rs1800471	19	41858875	Failed Cohort A and B
Angiogenesis	*VCAM1*	rs1041163	1	101183824	Included
Angiogenesis	*VEGFA*	rs2010963	6	43738349	Low genotyping Cohort B
Angiogenesis	*VEGFA*	rs3025042	6	43741450	Excluded MAF<1%
Angiogenesis	*VEGFA*	rs3024994	6	43743506	Included
Angiogenesis	*VEGFA*	rs2146323	6	43745094	Failed Cohort A and B
Angiogenesis	*VEGFA*	rs3025000	6	43746168	Included
Angiogenesis	*VEGFA*	rs3025047	6	43746409	Excluded MAF<1%
Angiogenesis	*VEGFA*	rs3025010	6	43747576	Low genotyping Cohort B
Angiogenesis	*VEGFA*	rs3025030	6	43750586	Included
Angiogenesis	*VEGFA*	rs3025033	6	43751074	Included
Angiogenesis	*VEGFA*	rs3025035	6	43751358	Included
Diabetes Mellitus	*ADIPOQ*	rs2241766	3	186570891	Included
Diabetes Mellitus	*ADIPOQ*	rs1501299	3	186571122	Included
Diabetes Mellitus	*AP1M2*	rs1821282	19	10692713	Failed Cohort A and B
Diabetes Mellitus	*CAPN10*	rs3792267	2	241531173	Failed Cohort A and B
Diabetes Mellitus	*CAPN10*	rs5030952	2	241542702	Failed Cohort A and B
Diabetes Mellitus	*ENPP1*	rs1044498	6	132172367	Included
Diabetes Mellitus	*GCKR*	rs1260326	2	27730939	Included
Diabetes Mellitus	*GRM3*	rs6465084	7	86403474	Included
Diabetes Mellitus	*HNF1A*	rs1800574	12	121416863	Low genotyping Cohort B
Diabetes Mellitus	*HNF4A*	rs2144908	20	42985716	Included
Diabetes Mellitus	*IGF1R*	rs2229765	15	99478224	Included
Diabetes Mellitus	*IGFBP3*	rs2854744	7	45961074	Low genotyping Cohort B
Diabetes Mellitus	*IRS1*	rs1801278	2	227660543	Low genotyping Cohort B
Diabetes Mellitus	*PPARA*	rs1800206	22	46614273	Included
Diabetes Mellitus	*PPARG*	rs4135352	3	12458207	Excluded MAF<1%
Drug Metabolism	*CYP11B2*	rs1799998	8	143999599	Included
Drug Metabolism	*GST*	rs3211206	1	110201643	Included
Drug Metabolism	*GSTO1*	rs4925	10	106022788	Included
Drug Metabolism	*GSTP1*	rs1695	11	67352688	Included
Drug Metabolism	*MGP*	rs1800801	12	15038787	Included
Drug Metabolism	*MTHFR*	rs1801131	1	11854475	Included
Drug Metabolism	*MTHFR*	rs1801133	1	11856377	Low genotyping Cohort B
Drug Metabolism	*PTGS1*	rs3842787	9	125133506	Low genotyping Cohort B
Drug Metabolism	*PTGS2*	rs20417	1	186650320	Failed Cohort A
Drug Metabolism	*TP53*	rs1042522	17	7579471	Failed Cohort A and B
Drug Metabolism	*VKORC1*	rs2359612	16	31103795	Included

Biological Process	Gene Symbol	dbSNP ID	Chr	Position	Genotyping
Fibrinolysis & Coagulation	*ANXA5*	rs11575945	4	122617744	Included
Fibrinolysis & Coagulation	*CYP2C9*	rs1057910	10	96741052	Included
Fibrinolysis & Coagulation	*F12*	rs1801020	5	176836531	Included
Fibrinolysis & Coagulation	*F13A1*	rs7740663	6	6319003	Excluded MAF<1%
Fibrinolysis & Coagulation	*F2*	rs1799963	11	46761054	Included
Fibrinolysis & Coagulation	*F3*	rs1361600	1	95007917	Failed Cohort A and B
Fibrinolysis & Coagulation	*F5*	rs6025	1	169519048	Included
Fibrinolysis & Coagulation	*F7*	rs6046	13	113773158	Failed Cohort A and B
Fibrinolysis & Coagulation	*F8*	rs1800291	23	154158284	Included
Fibrinolysis & Coagulation	*FGA*	rs6050	4	155507589	Included
Fibrinolysis & Coagulation	*FGB*	rs1800790	4	155483707	Included
Fibrinolysis & Coagulation	*GP1BA*	rs2243093	17	4835894	Failed Cohort A and B
Fibrinolysis & Coagulation	*GP3BA*	rs5918	17	45360729	Included
Fibrinolysis & Coagulation	*PLAT*	rs2020918	8	42072437	Included
Fibrinolysis & Coagulation	*PLAU*	rs2227564	10	75673100	Low genotyping Cohort B
Fibrinolysis & Coagulation	*PROC*	rs1799810	2	128176039	Low genotyping Cohort B
Fibrinolysis & Coagulation	*PROS1*	rs6123	3	93593118	Included
Fibrinolysis & Coagulation	*RETN*	rs1862513	19	7733792	Included
Fibrinolysis & Coagulation	*SERPINC1*	rs2227606	1	173881121	Excluded MAF<1%
Fibrinolysis & Coagulation	*SERPINE1*	rs1799768	7	100769706	Included
Fibrinolysis & Coagulation	*SERPINE1*	rs7242	7	100781444	Included
Fibrinolysis & Coagulation	*TAFI*	rs1926447	13	46629943	Included
Fibrinolysis & Coagulation	*THBD*	rs1042579	20	23028723	Low genotyping Cohort B
Fibrinolysis & Coagulation	*VWF*	rs1063856	12	6153533	Included
Hypertension	*ACE*	rs1987693	17	61565764	Excluded MAF<1%
Hypertension	*ACE*	rs4341	17	61565989	Low genotyping Cohort B
Hypertension	*ADRB2*	rs1042713	5	148206439	Included
Hypertension	*ADRB2*	rs1042714	5	148206472	Low genotyping Cohort B
Hypertension	*ADRB2*	rs1800888	5	148206884	Included
Hypertension	*ADRB3*	rs4994	8	37823797	Low genotyping Cohort B
Hypertension	*AGT*	rs699	1	230845793	Failed Cohort A and low Cohort B
Hypertension	*AGTR1*	rs5186	3	148459987	Included
Hypertension	*KCNJ11*	rs5217	11	17409054	Excluded MAF<1%
Hypertension	*KCNMB1*	rs11739136	5	169810795	Included
Hypertension	*SCNN1A*	rs5742912	12	6458349	Low genotyping Cohort B
Inflammation	*CD40*	rs1883832	20	44746981	Included
Inflammation	*CRP*	rs1205	1	159682232	Included
Inflammation	*CRP*	rs1130864	1	159683090	Included
Inflammation	*CRP*	rs1800947	1	159683437	Included
Inflammation	*IFNG*	rs2430561	12	68552519	Included
Inflammation	*IL10*	rs1800872	1	206946406	Included
Inflammation	*IL10*	rs1800896	1	206946896	Included
Inflammation	*IL13*	rs1295686	5	131995842	Included
Inflammation	*IL1A*	rs1800587	2	113542959	Included
Inflammation	*IL1B*	rs1143634	2	113590389	Included
Inflammation	*IL1B*	rs1143627	2	113594386	Included
Inflammation	*IL1B*	rs16944	2	113594866	Included
Inflammation	*IL4R*	rs1805015	16	27374179	Included
Inflammation	*IL4R*	rs1801275	16	27374399	Low genotyping Cohort B
Inflammation	*IL5*	rs2069812	5	131879915	Included
Inflammation	*IL5RA*	rs2290608	3	3151758	Included

Biological Process	Gene Symbol	dbSNP ID	Chr	Position	Genotyping
Inflammation	*IL6*	rs1800797	7	22766220	Included
Inflammation	*IL6*	rs1800796	7	22766245	Included
Inflammation	*IL6*	rs1800795	7	22766644	Included
Inflammation	*IL9*	rs2069885	5	135228164	Included
Inflammation	*ITGA2*	rs1126643	5	52347368	Included
Inflammation	*MCP1*	rs1024611	17	32579787	Included
Inflammation	*MIF*	rs755622	22	24236391	Failed Cohort A and low Cohort B
Inflammation	*MMP1*	rs1799750	11	102670495	Included
Inflammation	*MMP10*	rs486055	11	102650423	Included
Inflammation	*MMP12*	rs2276109	11	102745790	Included
Inflammation	*MMP13*	rs2252070	11	102826538	Included
Inflammation	*MMP2*	rs243864	16	55512321	Included
Inflammation	*MMP21*	rs10901425	10	127462524	Failed Cohort A and B
Inflammation	*MMP3*	rs3025058	11	102715952	Included
Inflammation	*MMP7*	rs11568818	11	102401660	Included
Inflammation	*MMP8*	rs1320632	11	102596062	Included
Inflammation	*MMP9*	rs3918248	20	44071031	Included
Inflammation	*MMP9*	rs2274756	20	44076518	Included
Inflammation	*MMP9*	rs8113877	20	44635044	Included
Inflammation	*MMP9*	rs3918278	20	44635653	Included
Inflammation	*MMP9*	rs3918241	20	44635734	Included
Inflammation	*MMP9*	rs3918243	20	44636363	Excluded MAF<1%
Inflammation	*MMP9*	rs3918280	20	44636681	Excluded MAF<1%
Inflammation	*MMP9*	rs3918249	20	44638135	Included
Inflammation	*MMP9*	rs3918253	20	44639510	Low genotyping Cohort B
Inflammation	*MMP9*	rs2274755	20	44639691	Included
Inflammation	*MMP9*	rs2236416	20	44640574	Low genotyping Cohort B
Inflammation	*MMP9*	rs3918256	20	44640958	Low genotyping Cohort B
Inflammation	*MMP9*	rs3787268	20	44641730	Included
Inflammation	*MMP9*	rs2250889	20	44642405	Included
Inflammation	*NOS2A*	rs1137933	17	26105931	Included
Inflammation	*NOS3*	rs7791889	7	22960361	Failed Cohort A and B
Inflammation	*NOS3*	rs4722204	7	22960596	Included
Inflammation	*NOS3*	rs10266564	7	22961111	Low genotyping Cohort A
Inflammation	*NOS3*	rs1800779	7	150689942	Low genotyping Cohort B
Inflammation	*NOS3*	rs2070744	7	150690078	Failed Cohort A and low Cohort B
Inflammation	*NOS3*	rs2257073	7	150883286	Low genotyping Cohort B
Inflammation	*NOS3*	rs2257090	7	150883760	Low genotyping Cohort B
Inflammation	*NOS3*	rs10275136	7	150883891	Failed Cohort B
Inflammation	*NOS3*	rs12703116	7	150887941	Included
Inflammation	*NOS3*	rs2243428	7	150888657	Low genotyping Cohort A and B
Inflammation	*NOS3*	rs2288649	7	150888787	Low genotyping Cohort B
Inflammation	*NOS3*	rs2435609	7	150890072	Included
Inflammation	*NOS3*	rs2487151	7	150890240	Included
Inflammation	*NOS3*	rs2435608	7	150890805	Low genotyping Cohort B
Inflammation	*NOS3*	rs6952465	7	150891640	Included
Inflammation	*NOS3*	rs310590	7	150892346	Included
Inflammation	*NOS3*	rs310589	7	150892598	Low genotyping Cohort B
Inflammation	*NOS3*	rs310588	7	150892949	Low genotyping Cohort B
Inflammation	*NOS3*	rs310586	7	150893628	Included
Inflammation	*NOS3*	rs310585	7	150895254	Failed Cohort B
Inflammation	*NOS3*	rs310584	7	150896222	Included
Inflammation	*NOS3*	rs6977933	7	150901590	Failed Cohort B

