SENSUS COMPETITION 2020

TEAM RESULTS DOCUMENT

LxUs
UNIVERSITY OF LISBON

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1 – Summary

The LxUs team's biosensor consists in a portable and immediate option to measure free valproic acid (fVPA) in plasma. Our market research involved conversations with patients, doctors, neuroscientists and biosensor experts and shed a light on the difficulty of adjusting medicine to children and teenagers due to their constant growth and weight changes, and on the consequent anxiety felt by patients and their parents, to which a biosensor can help. The principle of our assay comprises the protein complex STAT3-HDAC3 and ferrocene. VPA binds to HDAC3 and the complex STAT3-HDAC3 causes a specific inhibition. In these circumstances STAT3 does not dimerize, ferrocene reacts, and a current is generated through screen-printed gold electrodes and the selected potentiostat. To associate those currents with VPA concentrations, square-wave voltammetry should be used. Coupled to our biosensor, we have our own fully personalized app which allows patients to monitor the evolution of their health status and engage on a community of other users and neurologists. Our business model was built around the idea of a patient-centered environment, aiming not only at the improvement of patients' daily life but also at a meaningful contribution to the development of a preventive way of handling this complex disease.

2 - Biosensor system and assay

2.1 - Molecular recognition and assay reagents

Antibodies, aptamers, enzymes: Valproic Acid (VPA) is an indirect inhibitor of STAT3 through the inhibition of HDAC3. Literature suggests that VPA inhibits the catalytic activity of HDAC, inducing a complex formation between HDAC3 and STAT3 [1]. This interaction consequently inhibits STAT3 phosphorylation in Tyrosine 705 residue by the Src kinase [2]. Upon phosphorylation inhibition, STAT3 is not able to dimerize. **fVPA recognition:** Since VPA interacts with both Albumin and HDAC3, through its N-terminal, we guarantee that only fVPA binds to HDAC3 and consequently leads to signal generation. **Conjugation chemistries:** Ferrocene (Fc) can be bound to STAT3 through its C-terminal. Fc is commonly used in electrochemical biosensors as it can provide signals when the associated proteins suffer conformational changes. Thus, the existence or absence of dimerization can provide us an electrochemical signal [2]. **Screen-printed gold electrodes:** STAT3 protein, bound to ferrocene, is immobilized on the gold surface of the 220AT screen-printed gold electrode from Metrohm DropSensthat. Briefly, this protein is covalently linked via N-terminal to the N-hydroxysuccinimide active ester of lipoic acid (Lip-NHS) immobilized on the gold surface [2]. **(Bio)chemical reagents:** This strategy was inspired by the article "Electrochemical detection of the Fc-STAT3 phosphorylation and STAT3-Fc-STAT3 dimerization and inhibition" [2]. Hence, the reagents that would be used are 3 : 1% vv H_2SO_4:30% H_2O_2), water, KOH (0.5); H_2SO_4 (0.5 M);distilled ethanol; N_2 gas; N-hydroxysuccinimide activated ester of lipoic acid (Lip-NHS) solution (2 mM) in ethanol; Tris-HCl buffer, pH 7.4 containing 150 mM NaCl and 25% glycerol); ethanolamine solution (100 mM) in ethanol; mercaptohexanoic acid (2 mM) in ethanol; Src kinase reaction buffer that consists of the kinase assay buffer specific to the kinase, 200 mM Fc-ATP and 1 mgmL1 Src kinase protein. Sodium phosphate buffer (pH 7.4); 5 ug of STAT3; 5ug of HDAC3. In Figure 1 the molecular mechanism of recognition of fVPA is clarified.

