Project title:

IMPROVEMENT DIAGNOSIS AND FOLLOW-UP OF PATIENTS WITH THROMBOTIC MICROANGIOPATHY: DIFERENTIAL DIAGNOSIS OF HAEMOLITIC UREMIC SYNDROME AND THROMBOTIC THROMBOCITOPENIC PURPURA THROUGH ADAMTS13 FUNTIONAL ASSAY

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RESPONSIBLE PARTIES

A. Project initiator and funder

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B. Collaborators/Committees

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Andalusian Group of Thrombotic Microangiopathies (TMA) of the Andalusian Association of Haematology and Hemotherapy (AAHH). It will help the dissemination of the project and the collaboration of Andalusian hospitals from its work platform.

3. MILESTONES

Table 1 presents planned milestones for the project. These milestones are based on a timely review and approval of the project. Administrative changes to milestones due to delays in study preparation and enrolment do not require amendments to the protocol. Revised study timelines and milestones which do not constitute a need for a formal protocol amendment.

Table 1. Milestones

Milestone	**Planned date**
Start laboratory studies and data collection	2Q 2023
End of laboratory data collection	2Q 2024
Final report of study results	3Q 2024
ECAT accreditation	4Q 2024

4. RATIONALE AND BACKGROUND

A. Medical Background

Acquired Thrombotic thrombocytopenic purpura (aTTP) is a rare life-threatening haematological disease[1] characterised by thrombotic microangiopathy (TMA)[1,2] with an average prevalence of approximately 10 cases/million people and an annual incidence between 1.5 and 6.0 cases per million according to different studies conducted in France[3], United States[4,5] and in the United Kingdom[6,7].

The first acute episode of aTTP mostly occurs during adulthood (~90% of all aTTP cases), but some child and adolescent forms have been also described (~10% of all aTTP cases)[1]. aTTP is still considered a life-threatening disease with a mortality rate of 10-20% in spite of appropriate therapeutic management[1] and its clinical course is characterised by a relapsing tendency[1,2]. Some factors that can increase the risk of developing the disease are blood group O, African ethnicity, obesity and female sex[1,8].

aTTP is caused by severe ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) deficiency[8]. This protein reduces the size of the ultra large von Willebrand factor (VWF) multimers secreted by the endothelial cells. After secretion, long VWF multimers are released into the blood and some remain adherent to endothelial cells[2,8]. Platelets bind tightly to VWF on endothelial cells or at sites of vascular injury forming aggregates that can embolize and occlude downstream arterioles[1]. This effect is prevented by ADAMTS13 enzyme which cleaves a specific domain on VWF reducing its size. Thus, ADAMTS13 deficiency leads to a failure in VWF size regulation and the consequent dependent platelet adhesion causing the cardinal features of aTTP (thrombocytopenia and microangiopathic haemolytic anaemia and possible organ damage)[2].

The ADAMTS13 deficiency can be acquired via autoantibodies to ADAMTS13, which is the most common form of the disease, or congenital via recessively inherited biallelic mutations of the ADAMTS13 gene. This last form is rare and represents a 2% of all aTTP cases[1]. Independently of its origin, the lack of ADAMTS13 activity (less than 10%) is the only biologic marker specific for aTTP[1] and it is a crucial factor in the diagnosis.

aTTP is clinically defined as a severe thrombocytopenia persistence with microangiopathic haemolytic anaemia, without disseminated intravascular coagulation or another apparent cause and without acute renal failure at presentation[8]. This symptomatology is consistent in the 80%

of all patients but a small fraction of patients with severe ADAMTS13 deficiency do not meet this definition because they have uncommon symptoms like acute renal failure or organ ischemia or infarctions (mostly related to the brain)[8]. Also, some aTTP cases with asynchronous appearance and disappearance of signs and symptoms have been reported[2].

In conjunction with this, none of these symptoms are exclusive to aTTP and they may be present in other pathologies such as haemolytic uremic syndrome (HUS), other TMA syndromes or the Evans syndrome[1,8]. In addition, aTTP symptoms can be confused with isolated thrombocytopenia, isolated haemolytic anaemia or even ischemic manifestation linked to autoimmune diseases like systemic lupus erythematosus[1].

Due to this complex differential diagnosis, the algorithms based just on clinical or biological criteria always miscategorise some patients[1,8]. For this reason, the presence of a severe deficiency of ADAMTS13 in conjunction with the other criteria is necessary for a clear aTTP diagnosis (<10% ADAMTS13 activity confirms aTTP diagnosis).