Biological Process	Gene Symbol	dbSNP ID	Chr	Position	Genotyping
Inflammation	*SELE*	rs5355	1	169695869	Included
Inflammation	*SELE*	rs5361	1	169701059	Included
Inflammation	*SELP*	rs6133	1	169565345	Low genotyping Cohort A
Inflammation	*TIMP1*	rs11551797	23	47445939	Low genotyping Cohort B
Inflammation	*TIMP1*	rs2070584	23	47446518	Included
Inflammation	*TNF*	rs1800629	6	31543030	Low genotyping Cohort B
Inflammation	*TNFRSF1B*	rs1061622	1	12252954	Included
Lipid Metabolism	*APOB*	rs5742904	2	21229159	Excluded MAF<1%
Lipid Metabolism	*APOB*	rs1367117	2	21263899	Included
Lipid Metabolism	*APOC3*	rs2854117	11	116700141	Low genotyping Cohort B
Lipid Metabolism	*APOE*	rs405509	19	45408835	Low genotyping Cohort B
Lipid Metabolism	*APOE*	rs440446	19	45409166	Failed Cohort B
Lipid Metabolism	*APOE*	rs429358	19	45411940	Failed Cohort A and B
Lipid Metabolism	*APOE*	rs7412	19	45412078	Low genotyping Cohort B
Lipid Metabolism	*APOH*	rs1801692	17	64222163	Included
Lipid Metabolism	*CETP*	rs1800775	16	56995235	Low genotyping Cohort A and B
Lipid Metabolism	*CETP*	rs5882	16	57016091	Included
Lipid Metabolism	*FABP2*	rs179983	6	16350143	Excluded MAF<1%
Lipid Metabolism	*LDLR*	rs11669576	19	11222299	Failed Cohort A and B
Lipid Metabolism	*LEPR*	rs1137101	1	66058512	Included
Lipid Metabolism	*LIPC*	rs1800588	15	58723674	Included
Lipid Metabolism	*LPL*	rs268	8	19813528	Included
Lipid Metabolism	*LPL*	rs328	8	19819723	Included
Lipid Metabolism	*LRP1*	rs12307379	12	57518664	Included
Lipid Metabolism	*LRP1*	rs11172113	12	57527282	Included
Lipid Metabolism	*LRP1*	rs922963	12	57529292	Excluded MAF<1%
Lipid Metabolism	*LRP1*	rs17547610	12	57530340	Low genotyping Cohort B
Lipid Metabolism	*LRP1*	rs4759044	12	57530669	Included
Lipid Metabolism	*LRP1*	rs715948	12	57532981	Low genotyping Cohort B
Lipid Metabolism	*LRP1*	rs1799986	12	57535265	Failed Cohort A and B
Lipid Metabolism	*LRP1*	rs1800127	12	57539081	Low genotyping Cohort B
Lipid Metabolism	*LRP1*	rs7398375	12	57540847	Included
Lipid Metabolism	*LRP1*	rs10876966	12	57543571	Included
Lipid Metabolism	*LRP1*	rs12814239	12	57569477	Failed Cohort B
Lipid Metabolism	*LRP1*	rs2228187	12	57571248	Low genotyping Cohort B
Lipid Metabolism	*LRP1*	rs12368582	12	57582546	Low genotyping Cohort B
Lipid Metabolism	*LRP1*	rs1800168	12	57592556	Included
Lipid Metabolism	*LRP1*	rs1800159	12	57593893	Included
Lipid Metabolism	*LRP1*	rs7956957	12	57602814	Low genotyping Cohort B
Lipid Metabolism	*LRPAP1*	rs11940827	4	3533949	Failed Cohort B
Lipid Metabolism	*OLR1*	rs11053646	12	10313447	Included
Lipid Metabolism	*PPARGC1*	rs8192678	4	23815661	Included
Lipid Metabolism	*SREBF2*	rs2269657	22	42264268	Included
Myocardial Infarction	*ESR1*	rs9340799	6	152163380	Included
Myocardial Infarction	*ESR2*	rs1255998	14	64693870	Included
Myocardial Infarction	*ESR2*	rs1256065	14	64698931	Failed Cohort A and B
Myocardial Infarction	*ESR3*	rs1256030	14	64747169	Included
Myocardial Infarction	*PLA2G7*	rs1051931	6	46672942	Included
Myocardial Infarction	*PON1*	rs662	7	94937445	Included
Myocardial Infarction	*PON2*	rs7493	7	95034774	Included
Myocardial Infarction	*S100B*	rs11542310	21	48019250	Excluded MAF<1%
Myocardial Infarction	*S100B*	rs1051169	21	48022229	Included

Biological Process	Gene Symbol	dbSNP ID	Chr	Position	Genotyping
Stroke	*A2M*	rs669	12	9232267	Included
Stroke	*A2M*	rs1800433	12	9232350	Failed Cohort A and B
Stroke	*AACT*	rs4934	14	95080802	Low genotyping Cohort B
Stroke	*ADD1*	rs4961	4	2906706	Included
Stroke	*AIM1*	rs783396	6	106987369	Not attempted Cohort B
Stroke	*ALOX5AP*	rs10507391	13	31312095	Included
Stroke	*ALOX5AP*	rs4769874	13	31326440	Failed Cohort B
Stroke	*AMPD1*	rs17602729	1	115236056	Included
Stroke	*ANP*	rs5065	1	11906067	Included
Stroke	*ASTN2*	rs3761845	9	119770479	Not attempted Cohort B
Stroke	*ASTN2*	rs10817974	9	119775605	Not attempted Cohort B
Stroke	*CD14*	rs2569190	5	140012915	Included
Stroke	*CDH1*	rs16260	16	68771033	Included
Stroke	*CDK5*	rs11541602	7	150751131	Excluded MAF<1%
Stroke	*CX3CR1*	rs3732379	3	39307255	Included
Stroke	*DYX8*	rs1635712	1	35944751	Excluded MAF<1%
Stroke	*ELMO1*	rs11766187	7	36934572	Excluded MAF<1%
Stroke	*ELN*	rs17855988	7	73474824	Low genotyping Cohort B
Stroke	*FLT1*	rs7993418	13	28883060	Failed Cohort A and B
Stroke	*GJA1*	rs17653265	6	121768750	Included
Stroke	*GJA4*	rs1764391	1	35260768	Failed Cohort B
Stroke	*GNB3*	rs5443	12	6954874	Low genotyping Cohort B
Stroke	*HIF1AN*	rs2295778	10	102295835	Low genotyping Cohort B
Stroke	*HP*	rs587660	16	72094294	Excluded MAF<1%
Stroke	*HSPA6*	rs400835	1	161494731	Failed Cohort A and B
Stroke	*IMPA2*	rs7506045	18	11987271	Not attempted Cohort B
Stroke	*ITGB6*	rs10497212	2	160964699	Not attempted Cohort B
Stroke	*KCNIP4*	rs4697177	4	20760088	Not attempted Cohort B
Stroke	*KCNK17*	rs10947803	6	38989304	Not attempted Cohort B
Stroke	*KCNK17*	rs10807204	6	39273425	Not attempted Cohort B
Stroke	*LTA*	rs909253	6	31540312	Included
Stroke	*MGAT5*	rs2118844	2	135167599	Not attempted Cohort B
Stroke	*NEUROD1*	rs1801262	2	182543454	Included
Stroke	*P2RY1*	rs1065776	3	152553627	Included
Stroke	*PDE4D*	rs1396476	5	59396641	Included
Stroke	*PDE4D*	rs2910829	5	59469898	Included
Stroke	*PDE4D*	rs966221	5	59502519	Included
Stroke	*PDE4D*	rs702553	5	59736772	Included
Stroke	*PDE4D*	rs12188950	5	59783316	Not attempted Cohort B
Stroke	*PDE4D*	rs12153798	5	59787640	Not attempted Cohort B
Stroke	*PDE4D*	rs152312	5	59787815	Included
Stroke	*ROCK2*	rs9808232	2	11276571	Low genotyping Cohort A
Stroke	*SPATA13*	rs2793483	13	24759687	Not attempted Cohort B
Stroke	*SPTB*	rs229673	14	65281943	Not attempted Cohort B
Stroke	*TLR4*	rs4986790	9	120475301	Included
Stroke	*ZNF650*	rs10204475	2	170932521	Not attempted Cohort B
<Confused by rs1801177>	*Intergenic*	rs180117	17	67922657	Excluded Intergenic

Table S2

A)

	rs669	rs1801020	rs1130864	rs11575945	rs1799768	rs1800947	rs1926447	rs4341*	rs4934	rs6050
N	104	149	69	113	109	71	104	64	108	134
Kappa	0.849	0.966	0.887	0.667	0.835	0.945	0.829	0.835	0.719	0.622
K CI95%	0.754-0.943	0.920-1	0.604-0.833	0.504-0.831	0.743-0.927	0.838-1	0.733-0.925	0.709-0.961	0.604-0.833	0.494-0.749
MAF	0.30	0.18	0.35	0.12	0.47	0.05	0.30	0.42	0.43	0.22

B)

RS	Common name	Gene	Primer forward (5'-3')	Primer reverse (5'-3')	Reference
rs1130864	C1444T	*CRP*	AGCTCGTTAACTATGCTGGGGCA	CTTCTCAGCTCTTGCCTTATGAGT	[43]
rs11575945	-1C>T	*ANXA5*	GGGCACGAGTTGCAAATGGCG	GTCGCAGCATACAAAGTTGTG	[44]
rs1799768	4G/5G	*SERPINE1 (PAI1)*	TTACCATGGTAACCCCTGGT	AGCCACGTGATTGTCTAGGT	[45]
rs1800947	G1059C	*CRP*	ACTGGACTTTTACTGTCAGGGC	ATTCCCATCTATGAGTGAGAACCT	[43]
rs1801020	C46T / -4C>T	*F12*	CCAGTCCCACTATCTAGAAAAG	ATGGCTCATGGCTGTGATAG	[32]
rs1926447	Thr325Ile	*CBP2 (TAFI)*	GCTTTGTTCAGCATTGTCATAG	CAATTGTGATTGCCATAAAGTG	[46]
rs4341	LD with I/D *	*ACE*	TGGGACCACAGCGCCCGCCACTAC CTGGAGACCACTCCCATCCTTTCT	TCGCCAGCCCTCCCATGCCCATAA GATGTGGCCATCACATTGGTCAGAT	[47]
rs4934	Ala104Thr	*SERPINA3*	CAGAGTTGAGAATGGAGA	TTCTCCTGGGTCAGATTC	[48]
rs6050	Thr312Ala	*FGA*	CCTAGCAGTGCTGGAAGCTG	GGCTCCCAGGGTTTTGGT	[49]

C)