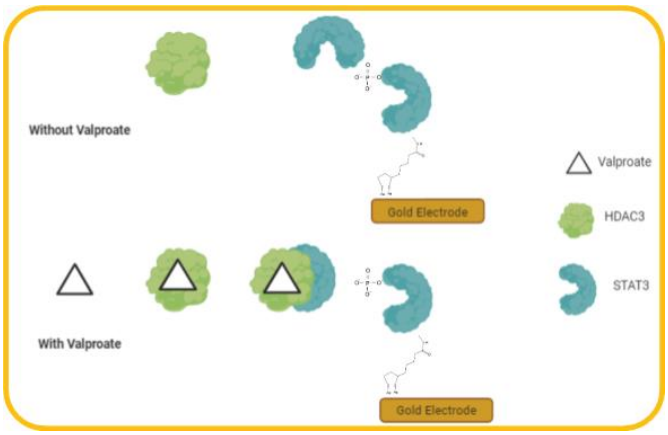


Figure 1 Molecular mechanism that occurs in the presence and absence of VPA in plasma. Without VPA, STAT3 protein dimerizes, after phosphorylation of the Tyrosine 705 residue. With VPA present in plasma, a complex form between VPA and HDAC3, inhibiting phosphorylation and consequently blocking STAT3 dimer formation.

2.2 - Physical transduction

For signal detection, our electrochemical biosensor uses the 220-AT screen-printed gold electrode from Metrohm DropSens [3]. Screen-printed electrodes (SPE) are electrochemical transducers that allow for miniaturization of sensors, making it possible to integrate the working, reference and auxiliary electrodes in the same chip [4]. The electroanalytical method used in this

project is a square-wave voltammetry which is carried out on the *Sensit Smart* device from PalmSens [5]. Voltammetry is organized in a way that provides information about the electrochemical reaction - oxidation of ferrocene - occurring in the working electrode which, due to the transfer of an electron, leads to signal generation when there is no STAT3 dimer formation [2]. The current flows between the working electrode and the auxiliary electrode, and the reference electrode maintains a constant potential that serves as a reference point to control the relative potential of the working electrode. In the voltammogram graph it is possible to see a curve representing the variation of the current versus the potential of the working electrode relative to the constant potential of the reference electrode. Square-wave voltammetry is one of the most advanced voltammetry techniques, and by combining a staircase potential-time function with small potential pulses leads to a decrease in the measured charging current, which is considered the limiting factor in terms of the sensitivity of voltammetry, consequently making the measurement more precise and efficient [6]. In this situation, when the potential is being increased towards the potential peak of the ferrocene reaction, a current is produced, and an oxidation peak is shown in the graph. After immobilization procedures on the SPE surface, measurements are performed in order to achieve a baseline. Therefore, samples with known concentrations of fVPA are used to prepare a calibration curve that can then determine the concentration of fVPA on an unknown sample. It is important to refer that higher concentrations of fVPA will lead to higher measured currents, since it indirectly inhibits STAT3 dimerization.

2.3 - Cartridge technology

In order to achieve a readable signal upon the presence or absence of dimerization, SPE should be previously modified in order to have STAT3 bound to ferrocene fixed as well as a solution with free STAT3 and HDAC3. Before inserting the SPE in the *Sensit Smart* device, the sample should be added with the Src kinase assay on the region of the SPE that contains the working electrode. After proceeding with connection of the SPE with the potentiostat, the measurement can be performed. The final concentration is shown in the smartphone of the user. Following the results, the SPE must be discharged, while the *Sensit Smart* can be used for future measurements.

2.4 - Reader instrument and user interaction

The size of our biosensor is approximately 77x25x11 mm and it contains an hardware part, the modified 220AT screen-printed gold electrode from Metrohm DropSens and the *Sensit Smart* device from PalmSens, and a software part containing the PSTrace software of PalmSens necessary for application of square-wave voltammetry, and the LxUsApp, which besides calculating the VPA concentration through the calibration curve,, has incorporated other features to provide an user-friendly interface. The users must collect their blood sample, mix it with the Src kinase kit, and simply insert the SPE inside the *Sensit Smart* (as shown in Figure 2) and then connect with their personal smartphone. In order to understand the new information provided, the patient should wait for the data to be processed with the objective of showing the sample concentration. This new information will appear in our LxUsApp which allows patients to self-monitor themselves. They will be able to create their own profile and keep track of their latest exam results, as well as a seizure diary where they can take note of any significant events/feelings they experienced during a seizure. When the results of the patient are different from what is expected, or when a new entry to the seizure journal is added, the neurologist will be updated in real time.