The first-line therapy for acute aTTP is based on daily therapeutic plasma exchange (TPE) for supplying ADAMTS13 deficiency[1] in conjunction with steroids as an adjunctive treatment due to the autoimmune nature of aTTP[1,2]. Another possible treatment is the use of humanized anti-CD20 monoclonal antibody (rituximab). This therapy is being discussed as frontline treatment for patients with a suboptimal response to aTTP conventional treatment due to the high response rates, the shorter hospitalization periods and the fewer relapses reported in some studies[1].

Cablivi® (caplacizumab), recently approved by the FDA and the EMA in some countries, is a humanized bivalent nanoantibody that binds to von Willebrand factor A1 domain preventing its union with platelets and it is indicated for adult patients with aTTP along with immunosuppression and treatment with TPE[9]. The data obtained in the HERCULES phase III clinical trial show that caplacizumab reduces the median time to normalization of the platelet count, the proportion of deaths related with aTTP and the percentage of patients who had a recurrence of TTP at any time during the trial[10]. Moreover, caplacizumab treatment reduced the average number of days of TPE, the volume of plasma used, the average length of stay in the Intensive Care Unit (ICU) and the average length of hospitalization[9,10].

In refractory or unresponsive aTTP, more intensive therapies including twice daily plasma exchange; pulses of cyclophosphamide, vincristine, or cyclosporine A are recommended, and in extreme cases even salvage splenectomy is considered[1]. Finally, there are some new

investigational drugs including N-acetylcysteine (inhibits platelet adherence to VWF), bortezomib (protease inhibitor) or recombinant ADAMTS13 that are showing promising results in the management of aTTP[1].

Despite of all these new therapies, TPE remains as the cornerstone of current aTTP management, and with prompt treatment initiation, the average survival rate from a first aTTP episode is 80-90%[1]. Due to this, the major challenge in aTTP is the persistent risk of an unpredictable, life-threatening relapse. Most relapses occur during the first or second year, but some of them can occur as late as 10 or 20 years after an episode of aTTP[1]. Relapses are associated with death in the worse cases and risk of chronic neurocognitive disability, arterial hypertension and other deficits in health related quality of life[2]. This relapses are always associated with recurrent or persistent severe ADAMTS13 deficiency, which suggests that monitoring ADAMTS13 during the follow-up period might help with in time relapse detection for preventive treatment[1,2] avoiding the need of unit care hospitalization.

B. Rationale

The lack of ADAMTS13 is the only biological marker that is specific for aTTP diagnosis[8] and the assessment of ADAMTS13 is of clinical importance because it is essential for the rapid differential diagnosis between aTTP and other TMA. Furthermore, monitoring of ADAMTS13 activity is useful to ensure biological remission (ADAMTS13 levels > 10%) as well as predicting relapses[8,11].

Due to the high mortality rate of aTTP, treatment should be started as soon as the disease is suspected, sometimes even before confirmation with the ADAMTS13 test results. This situation may lead to misdiagnose some patients and leave them without the appropriate treatment. In conclusion, ADAMTS13 activity assay is crucial for an early diagnosis and optimal management of acute aTTP and any delay in ADAMTS13 results will have a negative impact on the diagnosis, treatment and prognosis of the patient[12].

There are currently 2 techniques available for the ADAMTS13 activity determination, the fluorescence resonance energy transfer (FRET) and the Technozym chromogenic enzyme-linked immunosorbent assay (ELISA)[11,12]. Both are considered reference methods but they require considerable skill because they are highly manual and this increases the risk of error[11]. Furthermore, these methods are time-consuming, not widely available[11,12] and, in case of the ELISA method, it requires a new calibration at each run[11]. The inter-laboratory variability is also a challenge and therefore a validation and/or interpretation method could be needed.

Recently, a new and first fully automated method HemosIL AcuStar ADAMTS13 Activity assay (Instrumentation Laboratory, Bedford, Massachusetts, United States)[11,12] has been developed. HemosIL AcuStar ADAMTS13 Activity assay is a two steps chemiluminescent immunoassay (CLIA) with an analytical time of 33 minutes[11] for the quantitative measurement of ADAMTS13 activity in human-citrated plasma on the ACL AcuStar analyser. The immunoassay uses the GST-VWF73 substrate in combination with magnetic particles for rapid separation and chemiluminescence technology detection[11,12]. The ADAMTS13 present in the plasma sample cleavages the GST-VWF73 substrate and the detection of the generated fragments is based upon an isoluminol-labelled monoclonal antibody that specifically reacts with the cleaved peptide. The emitted light is proportional to the ADAMTS13 activity in the sample[12].