RS	Common name	Gene	Primer forward (5'-3')	Primer reverse (5'-3')	Exon
rs669	Val1000Ile	*A2M*	AGAGATCTTGTGTGGGTGGAA	AAGGACTCTAGGTTCATGCTTCA	E1
			GCAATGCCTCTCTTCCTTCA	TGACCCTGACTAAAAGAACCAA	E2
			ACAGCAAATAGGGGAGGAGA	CAGGTTCCCAGCCTGTTTAT	E3 and 4
			GCAAATACTAGCTGCTTTGCTT	AATGTCACCCATGGGAAATG	E5 and 6
			AGGAATTCCCCCAAAGTTGA	TTTTTGTGTTGCCACTGCTC	E7
			ATGGGGAGGTTCAGTGTCTG	TGCTGCAACTCACTGCTTTT	E8
			TCACACCGGTTATCTGACCA	CAGGCATGGTTTTAGCACAA	E9 and 10
			TGTTTGGTGCCTATTAGTTATTACC	TGGAAATTTTCTCACCCTTAGGT	E11
			TCTCAATCCCAGGAGGCTCT	TCCGTGGTGTAAGGCTGAAT	E12 and 13
			TGAGAGGAGAGAGGGCTTTG	CCCTTTTGGCCCTAATGTTC	E14
			ATGCATGGGTAAACCTGGAA	TCCAGTCATTTTTCCTTGCTC	E15
			TGGATCATGAGGTCAGGAGA	CCCATTACCCTCAACTAGGACA	E16
			TGGATTGCTTGTCAAATTCAA	TGTTTGTTAATTTTTGTCACATTGTA	E17
			TGGTGGCAACTATTACATTCTCTC	TCAGGGACCTCTATTGTTGCAT	E18
			GGGATTCTCTCCCTAGAAAGG	TTTTGCATACAAGAGTTTGCAGT	E19
			TCAAACCCATTCTCCCTCTG	CAAAAACCCCCAACAAAACA	E20 and 21
			TGGTCCCTCCATTAAAACACA	TCAAATGCATTGATGGTGCT	E22
			GCTTCTGGGTCATAGAGCTGTT	TGAAACCTACTGGGAAATCCA	E23 and 24
			TCTGCTTAATGACTTTGATAGATGA	GCTGGCACACAAAAAGAAGC	E24 and 25
			GCCTGTTGCTGTGAAGTGGT	TTTCAGACAGCAGGTCAGCA	E26 and 27
			GGGATGGATATGGGTAAGGA	GGATTTTTGCCATGCAGTTC	E27 and 28
			GGGCATGCAATTCTCATTTC	TCATTCAAATCTTTTGCTACCTGA	E29
			GTCCCTCACATGGGTCTTTG	TTCCCGAAAGAAGACTGGTG	E30 and 31
			GCTGTGGAATGCTTAACATGG	TGCTGGGGATACATACATCA	E32
			CCCCAGGGGTTTCATCTATT	CCTTCCCAGATGTTCCCTTA	E33
			AAACTAATTCAAAGAGAGAAGAAAGC	CCCCAACACATCTCCTAAACC	E34
			TCCCACAGATGTTTGCTTTCT	AAGTCCTTCAGCACTTGATTCC	E35 and 36

Table S3

	Cohort A (n=531)	Cohort B (n=354)	p-value A vs B	Cohort A+B (n=885)	Cohort C (n=287)	Cohort C' (n=98)	p-value C vs A+B	Cohort D (n=491)	p-value D vs A+B
Age mean ± S.D.	70.68±12.0	70.25±12.0	0.616	70.51±12.0	68.40±12.2	70.36±10.78	0.007*	69.17±13.8	0.274
Male gender	54.8% (289)	54.7% (181)	0.964	54.8% (470)	56.7% (161)	51.6% (49)	0.574	61.9% (304)	0.011*
Current smoker	26.1% (131)	20.1% (66)	0.044*	23.7% (197)	22.6% (60)	20.9% (19)	0.693	23.9% (116)	0.956
Hypertension	58.7% (305)	59.9% (197)	0.724	59.1% (502)	59.9% (167)	62.4% (58)	0.830	60.5% (293)	0.614
Diabetes Mellitus	22.9% (120)	18.7% (62)	0.143	21.0% (184)	24.3% (68)	30.9% (29)	0.297	28.8% (139)	0.002*
Atrial Fibrillation	39.3% (205)	29.6% (97)	0.004*	35.5% (302)	22.1% (62)	31.6% (30)	2.81 E-5*	18.7% (91)	7.73 E-11*
Dyslipidemia	33.1% (173)	32.7% (107)	0.899	33.0% (280)	37.7% (106)	43.2% (41)	0.146	37.1% (179)	0.125
Coronary disease	20.3% (103)	14.6% (48)	0.037*	18.0% (151)	16.2% (45)	17.2% (16)	0.482	14.1% (67)	0.066†
TOAST									
Cardioembolism	48.9% (242)	45.5% (157)	0.421	47.6% (399)	37.1% (104)	40.8% (40)	1.56 E-5*	24.6% (118)	1.28 E-18*
Large-artery atherosclerosis	22.6% (110)	21.7% (75)		22.1% (185)	17.1% (48)	8.2% (8)		22.3% (107)	
Other causes	28.5% (141)	32.8% (113)		30.3% (254)	45.7% (128)	51.0% (50)		53.1% (255)	
Hemorrhagic Transformation (HT)	22.9% (115)	20.9% (74)	0.495	22.1% (189)	20.7% (56)	22.4% (22)	0.610	4.9% (24)	7.47 E-17*
Hemorrhagic Infarction (HI)	12.9% (65)	12.0% (42)	0.693	12.5% (107)	12.3% (34)	11.2% (11)	0.865	3.3% (17)	1.00 E-8*
Parenchymal Hematoma (PH)	9.8% (50)	9.1% (32)	0.856	9.6% (82)	8.1% (22)	11.2% (11)	0.466	1.4% (7)	6.28 E-9*
Symptomatic HT	4.1% (21)	3.1% (10)	0.484	3.7% (31)	-	8.16% (8)	-	-	-
Median baseline NIHSS score	16 (0-29)	15 (0-30)	0.262	16 (0-30)	12 (0-41)	11(0-41)	2.74 E-4*	4 (0-16)	1.04 E-107*
Proximal occlusion	58.4% (241)	51.7% (109)	0.111	56.1% (360)	-	13.6% (8)	-	-	-
In-hospital mortality	10.5% (55)	10.6% (35)	0.974	10.6% (90)	-	12.2% (12)	-	-	-

Table S4

A)

SNP	Genotypes	HT(%) Cardioembolic (n=395)			HT(%) Atherothrombotic (n=185)			HT(%) Other etiologies (n=252)		
	HM1 / HT-HM2	HM1	HT-HM2	p-value	HM1	HT-HM2	p-value	HM1	HT-HM2	p-value
rs1801020	CC > T-carriers	34.7	18.4	0.001	16.5	21.7	0.408	15.7	7.1	0.076
rs669	AA > G-carriers	39.9	18.0	4.56E-06	21.4	14.4	0.229	16.5	10.1	0.151
rs1695	GG < A-carriers	17.5	31.3	0.072	8.7	19.2	0.377	3.6	14.2	0.140

B)

SNP	Alleles	HT(%) Cardioembolic (n=395)			HT(%) Atherothrombotic (n=185)			HT(%) Other etiologies (n=252)		
		Risk	No Risk	p-value	Risk	No Risk	p-value	Risk	No Risk	p-value
rs1801020	C > T	31.74	11.43	0.001	18.01	19.70	0.751	14.55	6.25	0.046
rs669	A > G	33.20	17.92	3.47E-05	20.33	13.51	0.123	14.60	9.42	0.131

Table S5

A)

	Death% Cohort A+B (n=885)		
	Present	Absent	p-value
Vascular Risk Factors			
Gender (female)	12.0% (46)	9.3% (43)	0.207
Smoking habit	7.6% (15)	10.9% (68)	0.181
Hypertension	13.1% (65)	7.0% (24)	0.005
Diabetes Mellitus	13.4% (24)	9.8% (65)	0.162
Dyslipidemia	8.6% (24)	11.1% (62)	0.253
Atrial Fibrillation	17.9% (53)	6.6% (36)	4.02E-7
Heart disease	10.7% (16)	10.2% (69)	0.843
Previous Stroke	20.0% (20)	8.9% (65)	0.001
Previous Treatments			
Antiplatelets	13.8% (37)	8.1% (44)	0.011
Statins	14.2% (15)	11.5% (45)	0.459
Oral hypoglycemics	11.9% (8)	11.4% (44)	0.904
Loop diuretics	20.1% (28)	7.9% (25)	1.70E-4
ACE-I	16.5% (15)	10.2% (39)	0.089
ARA-II	9.5% (4)	11.5% (49)	0.703
Radiological study			
Early ischemic signs	12.6% (33)	8.7% (41)	0.090
Clinical parameters			
Proximal occlusion (baseline TCD)	15.4% (53)	6.3% (17)	4.18E-4
Basilar artery occlusion	17.6% (9)	10.2% (80)	0.094
POCI (OCSP)	14.0% (8)	10.3% (77)	0.376
SITS-MOST protocol violations	14.1% (50)	6.6% (23)	0.001
HT occurrence	19.5% (36)	7.8% (51)	3.75E-6
Recanalization (1h after t-PA bolus)	10.6% (16)	13.6% (43)	0.300
TOAST classification			0.030
Cardioembolism	12.9% (51)	-	
Large Artery Atherosclerosis	10.4% (19)	-	
Others	6,4% (16)	-	

B)

	Cohort A+B (n=885)		
	Death	None	p-value
Age (years, mean±S.D.)	76.1±9.2	70.3±11.9	5.07E-6
Systolic blood pressure (mm, mean±S.D.)	157.7±33.4	153.3±25.8	0.449
Diastolic blood pressure (mm, mean±S.D.)	81.8±17.6	83.2±15.9	0.849
Baseline NIHSS (median, IR)	20 (18-20.25)	14 (9-19)	2.63E-14
Body temperature (ºC, median, IR)	36.0 (35.85-36.4)	36.2 (36.0-36.5)	0.147
Glycemia (mg/dl, median, IR)	135.5 (116.75-166)	118 (101-146)	0.003
Time from onset to treatment (min, median, IR)	160 (120-191.25)	155 (122-180)	0.792

C)

Variables in the Equation	Beta	S.E.	p-value	adj. OR	95% C.I.for adj. OR	
					Lower	Upper
Age	0.033	0.014	2.02E-02	1.033	1.005	1.062
Previous stroke	0.921	0.331	5.46E-03	2.511	1.312	4.807
Baseline NIHSS	0.184	0.032	7.10E-09	1.201	1.129	1.279
Atrial Fibrillation	0.723	0.277	9.15E-03	2.060	1.196	3.548
Presence of TH	0.900	0.274	1.03E-03	2.460	1.437	4.211
rs1801020 (C allele)	0.881	0.320	5.83E-03	2.414	1.290	4.517

Table S6

A)

Gene	SNP	Strata	OR	Wald	p-value	OR10	OR01	OR11	Mult. model	Add. model	Synergy Index	RERI	AP	Case test	Case p-value	Control test	Control p-value
LRP1-A2M	rs12307379-rs669	(3-3)	2.435	25.02	5.67E-07	0.812	1.232	2.877	1.001	1.045	42.146	1.832	0.637	0.663	0.416	2.92	0.087
F12-A2M	rs1801020-rs669	(3-3)	2.412	23.85	1.04E-06	1.955	2.485	4.456	4.859	3.44	1.416	1.016	0.228	1.047	0.306	3.287	0.070
KCNMB1-A2M	rs11739136-rs669	(2-3)	3.067	23.03	1.60E-06	0.906	1.689	3.85	1.53	1.595	4.792	2.255	0.586	2.924	0.087	2.285	0.131
APOH-A2M	rs1801692-rs669	(3-3)	2.27	21.50	3.53E-06	0.943	0.791	1.855	0.746	0.755	-3.213	1.121	0.604	0.41	0.522	3.003	0.083
CD40-A2M	rs1883832-rs669	(3-3)	2.382	21.25	4.04E-06	1.334	0.623	2.217	0.831	0.957	-28.322	1.26	0.568	4.209	0.040	3.099	0.078
APOB-A2M	rs1367117-rs669	(3-3)	2.331	21.10	4.37E-06	1.098	1.751	2.911	1.922	1.849	2.252	1.062	0.365	1.274	0.259	0.089	0.765
LRP1-A2M	rs10876966-rs669	(3-3)	2.26	21.06	4.46E-06	1.46	0.946	2.43	1.381	1.406	3.526	1.025	0.422	1.53	0.216	0.619	0.431
CRP-A2M	rs1800947-rs669	(3-3)	2.243	20.95	4.70E-06	4.032	5.655	8.591	22.8	8.687	0.988	-0.096	-0.011	1.165	0.280	0.244	0.621

B)