Figure 2 Representation of the biosensor design prototype.

3 - Technological feasibility

Considering the validation of some of our ideas, we entered the world of BioChemical Simulations using *PyMOL*. Throughout molecular docking methods, we first tried to understand the interaction between HDAC3 protein and valproate. To perform these simulations, we used Autodock tools to prepare both valproate and the HDAC3 monomer. We have then focused the conformational docking pose search on the binding site of this protein, which is composed by a zinc atom coordinated to 3 protein side chains. The structure used was clean from all water molecules and the acetate molecule which was coordinating the zinc atom. By using a genetic algorithm, we were able to consistently obtain the docking solution shown in Figure 3, one the carbonyl group directly interacts with the zinc atom, while the two carbon chains span over the binding site access pocket.

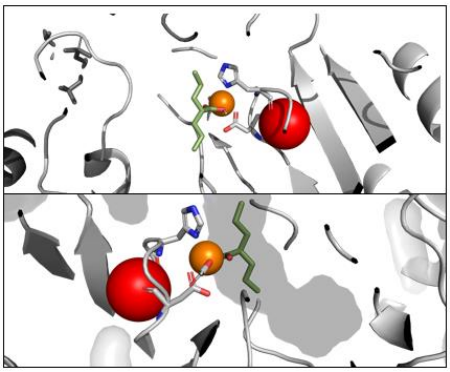


Figure 3 Docking results of valproate into the binding site of HDAC3 (two different perspectives). In green sticks we have the structure of valproate at the binding site of HDAC3, and as can be seen it docks in the optimal coordination position of the Zinc atom. Zinc is colored in red as a sphDoclere. The closest Potassium atom to the Zinc binding site is represented as a red sphere. The side chains of the aminoacids of the protein coordinating the Zinc atom are presented as sticks, colored in grey.

Secondly, we studied the interaction between ferrocene and its amine and carboxyl variants with STAT3. In order to try to identify the region where ferrocene and its amine and carboxyl variants interact at STAT3 protein's surface, we have also used Autodock software. Similarly, to what has been done in step one of this project, we have prepared both the monomer of STAT3 (PDB code 6NJS) and the three ferrocene molecules using Autodock tools. Afterwards, we have focused the conformational pose search at the C-terminal region of STAT3 with a grid simulation box using a grid spacing of 0,375 angstroms centered at the C-terminal of the protein. As can be seen in figure 4, ferrocene and ferrocene-NH_2 interact preferably with this protein in the same region, close to a surface pocket, which is quite evident in the figure 2-a. The interaction region found with these simulations is also close to the phosphorylated Tyrosine always referred to be important. The ferrocene-CO_2 binds in a different region of the C-terminal STAT3, but at a close distance to the previously reported site where the two other ferrocene variants bind.

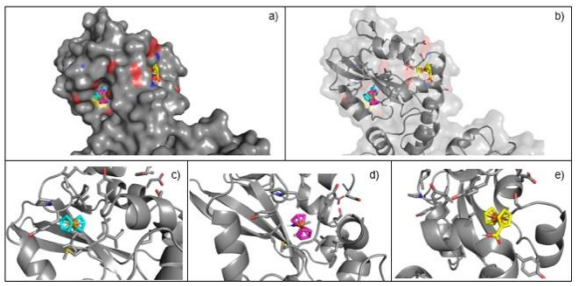


Figure 4 Here we present the results of the docking of ferrocene, ferrocene-NH_2 and ferrocene-CO_2 to the C-terminal of STAT3 protein. In a) we have the structure represented as a surface, while the three docked molecules are represented as sticks and can be found at the surface of the protein. As can be seen, ferrocene and its variant ferrocene-NH_2 bind at the same region of the C-terminal of STAT3, while ferrocene-CO_2 binds a close, but different region of this protein. In b), the same representation of a) is presented, but now with a transparency applied to the surface. In c) we have a zoom of the docking of ferrocene to C-terminal of STAT3, in d), a zoom of the docking of ferrocene-NH_2 to the C-terminal of STAT3 while in e) the zoom is at the region where ferrocene-CO_2 binds to this protein.