This new ADAMTS13 assay method has been compared with the other two available techniques in two different studies. First, Favresse *et al*. published the results of the comparison between Technozym activity ELISA assay and the new HemosIL AcuStar chemiluminescent assay[11]. On the other hand, Valsecchi *et al*. have recently published the results of validation of this new technique in comparison with ELISA and FRETS in 176 samples[12]. Both studies conclude that the new chemiluminescent ADAMTS13 activity assay showed a good correlation and excellent clinical performance for the diagnosis of severe ADAMTS13 deficiency with the FRETS-VWF73 assay and a commercial ELISA when considering only ADAMTS13 activity values below 10% (the internationally accepted cut-off for a diagnosis of severe ADAMTS13 deficiency typical of aTTP)[11,12]. Finally, Stratmann *et al.* have just published another study comparing the HemosIL AcuStar chemiluminescent assay with two commercially available ADAMTS13 assay kits using 24 paired test samples derived from 10 consecutively recruited patients[13] and their results corroborate the previously published data suggesting that the AcuStar assay could be a valuable and accurate tool for ADAMTS13 activity testing and aTTP diagnostic.

This favourable outcome along with the inherent advantages of automation, like time reduction and the fact that it does not require highly qualified personnel, make HemosIL AcuStar a good alternative for ADAMTS13 measuring. This is especially true among emergency situations when time is crucial and hospitals do not have time to send out samples to a reference laboratory for ADAMTS13 activity assay.

5. PROJECT AIMS

The **overall aim** of this project is **to improve the diagnostic, characterization and monitoring of the acquired thrombotic thrombocytopenic purpura (aTTP) in the reference hospitals from Andalucía to demonstrate its effectiveness for the global management of patients with aTTP in real-life and exclude the presence of other TMAs like haemolytic uremic syndrome.**

The secundary objective of this project is to achieve the European quality program ECAT (External quality Control of diagnostic Assays and Tests) certification that will accredit with the highest quality in the determination of the activity of ADAMTS13. To obtain the accreditation ISO/IEC 17043:2010 from the External Quality Control for Assays and Tests (ECAT). To incorporate the ECAT samples in the prospective validation of the chemiluminescence technique in the laboratories participating in the study. A sample of WHO ADAMTS13 standard plasma will be processed both in retrospective and prospective studies.

6. RESEARCH METHODS

6.1. Study design

This is an observational, prospective, multi-centre, non-interventional study.

The study will be carried out in the context of the usual clinical practice conditions, not imposing restrictions on the participating physician or influencing their normal clinical practice. In no case will any additional procedure be carried out, nor will any type of intervention, whether diagnostic or follow-up, be made other than the usual clinical practice.

6.2. Source of information and context

The study will be carried out Hospital Universitario Virgen del Rocio and patients will be selected through haematologists/nephrologist who routinely visit patients with suspected/confirmed TMA in Andalucía.
ADAMTS13 will be informed through regional laboratory results digital platform (MPA) and send to every investigator to help with the study of TMA and to treat patient according to routine clinical practice.

6.3. STUDY POPULATION AND SELECTION CRITERIA

Patients with clinical and laboratory suspicion of TMA (anaemia, thrombocytopenia, elevated haemolysis parameters and schistocytes in peripheral blood smear).

6.4. STUDY PROCEDURES

- **Schemes of samples:** The scheme of samples committed to carry out would be:
  - Diagnosis:

    Patients with a Plasmic Score greater than or equal to 5.

    Patients with Plasmic Score less than 5 with poor clinical evolution of TMA.
  - After 4 weeks of starting treatment
  - After 3 months of starting treatment
  - Six-monthly starting 9 months after starting treatment.
  - Any other study should be discuss with the reference center.
  - TMA related with neoplasm, drugs, transplant will be excluded from this protocol
  - Inhibitors wil be done only if ADAMTS13 is under 20% to confirmed immune TTP. It would be performed again in the same patient only 3 months later if there is no recovery of ADAMTS13 or in case of suspiction of relapse.