Gene	SNP	Strata	OR	Wald	p-value	OR10	OR01	OR11	Mult. model	Add. model	Synergy Index	RERI	AP	Case test	Case p-value	Control test	Control p-value
F12-PLA2G7	rs1801020-rs1051931	(3-2)	3.294	24.10	9.16E-07	2.217	1.248	5.613	2.767	2.465	3.148	3.148	0.561	0.929	0.335	0.356	0.551
VEGF-PLA2G7	rs3025010-rs1051931	(3-2)	4.774	19.97	7.89E-06	1.28	2.119	6.564	2.713	2.399	3.977	4.165	0.634	1.046	0.306	0.925	0.336

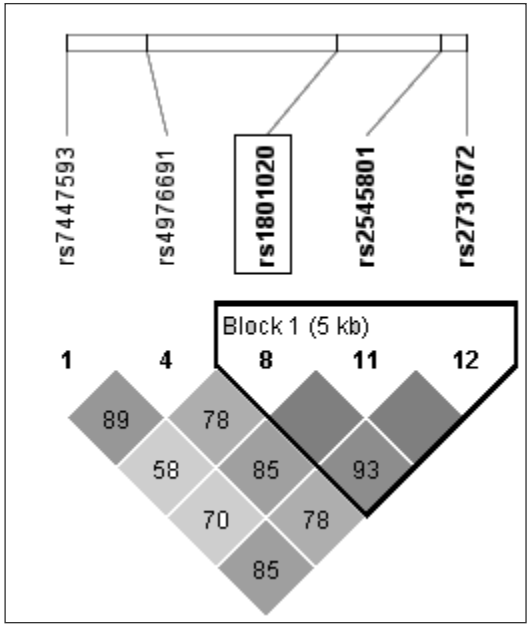
C)

Gene	SNP	Strata	OR	Wald	p-value	OR10	OR01	OR11	Mult. model	Add. model	Synergy Index	RERI	AP	Case test	Case p-value	Control test	Control p-value
SELE-ABCA1 **(HT)**	rs5361-rs2230806	(1-1)	8.9	4.51	0.034	0	0.465	10.155	0	0	-5.963	10.69	1.053	49.16	2.36E-12*	2.311	0.128
OLR1-TLR4 **(D)**	rs11053646-rs4986790	(2-2)	8.909	14.45	1.44E-04	0.111	1.415	8.333	0.157	0.526	-15.478	7.807	0.937	22.23	2.42E-06	0.271	0.603
OLR1-TLR4 **(D)**	rs11053646-rs4986790	(2-3)	0.132	5.27	0.0217	5.6	0.665	0.075	3.725	5.265	-0.217	-5.19	-69.52	20.38	6.36E-06	0.434	0.510

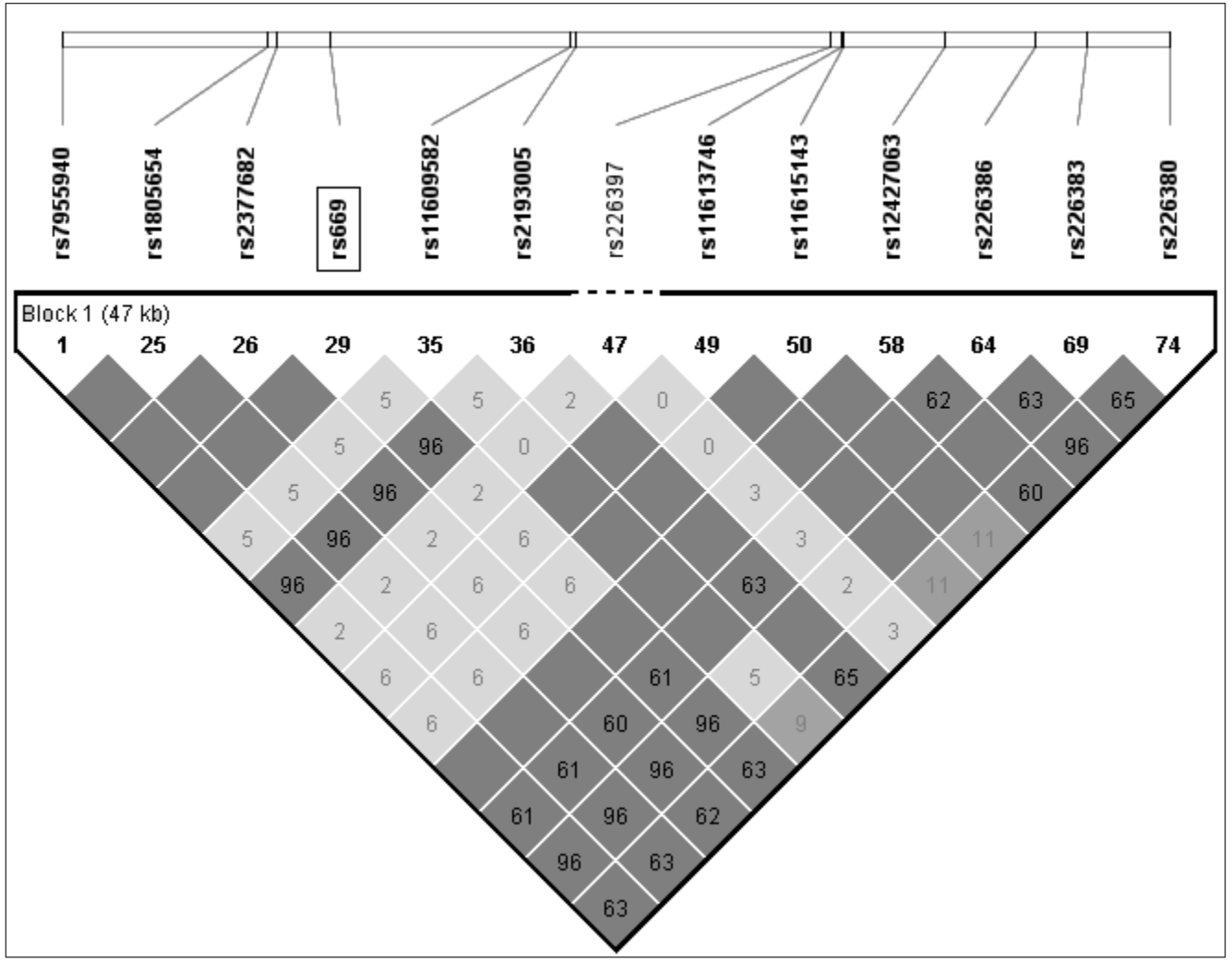
Figure S1

A) Hapmap linkage disequilibrium region

A.1)



A.2)



B) Alignment of V1000I:

Figure S2

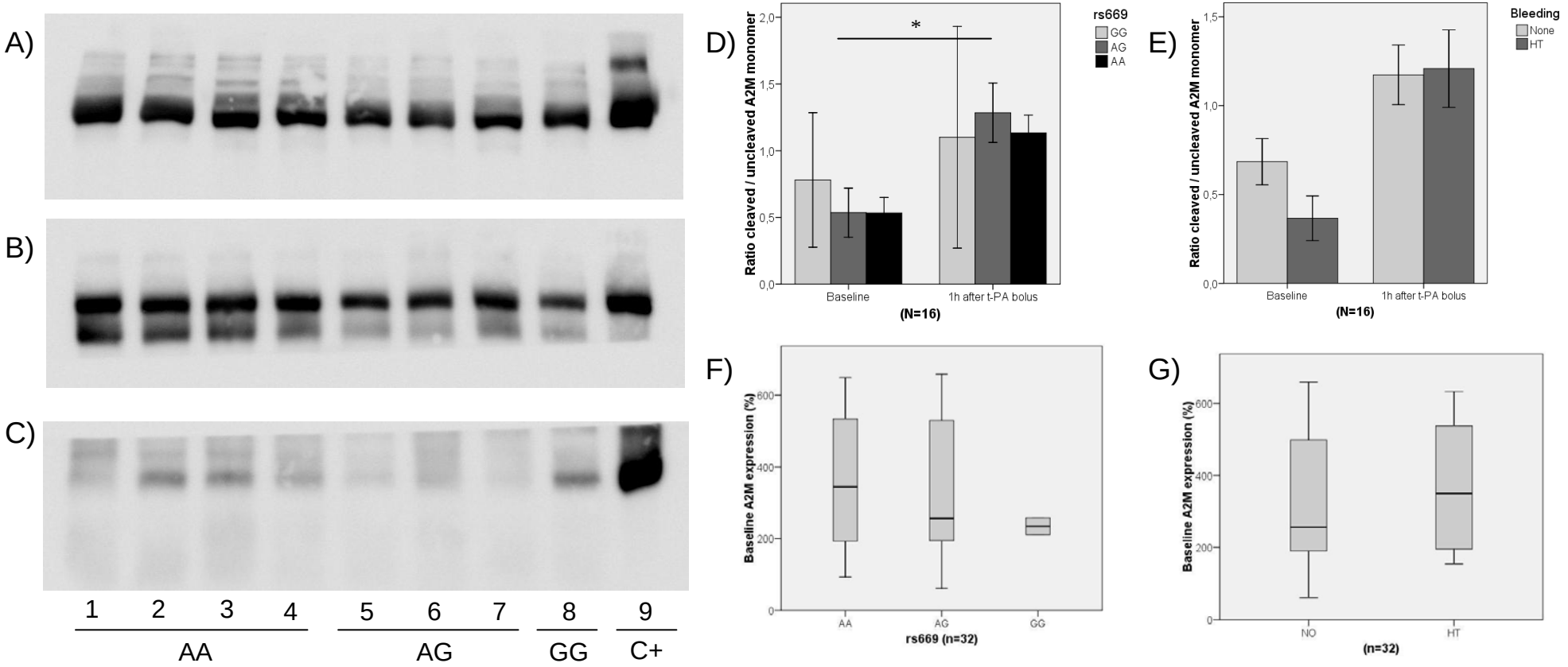

A)
B)
C)
1 2 3 4 5 6 7 8 9
AA
AG
GG
C+
D)
Ratio cleaved / uncleaved A2M monomer
2,0
1,5
1,0
0,5
0,0
*
Baseline
1h after t-PA bolus
(N=16)
rs669
GG
AG
AA
E)
Ratio cleaved / uncleaved A2M monomer
1,5
1,0
0,5
0,0
Baseline
1h after t-PA bolus
(N=16)
Bleeding
None
HT
F)
Baseline A2M expression (%)
600
400
200
0
AA
AG
GG
rs669 (n=32)
G)
Baseline A2M expression (%)
600
400
200
0
NO
HT
(n=32)