Lastly, by using Haddock web server our main objective was the identification of the binding interacting regions of HDAC3 with STAT3, which is shown in Figure 5. We have used a blind approach couplet to rigid body docking since we did not have any kind of information regarding how these two proteins interact. To perform these simulations, we have used one monomer of each protein.

The results show that the interaction between HDAC3 and STAT3 occurs close to the region where the interaction between the first and the second monomer of STAT3 occurs. It is also relevant to mention that it is very close to this region where the different ferrocenes bind with STAT3 protein and where Tyrosine 705 phosphorylates.

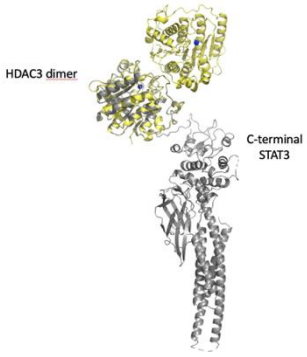


Figure 5 It is possible to see the interaction between HDAC3 and STAT3, which occurs closely to its C-terminal.

From those three different simulations, we can conclude that the interaction between HDAC3 protein and valproate, the interaction between HDAC3 and STAT3 protein, and the interaction between ferrocene and its amine and carboxyl variants with STAT3 are highly specific, which support our idea for measuring the fVPA concentration in plasma. The fact that the interaction between HDAC3 and STAT3 occurs in a region close to where the STAT3 dimer forms is very important since we can suppose that when HDAC3 binds to STAT3, the dimer formation is inhibited. In addition, since ferrocene is very close to this region, when the dimer is not formed, it oxidizes leading to signal generation. Finally, the simulations enabled us to understand that the different types of ferrocene interact preferably with the C-terminal of STAT3 which would lead us to use ferrocene-NH_2 and VPA fits perfectly in HDAC3 binding site. Although the fact that the different molecules fit very well, and their binding sites seem very specific, if the number of molecules used on the surface of the electrode was smaller, the concretization of our biosensor creation would be easier. Additionally, the need of adding the Src kinase kit to the blood sample collected before electrochemical measurement interferes with user action. We think that we can still improve our idea, in order to face these problems, but the impossibility of going to a lab delayed some of our findings. Regarding analytical performance, we are confident that the LxUs conceptual biosensor has the potential to achieve the required goal proposed by the SensUs Organization.

4 - Originality

The concepts behind our biosensor's construction are supported by the literature but the fundamental idea represents a novel approach. It has been described that valproate indirectly inhibits STAT3 phosphorylation and consequent dimerization through HDAC3 [1]. Furthermore, an electrochemical biosensor based on the direct inhibition of STAT3 phosphorylation and consequent dimerization inhibition was already developed [2]. On the contrary, an electrochemical biosensor to sense valproate in plasma through the indirect inhibition of STAT3 is a completely new design. Thus, our team combined different studies and concepts in order to reach the SensUs proposed objective. Considering the translation potential, our team developed a new environment built around the young patient with epilepsy, always trying to make this business benefit him/her and, at the same, be profitable. With that in mind, we created an environment full of different connections and data flows that can definitely change the life of an epileptic patient and also improve all the state-of-the-art related to this condition.