- **Type of samples and shipping address**:
  - Notify the Hospital Universitario Virgen del Rocio team in advance about the case to confirm criteria prior to sending it from Monday to Friday from 9 a.m. to 2 p.m. (telephone 955 01 20 00, indicate connection with a search for coagulation (758719) or a blood bank (756536).
  - 3 citrate tubes sent fresh if the sample can arrive the same morning of the extraction before 1:00 p.m. from Monday to Friday
  - If the sample cannot arrive in the time, freeze the plasma after simple centrifugation in 1-2 ml vials and send in dry ice.
  - The sample must be accompanied by a standard consultation sheet (see annex) indicating the contact telephone number of the responsible physician, Plasmic score, date of extraction, schistocyte percentage.
  - Shipping address: Hospital Virgen del Rocio, Avda. Manuel Siurot s/n, laboratory building, 5th floor, Coagulation. 41014. Seville.
  - For those centers unable to send by them own structure the samples, there is an MRW account (see attached document)

- **Results:** The results will be uploaded to MPA. Those hospitals that request it will be sent the report by official email anonymously. In emergency situation will be provided in 24 hours from Monday to Friday. In follow up in 4-5 days.

An excel will be saved with the samples per NHUSSA identification and time of extraction per patient received, to be able to make an annual activity report.

6.5. TECHNIQUE SELECTION

Blood sample collection and storage

Samples sent for storage will include peripheral blood in citrated tubes. Samples will be identified with standard number labels as any other sample in the routine laboratory of Haemostasis in the University Hospital Virgen del Rocio. Storage of samples until the study will be the same as any other coagulation study in the hospital, in coagulation fridges.

- **Citrated Blood Samples:**

Three citrated blood samples will be taken from patients with aTTP either at presentation or during follow after IC process. For plasma separation, samples are centrifuged at 2000g for 15 minutes, the top two-thirds of the plasma are removed into a labelled polypropylene tube and then centrifuged for a further 15 minutes at 2000 g[21]. The top two-thirds of this plasma will be stored in 500µL aliquots into cryovials and frozen at −80°C for storage. For each patient, a minimum of three 500µL aliquots will be needed for perform all ADAMTS13 assays.

- **Sample transportation**

For these purposes, sample shipment will be performed according to usual clinical practice and using carbonic snow for ensure sample conservation. An appropriate transporting services will be hired for this purpose in each center.

ADAMTS13 procedures

HemosIL AcuStar ADAMTS13 Activity Assay (Instrumentation Laboratory, Bedford, Massachusetts, United States)

The HemosIL AcuStar ADAMTS13 Activity assay is a fully automated, two-step chemiluminescent assay with 33 minutes of duration. The technique is based on a reagent composed of magnetic particles coated with the VWF73 peptide which contains the ADAMTS13 cleavage site. These particles are incubated with the plasma sample and then a magnetic separation and a wash step is carried out[12].

The detection of the VWF fragments generated by ADAMTS13 activity is based upon an isoluminol-labelled monoclonal antibody that specifically reacts with the cleaved peptide. So, a second incubation step with the antibody is needed with the corresponding magnetic separation and a wash step that follows[12].

Finally, the reagents that trigger the chemiluminescent reaction are added and the emitted light is considered proportional to the ADAMTS13 activity in the plasma sample. The chemiluminescent activity is measured as relative light units by the ACL AcuStar analyzer[12]. Results will be expressed as % of normality of the ADAMTS13 activity.

ADAMTS13 Inhibitor Assays

HemosIL AcuStar ADAMTS13 Inhibitor Assay (Instrumentation Laboratory, Bedford, Massachusetts, United States)

The principle of HemosIL AcuStar ADAMTS13 Inhibitor Assay is the same as the basis for the activity assays but in this case the presence of anti-ADAMTS13 inhibitors is determined using a previous mixing procedure step. Heat-inactivated patient samples and heat-inactivated HemosIL Normal Control (pooled normal plasma), that is used as a control, are mixed 1:1 with HemosIL Normal Control. After that, control and sample vials are incubated for 30 min at 37°C and finally residual ADAMST13 activity of each sample mixture is measured with the HemosIL® AcuStar ADAMTS13 Activity assay[26].

Residual ADAMTS13 activity is calculated as 100 x Activity of Patient Mixture / Activity of Control Mixture and residual activity values between 25-75% are used for calculation of the inhibitor titer[26]. This calculation is made according to the Bethesda method (BU/mL) where one Bethesda unit is the amount of inhibitor in 1 mL of plasma that will neutralize 50% of the clotting factor activity (residual activity=50%), and zero Bethesda units represent 100% residual activity[27].