Figure S3

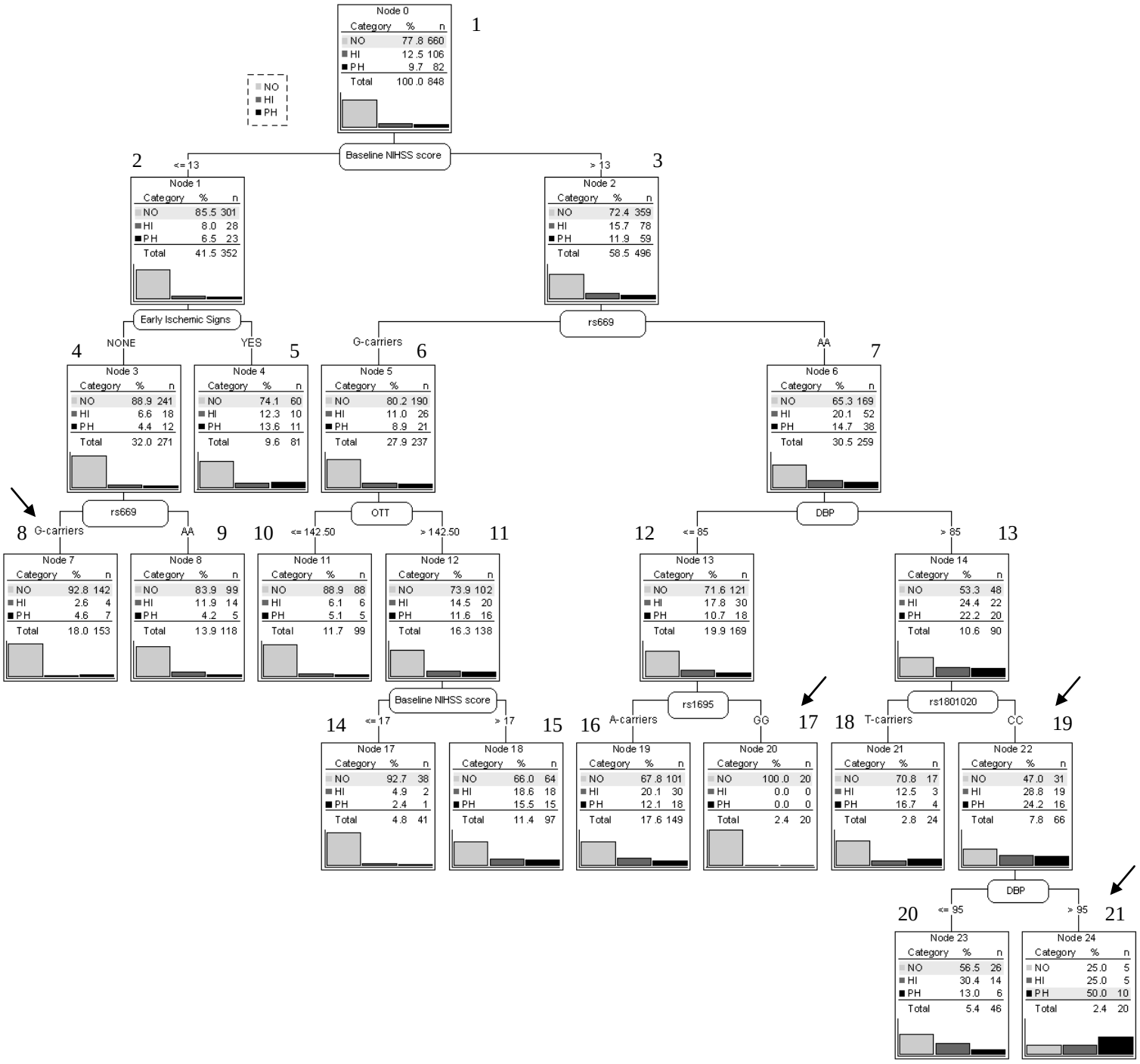

NO
HI
PH
Node 0
Category % n
NO 77.8 660
HI 12.5 106
PH 9.7 82
Total 100.0 848
1
Baseline NIHSS score
<= 13
> 13
2
3
Node 1
Category % n
NO 85.5 301
HI 8.0 28
PH 6.5 23
Total 41.5 352
Node 2
Category % n
NO 72.4 359
HI 15.7 78
PH 11.9 59
Total 58.5 496
Early Ischemic Signs
rs669
NONE
YES
G-carriers
AA
4
5
6
7
Node 3
Category % n
NO 88.9 241
HI 6.6 18
PH 4.4 12
Total 32.0 271
Node 4
Category % n
NO 74.1 60
HI 12.3 10
PH 13.6 11
Total 9.6 81
Node 5
Category % n
NO 80.2 190
HI 11.0 26
PH 8.9 21
Total 27.9 237
Node 6
Category % n
NO 65.3 169
HI 20.1 52
PH 14.7 38
Total 30.5 259
rs669
OTT
DBP
8
9
10
11
12
13
G-carriers
AA
<= 142.50
> 142.50
<= 85
> 85
Node 7
Category % n
NO 92.8 142
HI 2.6 4
PH 4.6 7
Total 18.0 153
Node 8
Category % n
NO 83.9 99
HI 11.9 14
PH 4.2 5
Total 13.9 118
Node 11
Category % n
NO 88.9 88
HI 6.1 6
PH 5.1 5
Total 11.7 99
Node 12
Category % n
NO 73.9 102
HI 14.5 20
PH 11.6 16
Total 16.3 138
Node 13
Category % n
NO 71.6 121
HI 17.8 30
PH 10.7 18
Total 19.9 169
Node 14
Category % n
NO 53.3 48
HI 24.4 22
PH 22.2 20
Total 10.6 90
Baseline NIHSS score
rs1695
rs1801020
14
15
16
17
18
19
<= 17
> 17
A-carriers
GG
T-carriers
CC
Node 17
Category % n
NO 92.7 38
HI 4.9 2
PH 2.4 1
Total 4.8 41
Node 18
Category % n
NO 66.0 64
HI 18.6 18
PH 15.5 15
Total 11.4 97
Node 19
Category % n
NO 67.8 101
HI 20.1 30
PH 12.1 18
Total 17.6 149
Node 20
Category % n
NO 100.0 20
HI 0.0 0
PH 0.0 0
Total 2.4 20
Node 21
Category % n
NO 70.8 17
HI 12.5 3
PH 16.7 4
Total 2.8 24
Node 22
Category % n
NO 47.0 31
HI 28.8 19
PH 24.2 16
Total 7.8 66
DBP
20
21
<= 95
> 95
Node 23
Category % n
NO 56.5 26
HI 30.4 14
PH 13.0 6
Total 5.4 46
Node 24
Category % n
NO 25.0 5
HI 25.0 5
PH 50.0 10
Total 2.4 20

Figure S4

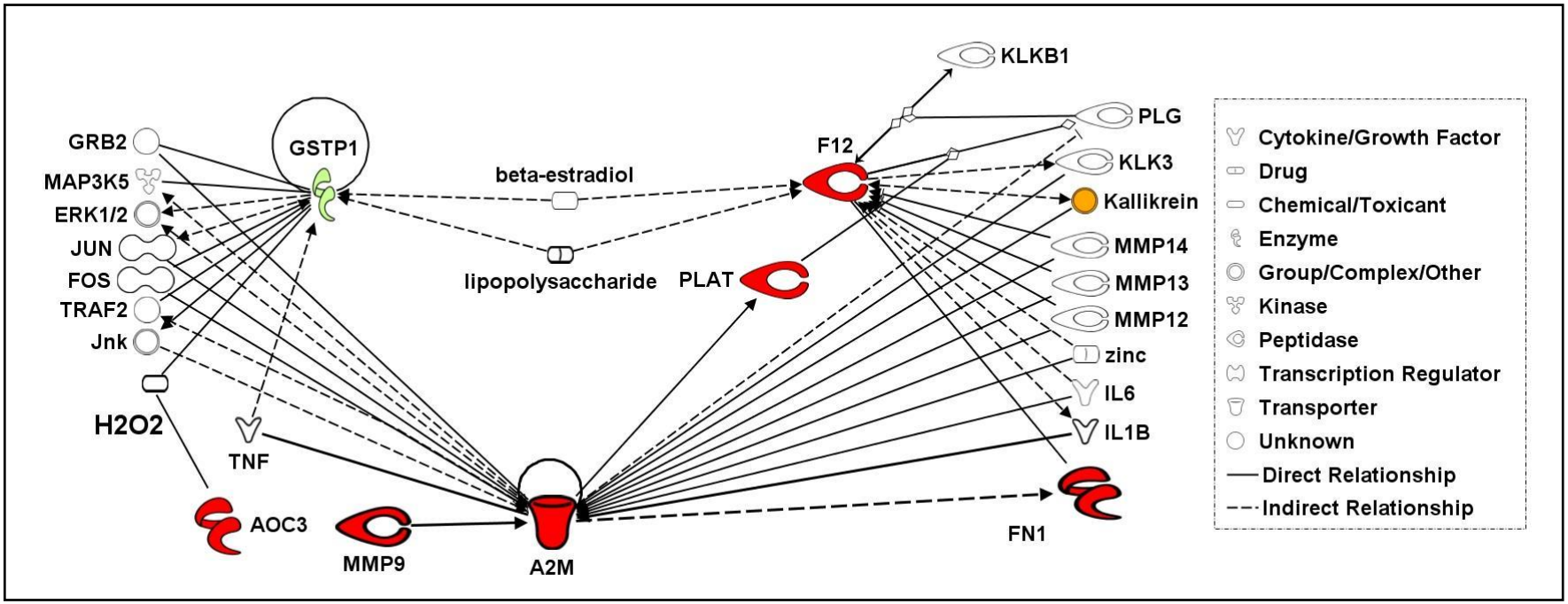
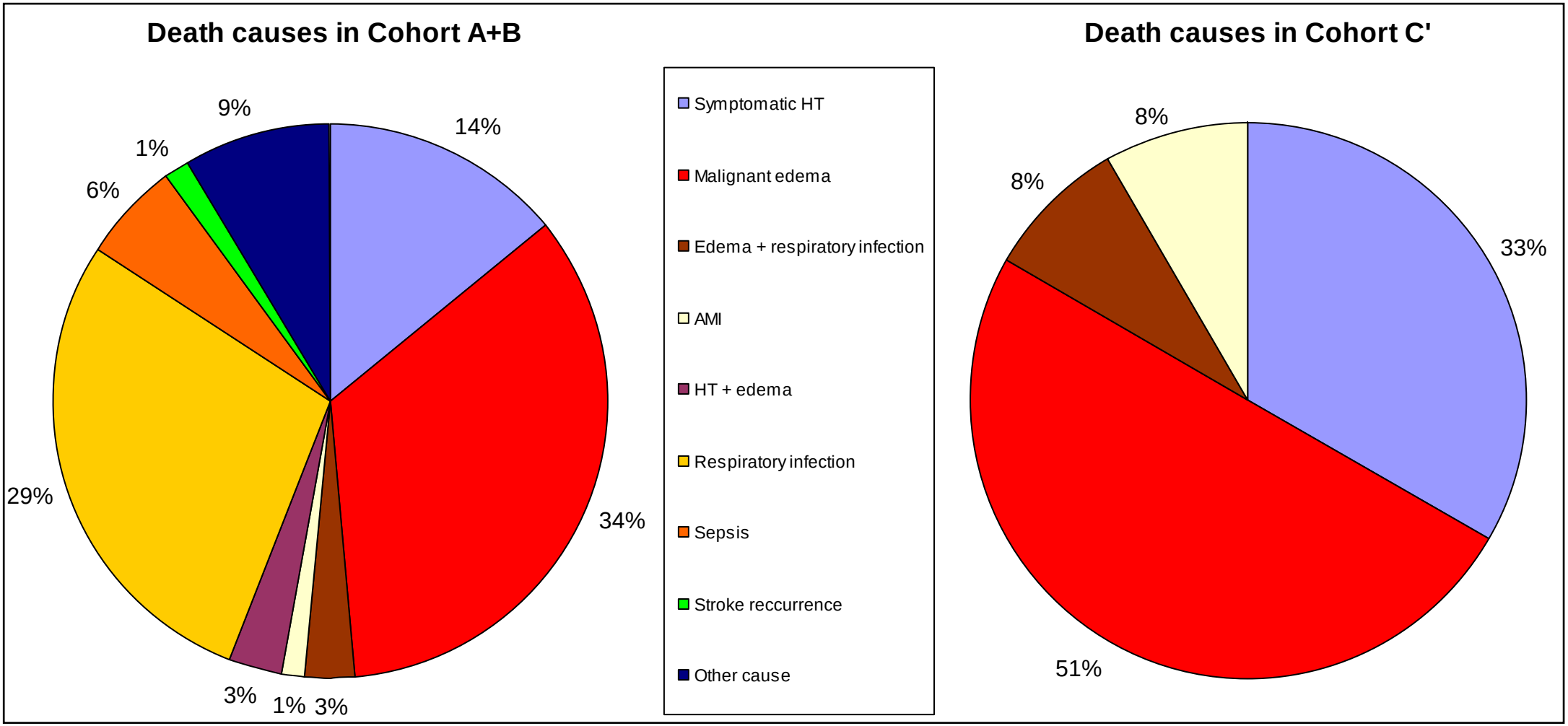