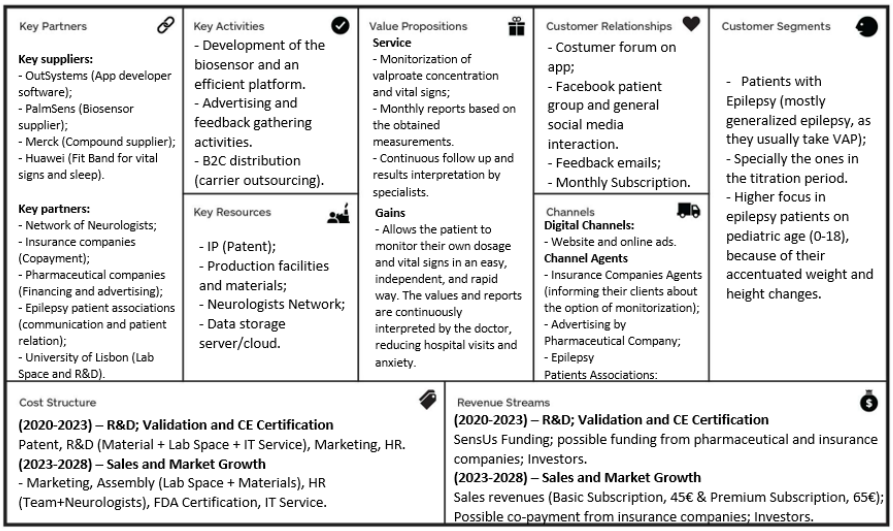
As LxUs supervisor, I can confirm that this group of students worked hard and notoriously in various aspects of this project, discussing their ideas with different SensUs' Partners (PalmSens, Huawei, Future Diagnostics and Saltro) and also validating those ideas with scientific and clinical experts as well as with patients and their families/carers. Regarding the scientific and prototype domains, the LxUs team engaged strongly with PalmSens with regard to the *Sensit Smart* device that was used as the reference biosensor measurement unit. The team also developed an innovative approach to the charge transfer reaction that could effectively measure fVPA in blood plasm by making use of the STAT3-HDAC3 complex: the VPA free in plasma inhibits the STAT3 dimerization and then generates a current through the ferrocene reaction. This approach was validated by experts from this field, like Prof. Ana Viana and Prof. Bruno Victor, from the Department of Chemistry of the Faculty of Sciences of the University of Lisbon, regarding (electro)chemical reactions the binding sites on the sensor surface, fundamental for defining reagents, quantities needed, and predicted biosensor performance. Besides the biosensor per se, the team extended the concept of the project in an innovative way to a digital health platform that combines the biosensor with an app and with a wearable (wristband). The proposal is that the wristband (e.g. Fitbit-like with a inertial measurement unit – accelerometer, gyroscope, magnetometer – and/or with pulse sensor) would provide data on sleep, daily activities, and eventual seizures that could be correlated with fVPA measurements such that, along time and with sufficient data, prediction of the need of changing VPA dosages would be possible and therefore seizures could be greatly prevented. Additionally, the team proposes the platform, and the app in particular, could provide teaching contents to patients and their families about the disease, as well as facilitate communication with clinicians/healthcare professionals (e.g. via chat/videoconference), and community building (communication between parents themselves and patients themselves).

I believe this to be a quite innovate approach, which they were able to reflect in an innovative business model, considering not only a B2C approach (selling directly to patients and their families/carers; actually they developed their solution to address pediatric patients, based on what they learned by interviewing the different stakeholders) but also a B2B approach (selling to pharma and/or medical device companies) with big data monetization coming out of the platform, which could provide further avenues for treatment research and optimization in epilepsy.

LxUs Team Supervisor, Hugo Alexandre Ferreira, MD, MScEng, PhD,

5 - Translational Potential

5.1 - Business Model Canvas



5.2 - Stakeholder desirability

The contemporary reality of valproic acid dosage monitoring stands on a neither preventive nor efficient approach. Since the beginning of our journey, we conducted surveys and interviews which led us to the understanding that during the titration period, there is a present anxiety felt by patients and their families. This is mainly due to the extensive time span between doctor's appointments, especially on public services, and the consequent possibility of children of pediatric age (0-18y) having seizure episodes when parents are not present. Their rapid weight and height changes can lead to underdosing, and therefore a more continuous monitoring of the dosage during the titration period is of high interest for the patient and their family. Furthermore, it also saves money (in the case of patients of private hospitals) and time, by reducing hospital visits.