6.6. Estimated Number of samples

- HUS incidence: 6-170 per million inhabitants.
- aTTP incidence: 1 per million inhabitants
- Incidence in Andalusia in low range 120 cases per year.
- Estimated number of samples:
    1. PTT exclusion: 125 samples
    2. PTT follow up: 160 samples (5 per patient a year)

3. Expenditure on high and low controls and calibration: 25 samples

The inclusion of 300 blood samples per year are planned to be analysed. However, the study will continue for a period of 12 months.

6.7. Protection of Human Subjects

6.7.1. Patient information and consent

There is no need of informed consent or patient special information because the determination of ADAMTS13 is key for aTTP diagnoses in routine clinical practice. The objective is to centralize the study and to guarantee the access to ADAMTS13 levels to every hospital improving patient care. All study personnel will guarantee the protection of the patient's personal data and will not include the names of the patients in any of the study forms, reports, articles or other disclosures, except for legal requirements. If data transfers occur, patient will be consult and strict standards of confidentiality and protection of the patient's personal data will be followed.

6.7.2. Benefit-risk assessment for the patient

The study will be carried out in the context of routine clinical practice and does not impose restrictions on the participating physician or influence the treatment and monitoring of the patient. This is an observational study, so the patients participating in this study will not undergo any clinical test that endangers their health.

6.7.3. Ethical board review and favourable opinion/approval

It is the responsibility of the investigator to obtain approval of the study protocol, possible amendments to the protocol, IC forms and any other relevant document from the EB.

7. A QUALITY ACCREDITATION PROCESS

The objective of the ECAT Foundation is to provide an international External Quality Assessment Programme (EQAP) for laboratories working in the field of haemostasis and thrombosis worldwide.

The laboratories participating in the EQAP of the ECAT receive freeze-dried plasmas on a regular basis. Each survey contains abnormal and/or normal plasma samples. The samples are safely packed by the ECAT Foundation and distributed to participating laboratories by postal service

with the corresponding instructions related to storage and the reconstitution of each type of plasma sample.

Each laboratory should perform all the different assays included in the ECAT programme certification in which they are inscribed using methods routinely used in their laboratory and then send the obtained results. Results can be returned via internet, fax or postal service. The deadline is usually 4 to 5 weeks after dispatch of the samples.

Finally, laboratories receive a report that shows each participating laboratory and how their results compare with those of other participants. These results are differentiated according to methodology subgroups as far as possible.

The project aim is to acquire the accreditation ISO/IEC 17043:2010 from the External Quality Control for Assays and Tests (ECAT). The requested EQAP will be in this case for the main programme with includes Von Willebrand Factor parameters and ADAMTS13 determination: ADAMTS13 - I (activity and antigen) and ADAMTS13 - II (antibodies). According with the provided instructions, ECAT samples will be incorporated in the prospective validation of the chemiluminescence technique in the laboratories participating in the study.

8. FUNDING

Sanofi and Alexion provide budget support material reactive for the study. Nor Alexion or Sanofi will receive any kind of information regarded to ADAMTS13 levels or patient management or evolution.

9. BUDGET

- HUS incidence: 6-170 per million inhabitants.
- aTTP incidence: 1 per million inhabitants
- Incidence in Andalusia in low range 120 cases per year.
- Estimated number of samples:
    4. PTT exclusion: 125 samples
    5. PTT follow up: 160 samples (5 per patient a year)
    6. Expenditure on high and low controls and calibration: 25 samples

billable concept	**nº samples**	**€/study**	**Total**
Cost per dosage (does not include displacement) Including indirect costs (IVA excluded)	300	200,00	60.000,00 €[1]
		TOTAL	60.000,00 €

This budget has been covered by hospital until, now. Thanks to Sanofi and Alexion collaboration, ADAMTS13 studies from all Andalucia will be able to be perform, without economic impact in University Hospital Virgen del Rocio, saving money we are spending now.