KLKB1
PLG
KLK3
Kallikrein
MMP14
MMP13
MMP12
zinc
IL6
IL1B
FN1
F12
GSTP1
GRB2
MAP3K5
ERK1/2
JUN
FOS
TRAF2
Jnk
H2O2
TNF
AOC3
MMP9
A2M
beta-estradiol
lipopolysaccharide
PLAT
Cytokine/Growth Factor
Drug
Chemical/Toxicant
Enzyme
Group/Complex/Other
Kinase
Peptidase
Transcription Regulator
Transporter
Unknown
Direct Relationship
Indirect Relationship

Figure S5


Death causes in Cohort A+B
9%
14%
1%
6%
29%
34%
3%
1%
3%
Symptomatic HT
Malignant edema
Edema + respiratory infection
AMI
HT + edema
Respiratory infection
Sepsis
Stroke reccurrence
Other cause
Death causes in Cohort C'
8%
8%
33%
51%

ARTICLE 5

Hum Genet. 2010; 127:473–474

Input for Missense/Nonsense Mutations (single base-pair substitutions)

Accession	Codon number	Nucleotide substitution	Amino acid substitution
HM090052	480	TAT-TGT	Tyr-Cys

Comments: Y480C

Gene symbol: SLC3A1

Disease: Cystinuria

Luigi Bisceglia, Pietro Stanziale
Foggia, Servizio di Genetica Medica, IRCCS Casa Sollievo della Sofferenza, Viale Cappuccini, 1, 71013, San Giovanni Rotondo, Italy, Tel.: +0039 0882 416347, Fax: +0039 0882 411616, E-mail: l.bisceglia@operapadrepio.it

Input for Missense/Nonsense Mutations (single base-pair substitutions)

Accession	Codon number	Nucleotide substitution	Amino acid substitution
HM090053	81	cCGC-TGC	Arg-Cys

Comments: The R81C mutation was found in a child showing a cystinuria Type non-I heterozygous phenotype, carrying the mutation R333W in the SLC7A9 gene.

Gene symbol: NR0B1

Disease: Adrenal hypoplasia

O. Thomas Mueller, A. Coovadia
Pathology and Laboratory Medicine, All Children's Hospital, 6th Street South, 801, 33701, Saint Petersburg, USA, Tel.: +011-1-727-767-8985, E-mail: muellert@allkids.org

Input for small deletions (<21 bp)

Accession	Codon number	Deletion
HD090009	454	CATCATC^GGCacAGTCAGCATG

Comments: An abnormality in the DAX1 [NR0B1] gene associated with X-linked congenital adrenal hypoplasia were detected in this patient. A deletion of two bases in the second exon was found which causes frameshift substitutions in codons 455–458 and a nonsense change (premature termination) at codon 459. This deletion has not been previously described but is likely to be pathogenic based on several reports of frameshift deletions in this region of NR0B1 that were reported to be pathogenic (HGMD). Genetic counseling and additional testing for other family members may be indicated. Patient was a 3 year old male with adrenal insufficiency and speech delay.

Gene symbol: NOTCH3

Disease: CADASIL

A. del Río-Espínola, I. Fernández-Cadenas, M. Mendióroz, M. Gutiérrez-Agulló, M.T. Fernández, J. Fernández-Morales, P. Delgado, S. Domingues-Montanari, E. Solé, J. Montaner
Neurology Department, Universitat Autònoma de Barcelona, Neurovascular Research Laboratory and Neurovascular Unit, Vall d'Hebrón Hospital, Passeig Vall d'Hebrón, 119, 08035 Barcelona, Spain, Tel.: +34-934894073, E-mail: adelrio@ir.vhebron.net

Input for Missense/Nonsense Mutations (single base-pair substitutions)

Accession	Codon number	Nucleotide substitution	Amino acid substitution
HM090054	379	cTGT-CGT	Cys-Arg

Comments: We believe that C379R is pathogenic due to four main points:(1) It is a typical mutation of CADASIL patients, involving a cysteine residue substitution.(2) No other pathogenic mutation previously reported was seen by direct sequencing of exons 1–25 of NOTCH3 gene.(3) This mutation was not seen in 53 population-matched controls, accounting for 106 alleles.(4) A mutation involving the same cysteine residue (C379S) was reported as pathogenic. "Opherk et al, Long-term prognosis and causes of death in CADASIL: a retrospective study in 411 patients. Brain 2004; 127:2533–9".

Gene symbol: NOTCH3

Disease: CADASIL

A. del Río-Espínola, I. Fernández-Cadenas, M. Mendióroz, M. Gutiérrez-Agulló, MT Fernández, J. Fernández-Morales, O. Maisterra, S. Domingues-Montanari, V. García-Patos, E. Solé, J. Montaner
Neurology Department, Universitat Autònoma de Barcelona, Neurovascular Research Laboratory and Neurovascular Unit, Vall d'Hebrón Hospital, Passeig Vall d'Hebrón, 119, 08035, Barcelona, Spain, Tel.: +34 934894073, E-mail: adelrio@ir.vhebron.net

Input for Missense/Nonsense Mutations (single base-pair substitutions)

Accession	Codon number	Nucleotide substitution	Amino acid substitution
HM090055	1250	TGCg-TGG	Cys-Trp

Comments: We believe this mutation is pathogenic based in four main points:(1) It is a typical CADASIL mutation, involving a cysteine residue substitution(2) No other previously reported pathogenic mutation was found after direct sequencing of exons 1–25 of NOTCH3 gene(3) The patient has a definitive diagnosis of CADASIL, as granular osmiophilic material (GOM) accumulations were found by electron microscopy of a skin biopsy sample(4) This mutation was not found in 53 population-matched controls, accounting for 106 alleles

Gene symbol: UROD

Disease: Porphyria, cutanea tarda

Maria Savino, Maria Garrubba, Leopoldo Zelante, Filippo Aucella, Claudio Carmine Guida, Stefano Angelo Santini
FG, IRCCS Hospital CSS, Cappuccini, 1, 71013 San Giovanni Rotondo, Italy, Tel.: +39 882 410291, Fax: +39 882 410291, E-mail: mg.savino@operapadrepio.it

Input for splicing mutations (single base-pair substitution)

Accession	Nucleotide substitution	Intron designation, number or letter	Donor/acceptor	Relative location
HS090005	G-A	9	Donor	+1

Comments: The 942 + 1G > A or IVS9 + 1G > A at 5′ donor site of exon 9 is a splicing site defect causing the exon 9 deletion and a premature stop codon within exon 10.

ARTICLE 6

Hum Genet. 2010; 127:474

Input for Missense/Nonsense Mutations (single base-pair substitutions)

Accession	Codon number	Nucleotide substitution	Amino acid substitution
HM090054	379	cTGT-CGT	Cys-Arg

Comments: We believe that C379R is pathogenic due to four main points:(1) It is a typical mutation of CADASIL patients, involving a cysteine residue substitution.(2) No other pathogenic mutation previously reported was seen by direct sequencing of exons 1–25 of NOTCH3 gene.(3) This mutation was not seen in 53 population-matched controls, accounting for 106 alleles.(4) A mutation involving the same cysteine residue (C379S) was reported as pathogenic. "Opherk et al, Long-term prognosis and causes of death in CADASIL: a retrospective study in 411 patients. Brain 2004; 127:2533–9".

Gene symbol: NOTCH3

Disease: CADASIL

A. del Río-Espínola, I. Fernández-Cadenas, M. Mendióroz, M. Gutiérrez-Agulló, MT Fernández, J. Fernández-Morales, O. Maisterra, S. Domingues-Montanari, V. García-Patos, E. Solé, J. Montaner
Neurology Department, Universitat Autònoma de Barcelona, Neurovascular Research Laboratory and Neurovascular Unit, Vall d'Hebrón Hospital, Passeig Vall d'Hebrón, 119, 08035, Barcelona, Spain, Tel.: +34 934894073, E-mail: adelrio@ir.vhebron.net

Input for Missense/Nonsense Mutations (single base-pair substitutions)

Accession	Codon number	Nucleotide substitution	Amino acid substitution
HM090055	1250	TGCg-TGG	Cys-Trp

Comments: We believe this mutation is pathogenic based in four main points:(1) It is a typical CADASIL mutation, involving a cysteine residue substitution(2) No other previously reported pathogenic mutation was found after direct sequencing of exons 1–25 of NOTCH3 gene(3) The patient has a definitive diagnosis of CADASIL, as granular osmiophilic material (GOM) accumulations were found by electron microscopy of a skin biopsy sample(4) This mutation was not found in 53 population-matched controls, accounting for 106 alleles

Gene symbol: UROD

Disease: Porphyria, cutanea tarda

Maria Savino, Maria Garrubba, Leopoldo Zelante, Filippo Aucella, Claudio Carmine Guida, Stefano Angelo Santini
FG, IRCCS Hospital CSS, Cappuccini, 1, 71013 San Giovanni Rotondo, Italy, Tel.: +39 882 410291, Fax: +39 882 410291, E-mail: mg.savino@operapadrepio.it

Input for splicing mutations (single base-pair substitution)

Accession	Nucleotide substitution	Intron designation, number or letter	Donor/acceptor	Relative location
HS090005	G-A	9	Donor	+1

Comments: The 942 + 1G > A or IVS9 + 1G > A at 5′ donor site of exon 9 is a splicing site defect causing the exon 9 deletion and a premature stop codon within exon 10.

ARTICLE 7

Neurology. 2010; 75(22):2033-5.

A missense *HTRA1* mutation expands CARASIL syndrome to the Caucasian population

M. Mendioroz, I. Fernández-Cadenas, A. del Río-Espinola, et al.

Neurology 2010;75;2033
DOI 10.1212/WNL.0b013e3181ff96ac

Clinical/Scientific Notes

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J. Montaner, MD, PhD

A MISSENSE *HTRA1* MUTATION EXPANDS CARASIL SYNDROME TO THE CAUCASIAN POPULATION

Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) (MIM 600142) is a heritable small vessel disease clinically characterized by nonhypertensive leukoencephalopathy associated with alopecia and spondylosis.[1-3] First described by Maeda et al.[2] in 1965, cases exclusively come from Japan and China.[3,4] Beginning in young adulthood, patients develop progressive motor and cognitive impairment and usually die within 10 years.[1] Centrifugally enlarged arteries with widespread loss of both medial smooth muscle cells and mural extracellular matrix are observed in the white matter and leptomeninges.[5] In July 2009, Hara et al.[6] described the HtrA serine protease 1 gene (*HTRA1*) as the causative gene of CARASIL. Here we report for the first time a Caucasian patient with CARASIL harboring a novel *HTRA1* mutation.