In order to target these patient's needs, we created a biosensing system that stands on three main pillars. Firstly, our biosensor that, as mentioned, will enable the patient to make measurements of fVPA in blood plasma at the comfort of their own home. On the other hand, we offer the possibility, on a premium package, of integrating a wearable that gathers important information about cardiac and sleep monitoring in order to obtain a well detailed monthly report. Additionally, both devices are connected to a user-friendly app, where the patients can not only see their values but also report any side effects, keep a seizure diary, and engage on a community forum with other users of our system.

Finally, we count on a network of neurologists as the last crucial pillar and one important stakeholder of our system, which closely follow each patient and analyze their results.

We propose not only a device but a whole patient-driven environment, offering peace of mind and time, as well as a better understanding of their own disease and access to a centralized community of patients and health professionals.

Our solution has the potential to lower the incidence of seizure crisis, and consequently the number of hospital visits and long-term damages. Therefore, insurance companies (public and private

ones) constitute one of our strongest potential stakeholders, in a copayment and possible financing basis, as this decrease is of their economic interest. Additionally, our value proposition for pharmaceutical companies which commercialize valproate is to create a safer and more controlled use of this drug, in a time where its prescription and usage is fairly questioned due to its side effects. Furthermore, the data collected throughout the years is valuable for the research done by these companies and by other research centers, which aim to achieve a better data-driven understanding of epilepsy. Finally, for neurologists (particularly pediatric neurologists), our biosensor system will benefit their clinical practice, as they are able to ask for immediate measurements of dosage and to communicate easily with the patient, along with the extra income received for each patient followed on the platform.

5.3 - Business feasibility

With this purpose, we will count on a multidisciplinary team working together. Apart from all the agents required to complete the process/agilize, the sale process is mentioned in Table 7, our team will count on the essential work of: a Bio-tech team responsible for the development and further production of the sensor; a Web/App Developer responsible for both the website development and the app; a Management team responsible for establishing contact with potential buyers and key partners, but also for establishing the doctor-patient bond; and a Doctors' network.

Beyond the human resources, our main logistics concerns the lab, where the sensor is developed itself, and also shipping devices. Between 2020 and 2021 we will develop our biosensor for the pilot study and clinical trial at TecLabs, the incubator of the Faculty of Sciences of the University of Lisbon, where we will pay 100 € per year for each lab station [7]. Different options of lab locations were considered taking into account the different countries addressed, but this turned out to be the most economically viable. In terms of shipping, to deliver our device to our customers, we will work with CTT, the main Portuguese delivery company. It works inside and outside Europe at an affordable cost for our product, including insurance in case of loss or damage.

To generate value propositions and revenues, our key resources will have an important role to make the business model work. To increase the strength of the business model, parts such as a patent and data storage will be key components. Finally, the network of neurologists is the bridge between us and the patient and, from the point of view of physical assets, production facilities and materials will be also very important to our strategy. Regarding our commercialization strategy, it will consist of a subscription service: that is, the patient pays a specific amount per month, instead of immediately paying the total amount of the device. We believe this strategy is ideal, considering the fact that it may make sense for a patient to use this device in a specific time-window of their treatment (mainly upon first diagnosis).

The development of our device wouldn't be possible without our partners, where each one has a key role: OutSystems is the Low code software used in our app; PalmSens is the biosensor supplier, Merck is responsible for supplying compounds and Huawei provides the fit band included in the premium subscription (Huawei Band 4). Thus, focused on keeping our partners and patients constantly updated on our work and our device, we will work on various initiatives: smart marketing to capture the attention of partners and the development of an annual online magazine in order to share with our community the objectives achieved, the future challenges and the testimonials of doctors and patients. Finally, we considered that attending international conferences of health professionals will be a good way to recruit doctors and specialists who can believe in our device and want to help their patients in this constant struggle.