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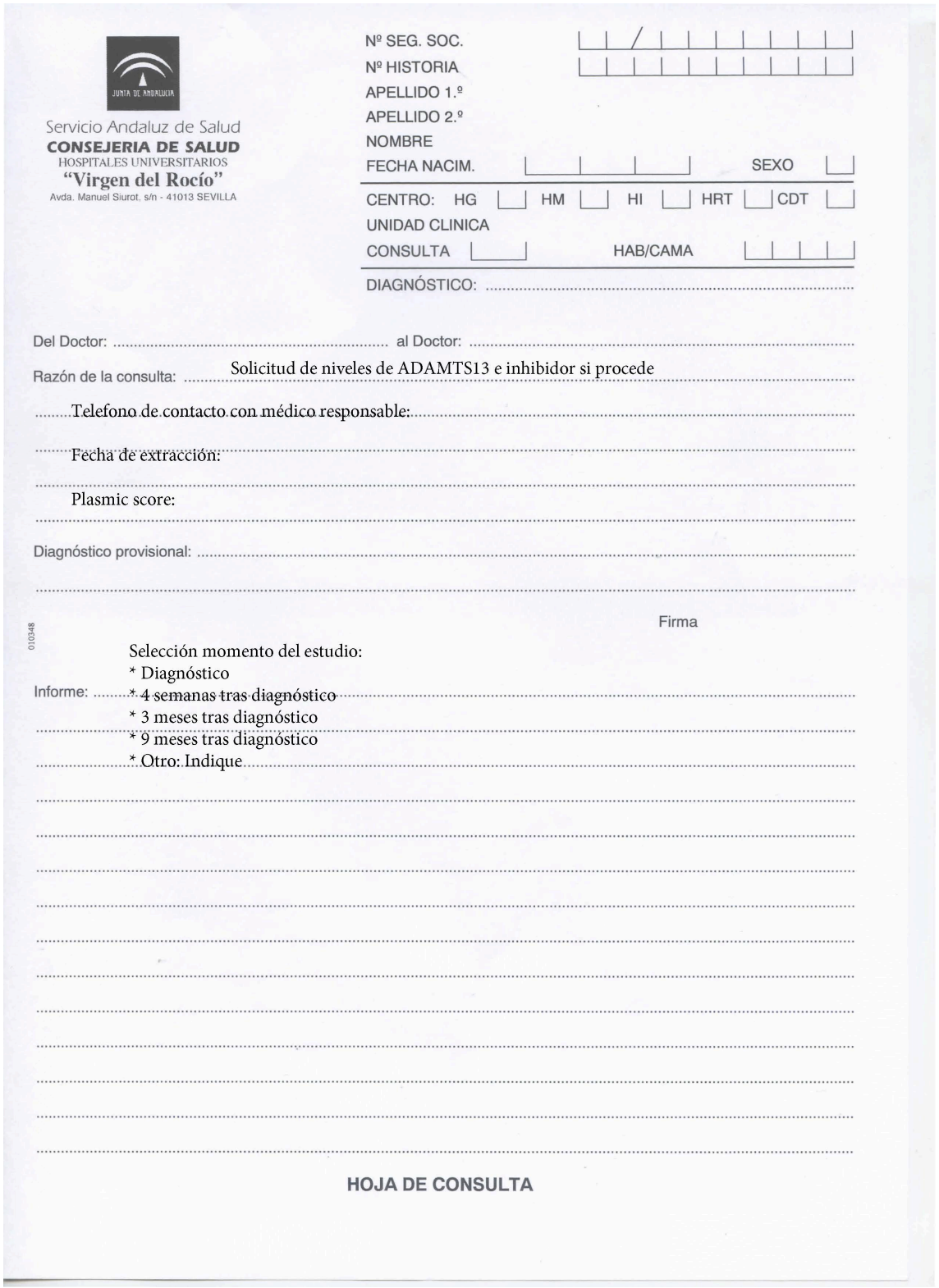
ANEXO



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Nº HISTORIA | | | | | | | | | | |
APELLIDO 1.º
APELLIDO 2.º
NOMBRE
FECHA NACIM. | | | | | | SEXO |
CENTRO: HG | | HM | | HI | | HRT | | CDT |
UNIDAD CLINICA
CONSULTA | | | HAB/CAMA | | | | |
DIAGNÓSTICO: .....

Del Doctor: ..... al Doctor: .....

Razón de la consulta: Solicitud de niveles de ADAMTS13 e inhibidor si procede

Telefono de contacto con médico responsable:

Fecha de extracción:

Plasmic score:

Diagnóstico provisional: .....

Firma

010348

Informe: Selección momento del estudio:
* Diagnóstico
* 4 semanas tras diagnóstico
* 3 meses tras diagnóstico
* 9 meses tras diagnóstico
* Otro: Indique

HOJA DE CONSULTA