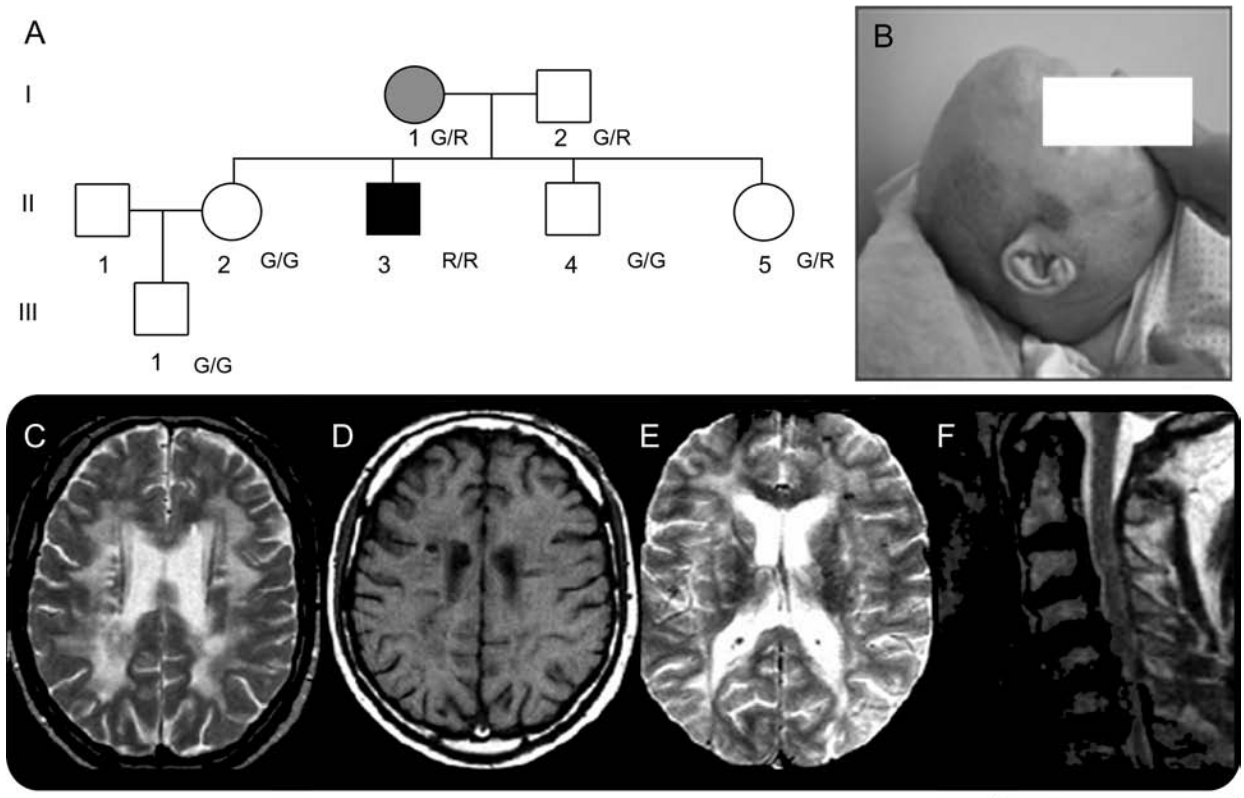
Case reports. The proband (II-3) was a 34-year-old man who in 2006 presented to the Neurology Department because of unsteady gait, urinary urgency, and slurred speech. He had a history of 150-pack-year cigarette smoking and alcohol and cocaine abuse for 8 years. No hypertension, diabetes mellitus, or dyslipidemia was recorded. Before 18 years of age, he had developed severe alopecia with male-pattern baldness. On examination, cognitive function was preserved but he showed dysarthria, dysphagia, emotional instability, and spastic gait. On the lower limbs, deep-tendon reflexes were slightly increased with no clonus, and Babinski sign was bilaterally present. During the following year, cognitive impairment with dysexecutive syndrome and progressive upper limbs weakness were noticed. Later on, the pseudobulbar syndrome and tetraparesis progressed until he was bedridden at age 43. Hemogram, biochemistry, and serologic analyses were normal. T2- and T1-weighted MRI of the brain showed a diffuse leukoencephalopathy involving periventricular and deep white matter, including anterior temporal lobes and external capsules, with multiple lacunar infarcts concerning the deep white and gray matter of both brain hemispheres and brainstem (figure, C and D). Transverse T2*-GE images revealed multiple microbleeds on pons, basal ganglia, and hemispheric subcortical white matter (figure, E). In addition, cervical spine MRI demonstrated prominent multilevel degenerative changes causing moderate to severe stenosis of the cervical canal (figure, F). Latencies of visual evoked potentials were not prolonged and CSF analysis showed no oligoclonal bands. Granular osmiophilic materials (GOMs), the pathologic hallmark of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (MIM125310), were not found by electron microscopy examination of skin biopsy.

Moreover, 6 relatives were examined and none of them showed complete CARASIL phenotype, although nonhypertensive leukoencephalopathy was found in the mother of the proband and a sister (II-1) showed cervical and lumbar arthrosis. They belonged to a Caucasian family with Spanish ancestry.

Direct sequencing of exons 1–25 of *NOTCH3* was performed in the proband and his mother to rule out CADASIL. Afterwards, sequencing of the coding region of *HTRA1* in the proband revealed a homozygous G to A transition at position 883 (c.883G>A) in exon 4. The heterozygous c.883G>A mutation was found in both parents and a sister (II-5) and no other mutations were found in the mother after complete sequencing of *HTRA1*. Furthermore, this mutation was not found in 80 healthy subjects used as controls (PCR conditions available upon request). All subjects gave informed, written consent and the study was approved by the Ethics Committee of our institution.

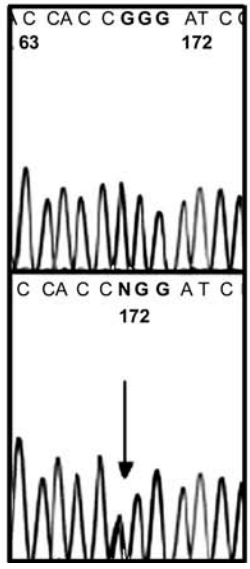
Discussion. We report for the first time a Caucasian patient with a CARASIL phenotype harboring a new missense mutation in the *HTRA1* gene. The deleterious effect of this mutation is supported by several arguments. First, the change occurs in the binding pocket of the protease domain of HtrA1, a highly conserved region essential for the activity of HtrA1 (figure G), where other CARASIL mutations were previously described (V297M and A252T)[6] and leads to the substitution of a noncharged polar amino acid (glycine) for the most polar positively charged residue (arginine) (p.Gly295Arg), predicted as

Figure Summary of results of the patient with cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL)



G

Species	Gene			252			295		297		
Homo sapiens	*HTRA1*	D	I	A	L	I	G	I	V	S	T
Homo sapiens	*HTRA2*	D	I	A	T	L	G	I	V	S	S
Homo sapiens	*HTRA3*	D	I	A	T	I	G	I	V	S	T
Homo sapiens	*HTRA4*	D	L	A	V	I	G	I	V	S	T
Pan troglodytes	*HTRA1*	D	I	A	L	I	G	I	V	S	T
Bos taurus	*HTRA1*	D	I	A	L	I	G	I	V	S	T
Mus musculus	*HTRA1*	D	I	A	L	I	G	I	V	S	T
Rattus norvegicus	*HTRA1*	D	I	A	L	I	G	I	V	S	T
Cavia porcellus	*HTRA1*	-	-	-	-	-	G	I	V	S	T
Danio rerio	*HTRA1*	D	I	A	L	I	G	I	V	S	T
Drosophila melanogaster	*HTRA1*	D	I	A	L	I	G	I	V	S	T
Arabidopsis thaliana	*HTRA1*	D	I	A	L	V	G	I	V	S	C
Saccharomyces cerevisiae	*HTRA1*	N	F	A	I	V	D	I	S	S	V
Escherichia coli	*HTRA1*	D	I	A	L	I	G	I	V	S	A
New CARASIL mutation *HTRA1*							R				



(A) *HTRA1* mutation distribution in the pedigree; the black symbol indicates complete CARASIL phenotype and the gray symbol only leukoencephalopathy. Aminoacids in position 295 are noted as glycine (G) or arginine (R). (B) The proband showed severe alopecia. (C–E) Brain transverse MRI revealed an extensive leukoencephalopathy involving anterior temporal lobes, external capsules, and semiovale centers (T2-weighted) (C); ancient lacunar infarctions (T1-weighted) (D); and subcortical microbleeds (T2*GE) (E). (F) Cervical spine sagittal T2-weighted MRI showed multilevel degenerative disease producing moderate to severe stenosis of the central canal. (G) Sequence analysis of *HTRA1* revealed a transition in exon 4 (c.883 G>A), leading to the replacement of an evolutionary conserved glycine to arginine at position 295. Mutated glycine residue is shown in black; pathogenic mutations reported by Hara et al. are shown in gray.

pathogenic with the Polyphen-2 software[7] (score 0.996; sensitivity 0.44/specificity 0.97). Finally, the p.Gly295Arg mutation is not a polymorphism since it was not detected in 160 control chromosomes and it was not described in SNP databases.

To date, CARASIL has only been described among Asians.[3,4] Therefore, this report expands CARASIL to white Caucasians and from now on, this condition should be considered in the differential diagnosis of small vessel disease in this popula-

tion. Although CARASIL resembles CADASIL, paying attention to extraneurologic signs could help to reach a correct diagnosis. Interestingly, a moderate leukoencephalopathy was also observed in a heterozygous carrier of the p.Gly295Arg mutation, in absence of hypertension or CADASIL disease. The actual prevalence of this condition merits further investigation.

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Disclosure: Dr. Mendioroz receives research support from the Instituto de Salud Carlos III (Spanish Ministry of Health) (FIS 05/00081). Dr. Fernández-Cadenas reports no disclosures. A. del Río-Espínola receives research support from Institut de Recerca Vall d'Hebrón. Dr. Rovira has received speaker honoraria from Bayer Schering Pharma and Teva Pharmaceutical Industries Ltd.; serves on the editorial boards of the American Journal of Neuroradiology *and* Neuroradiology*; and has served on the speakers' bureau for Bayer Schering Pharma. Dr. Solé, Dr. Fernández-Figueras, and Dr. García-Patos report no disclosures. Dr. Sastre-Garriga serves on scientific advisory boards for Bayer Schering Pharma, Biogen Idec, Novartis, Merck Serono, Pharmaceutical Industries Ltd., and the European MS Platform; and has received funding for travel and speaker honoraria from Bayer Schering Pharma, Biogen Idec, Novartis, Merck Serono, Pharmaceutical Industries Ltd., Almirall, and the European MS Platform. S. Domingues-Montanari, Dr. Alvarez-Sabin, and Dr. Montaner report no disclosures.*

Received April 9, 2010. Accepted in final form June 16, 2010.

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LACK OF ASSOCIATION BETWEEN VEGF GENE POLYMORPHISMS AND PLASMA VEGF LEVELS AND SPORADIC ALS

The connection between the AAG/AAG and AGG/AGG haplotypes of the 3 single nucleotide polymorphisms (SNPs) (−2578C/A, −1154G/A, and −634G/C) of the vascular endothelial growth factor (VEGF) gene and sporadic amyotrophic lateral sclerosis (sALS) was documented in some, but not all populations.[1] It was also shown that plasma VEGF levels in patients with sALS were significantly lower than in the controls.[1] A recent meta-analysis comprising 7,082 participants did not confirm the association between risk haplotypes and sALS.[2] Other studies did not confirm an association between plasma or serum VEGF levels and the risk of sALS.[3–5] We studied the significance of the AAG/AAG and AGG/AGG haplotypes of the 3 SNPs of the *VEGF* gene as well as the significance of plasma VEGF levels in Polish patients with sALS.

Methods. We included 271 unrelated patients with definite or probable sALS (El Escorial Criteria) admitted to the MND Clinic, Jagiellonian University, Poland. The controls consisted of 464 age- and gender-matched white, neurologically intact subjects. Additionally, 60 patients with sALS and 75 controls had plasma VEGF levels measured. All subjects gave written informed consent to participate in this study. The local ethical committee approved the study protocol.

Genotyping was performed by RT-PCR or restriction fragment length polymorphism methods.[6] Plasma VEGF levels were detected by ELISA (Quantikine; R&D Systems).[1]

Comparisons between groups were made with χ^2 or Student *t* tests. Hardy-Weinberg equilibrium was checked using χ^2 test. LD was evaluated with the software SAS Genetics and expressed as D≪. The association of the studied genotypes with sALS was assessed under assumptions of the dominant, recessive, or additive effects. Associations between the formed haplotypes were tested using the software SAS Genetics. Our results were combined with those of the previous studies assessing the significance of

C. Resum de resultats i discussió

1) Eficàcia del tractament amb t-PA

S'han identificat 3 polimorfismes, rs1799768 ó 4G/5G del gen *PAI-1* (4G/4G: 12.5% reoclusió 4G/5G-5G/5G: 2.7% reoclusió; p=0.025), rs1883832 ó -1C>T (T: 14.6% reoclusió C: 5.5% reoclusió; p=0.003) del gen *CD40* i rs1800801 ó -7A>G (AA: 17.2% reoclusió AG-GG: 5.6% reoclusió; p=0.042) del gen *MGP* associats amb una taxa més elevada de reoclusions arterials després de la recanalització de l'artèria per efecte del t-PA.

Els polimorfismes associats amb la reoclusió arterial i el model predictiu que se'n deriva s'han de validar en cohorts independents per confirmar la seva validesa. Aquest fet va ésser impossible per la dificultat d'aconseguir dades i mostres d'ADN d'una sèrie de pacients tractats amb t-PA amb un seguiment per Doppler Transcraneal tan exhaustiu.

Els estudis presentats en aquesta tesi són les primeres associacions genètiques amb el fenotip de reocclusió arterial. Un cop les dades hagin estat validades, s'ha de continuar amb extensius estudis funcionals en models animals d'isquèmia cerebral de tipus embòlic (inicialment en rata i ratolí), per determinar si els nivells elevats de PAI-1 **(article 1)**, CD40 o MGP **(article 2)** provoquen la reoclusió del vas.

Si els resultats són positius, hauríem de plantejar-nos la monitorització exhaustiva dels pacients amb els genotips de risc i adaptar el tractament fibrinolític al seu perfil de risc, administrant inhibidors específics per PAI-1 o CD40 o potser canviant el tractament preventiu secundari (MGP i selecció de l'inhibidor de la vitamina K més adequat).

D’altra banda, s’han identificat 4 variants associades a la recanalització ràpida del vas: rs1063856 ó Thr786Ala del gen *VWF* (GG: 52.6% Rec1h vs A-carriers: 30.1% Rec1h, p=0.001), rs1143627 (-511T>C) (CC: 53.2% Rec1h vs T-carriers: 30.9% Rec1h; p=0.002) i rs16944 (-31C>T) (TT: 50% Rec1h vs C-carriers: 30.2% Rec1h; p=0.005) del gen *IL1B* i rs2070584 (+4840G>T) (T:39.5% Rec1h vs G:28.5% Rec1h; 0.001) del gen *TIMP1*. Hem aconseguit crear un model predictiu de la recanalització emprant 3 d’aquests polimorfismes (rs1063856, rs1143627, rs2070584) que poden combinar-se amb les dades clíniques d’interès (classificació TOAST de fase aguda o fibrilació auricular) per discriminar quins pacients recanalitzaran més ràpidament (àrea sota la corba ROC 0.6-0.7). No s’ha pogut determinar un efecte funcional de les variants a la fase aguda de l’ictus, abans de rebre el tractament trombolític.

Tot i que hem aconseguit replicar els resultats **(article 3)**, cal expandir els resultats a altres grups ètnics i manca per identificar el significat funcional d'aquestes variants a la fase aguda. Caldria incrementar el tamany mostral, recollir mostres a diferents temps entre la mostra basal i el final de la infusió de t-PA, o fent servir estudis de coagulació i fibrinòlisi “in vitro” com el Clot Lysis.

L'estudi Clot Lysis estudia la coagulació (sense t-PA) o la coagulació i posterior lisi (amb t-PA) de la sang de cada pacient i s'ha demostrat que aquests paràmetres són fortament heretables, indicant que la genètica juga un paper important en aquest procés (Carter et al, 2007). Per a l'estudi, podrien seleccionar-se sangs dels genotips adequats o, complementariament, afegir proteïna recombinant de cadascuna de les molècules a provar.

Com s'indica a l'**article 3**, rs1063856 regularia els nivells de vWF i FVIII abans de l'ictus i

podria afectar a la composició del coàgul. Les variants rs1143627 i rs16944, per trobar-se a la regió promotora, han d'afectar a la transcripció de la IL-1β, però caldria un nombre de mostres més elevat per apreciar diferències significatives a la fase aguda o, d'altra banda, és possible que el seu efecte tingui lloc en altres teixits apart de la sang perifèrica.

El polimorfisme rs207584 de TIMP-1 s'ha de confirmar en estudis independents perquè només es va confirmar a la validació interna **(article 3)**. Com es troba a la regió 3' UTR del gen, la seva funcionalitat ha d'estar lligada a mecanismes regulatoris o bé la variant funcional és un altre SNP en desequilibri de lligament. En qualsevol cas, els nivells basals de TIMP-1 són predictors de l'evolució funcional dels pacients i caldrà determinar si és només per la seva activitat inhibidora de MMPs.

Finalment, de les variants anteriorment publicades, la variant I/D de l'ECA semblava predir la recanalització arterial en aquesta nova sèrie a la cohort A, tot i que el genotipatge va fallar a la cohort B i no la vam incloure als models predictius. El polimorfisme Thr325Ile del TAFI, per la seva banda, no es va associar a la recanalització arterial en aquestes dues cohorts.

2) Seguretat del tractament amb t-PA

S'han identificat 3 polimorfismes associats amb la Transformació Hemorràgica iatrogènica posterior al tractament amb t-PA: rs669 (V1000I) (A: 24.7% TH vs G: 14.6% TH; $p=10^{-5}$) del gen *A2M*, rs1801020 (-4C>T) (C: 23.5% TH vs T:14.6% TH; p=0.001) del gen *F12* i rs1695 (V104I) (GG:10.6% TH vs A-carriers: 23.6%; p=0.004) del gen *GSTP1*. A més, la variant rs1801020 era un predictor independent de mortalitat intrahospitalària (C: 12.4% mort vs T: 5.2% mort; $p=4.8 \times 10^{-4}$). Dos d'aquests polimorfismes (rs669 i rs1801020) s'han combinat amb dades clíniques (NIHSS basal, fibrilació auricular, OTT i pressió arterial

diastòlica) per generar un model predictiu d'hemorràgies (àrea sota la corva ROC=0.720), que també s'associa amb la mortalitat intrahospitalària i el pronòstic funcional al tercer mes. Aquest model té més capacitat predictiva que l'anteriorment publicat HAT score (0.720 vs. 0.603; p=0.0044) i va ésser validad en una sèrie independent.

Cal extendre els resultats a sèries més àmplies amb gran nombre de transformacions hemorràgiques simptomàtiques, per determinar si el model validat és extrapolable a altres poblacions.

Les 3 variants associades presentaven una associació amb els nivells o l'activitat de la proteïna abans i després del tractament amb t-PA. Per poder modificar la intervenció terapèutica pel risc de sagnat, s'haurien d'iniciar estudis funcionals en models embòlics d'isquèmia i alhora completar els estudis en humans en tamanys mostrals més grans.

Cal determinar si l'activitat de l'A2M controla l'apertura de la BHE **(article 4)**: amb WB més extensos, ELISAs específics per les dues conformacions (nativa i proteolitzada) i assaigs d'activitat "in vitro" amb les variants Valina i Leucina de la proteïna. S'ha de descartar també problemes a la producció de l'A2M, que podria solventar-se en cultius cel·lulars transfectats amb cadascuna de les variants.

Els nivells de FXII podrien ésser més informatius i presentar una millor correlació amb el genotip que l'activitat coagulativa **(article 4)**, pero la seva rellevància clínica seria més dubtosa. Ara bé, qualsevol alteració a la cascada de la coagulació es compensa per la resta de factors i els efectes podrien venir per altres funcions, com l'edema vasogènic. Notòriament, el fragment del FXII després d'un segon tall proteolític per la kallikreína (FXIIf), conserva la seva capacitat d'activar la via de les quinines i el complement, mentre

ja no activa la coagulació intrínseca a través del FIX. Si la meva hipòtesi és correcta, la mesura dels nivells d'aquest fragment podria ésser un biomarcador útil a la pràctica clínica.

Pel que fa al GSTP1 **(article 4)**, cal identificar els seus substrats rellevants durant la isquèmia (ex. peròxid d'hidrògen), ja sigui en models animals o en cultius sotmesos a deprivació d'oxígen i glucosa. S'ha de determinar si la modificació d'especificitat de substrat justifica els canvis als nivells plasmàtics i identificar en quin(s) fenòmen(s) relacionat(s) a la ruptura del vas participa; els processos més versemblants serien el *stress* oxidatiu i la infiltració leucocitària.

3) Diagnòstic genètic de les malalties de CADASIL i CARASIL

S'han identificat 2 mutacions noves causants de la malaltia de CADASIL: c.1135C>T ó C379R a l'exó 7 **(article 5)** i c.3750C>G ó C1250W a l'exó 23 **(article 6)**. Ambdúes afecten a residus de cisteïna, com és habitual a la patologia.

Les mutacions han estat identificades en una sola familia i la seva representació dins del conjunt de malalts de CADASIL pot ésser molt reduida. Podria realitzar-se un genotipatge més extens de controls sans, tot i que ambdues mutacions semblen clarament patogèniques.

Una mutació al mateix codó de l'exó 7 **(article 5)** havia estat identificada amb anterioritat, canviant per serina enlloc d'arginina (Opherk et al, 2004). La mutació de l'exó 23 **(article 6)**, amb 2 membres afectes, cosegregava amb les hiperintensitats a la resonància magnètica.

Notablement, les dues mutacions descrites es troben fora de les regions més freqüents, indicant que caldria fer un esforç diagnòstic i seqüenciar la regió EGF complerta (exons 2 al 24) més sovint, perquè ens permetria diagnosticar correctament molts més malalts de CADASIL.

D'altra banda, hem identificat el primer cas en població caucàsica de CARASIL o Síndrome de Maeda, portador de la mutació c.883G>A ó G295R. El pacient presentava un fenotip complert (calvicie, ictus i problemes cervicals). El curs de la malaltia era força més agressiu que a la CADASIL.

Només existia un pacient afecte dins la familia estudiada. A més, trobàvem fenocopies amb un fenotip incomplert dins de la mateixa familia (mare i germana gran). Podria realitzar-se un genotipatge més extens de controls sans, tot i que la localització i grau de conservació de la mutació ens fa pensar que és clarament patogènica.

Aquest cas clínic **(article 7)** mostra com la malaltia de CARASIL també es troba present en població caucàsica i l'hem de considerar pel diagnòstic diferencial de les malalties de petit vas. Totes les mutacions descrites fins ara al gen *HTRA1* es localitzen al domini proteasa de l'enzim, encara que no afecten el seu centre actiu. Sembla que aquest pot ésser el punt on les mutacions són més freqüents *("hot spot")*, però la curta llargada del gen (9 exons) i l'abaratiment progressiu de les tècniques de genotipatge fan molt més recomanable seqüenciar el gen complert en tots els pacients amb simptomatologia compatible.

D. Conclusions

1) Several polymorphisms associated with t-PA efficacy have been identified: variants 4G/5G (rs1799768) of *PAI-1* gene, -1C>T (rs1883832) of *CD40* gene and -7A>G (rs1800801) of *MGP* gene are associated with higher reocclusion risk after effective thrombolytic therapy.

Variants Thr786Ala (rs1063856) of *VWF* gene, -511T>C (rs1143627) and -31C>T (rs16944) of *IL1B* gene and +4840G>T (rs2070584) of *TIMP1* gene are associated with early artery recanalization after t-PA infusion.

2) Several polymorphisms associated with t-PA safety have been identified: variants Val1000Ile (rs669) of *A2M* gene, -4C>T (rs1801020) of *F12* gene and Val104Ile (rs1695) of *GSTP1* gene are associated with Hemorrhagic Transformation and -4C>T (rs1801020) of *F12* gene with in-hospital mortality.

3) Two new mutations (C379R i C1250W) causatives of CADASIL were identified in *NOTCH3* gene and the first caucasian case of CARASIL disease was diagnosed, an individual homozygous for G295R mutation in *HTRA1* gene.

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