5.4 - Financial viability

The business model described above is backed by two important financial concepts that allow us to have the financial viability required for this type of project.

The first concept is about the two different subscriptions we have available for our patients - Basic and Premium. The Basic Subscription allows the patient to have our biosensor in a leasing type of business, one measurement of VPA every month and a constant feedback from one of the neurologists in our network who will be monitoring the patient throughout the whole month. Regarding the leasing of the biosensor, which is in fact our second important concept, the patient will have to return it when they decide that they no longer want to use our services, allowing our company to have a profitable way of handling expensive components in our business model, without increasing the value of our service to non-practicable values. The Premium Subscription allows the patient to have exactly the same kind of experience as the Basic one, but additionally they will receive a fit band that has two important features - cardiac and sleep monitoring - allowing our Data Analysts and Neurologists to develop a monthly report about the health of our epileptic patients, associating the values and symptoms/seizures submissions chronologically. This will also enable another revenue stream to emerge, based on the fact of starting to obtain important data and information that, as mentioned before, could be deeply useful for pharmaceutical companies or other health-related companies/research centers.

The cost and sales estimations made for this model were based on a bottom-up approach, where we defined at the first stage the number of new young patients with epilepsy that start taking VPA every year. Additionally, we defined the path through which a customer has to go to buy our service, as well as the respective conversion rates for each transition (Appendix - Table 3,4,5), getting the final number of clients that are indeed committed to buy our product. By the same token, we started building our cost and time estimation with the objective of getting to those committed patients we get every year. Using this approach, we first started to get the HR needed and consequently the costs to maintain them and, after that, we got the materials costs (Appendix – Table 13) considering the number of clients we would have. All of this was important to understand which would be a viable price for our subscriptions: We concluded that the Basic Subscription would have to be 45€/month (except for Europe w/o Portugal and U.S.A. - 50€/month) and the Premium Subscription 85€ for the first month, which includes the acquisition of the wearable, and 65€/month for the remaining ones (except for Portugal - 60€/month).

Reckoning all the revenues and costs throughout the years (Appendix – Table 15,Fig 6), we can conclude that for the first three years until entering the market in Portugal (2023), we will need to have an initial investment of 450k € that allows us to work in R&D and assuring we can conclude the CE certification needed to move on with this business. After that, we plan on having two more markets entries, which are firstly in a group of European countries (Germany, Sweden and Denmark - 2024) which were considered based on relevant factors, such as: the prevalence in the younger population, the number of health professionals in the area, the way this condition is handled in the specific countries and how easy it is to both introduce and operate a new device in the country. Later on, we will move on to enter the U.S. market (2026), which will have an additional cost considering the FDA approval and all the different logistic work that has to be done to enter the desired market. After that last entry, it can be observed that, not only will the net income be exponentially profitable, but we would still have room to improve regarding our growing market share in terms of number of patients per year and also other types of revenues considering the data we would collect from our patients.

6 - Team and support

6.1 - Contributions of the Team Members

Carolina Piçarra (Team Captain): Carolina was one of the leaders of our team and also integrated the Management team, playing a crucial role in the organization of our project.

Filipa Baltazar (Team Captain): Filipa was also a leader, constantly motivating every colleague. Additionally, she was a member of the Management team and participated actively in SensUs Connect.

Duarte Saraiva: Duarte was a member of the BioTech team. He not only contributed to the design of the biosensor but also to the physical transduction to obtain a measurable signal.

Luísa Benavente: Luísa was dedicated to the BioTech team and, with her biochemistry knowledge, we were able to better understand our molecular interactions.

Melissa Teixeira: Melissa integrated the BioTech team and played an important role regarding the molecular recognition strategy as well as with her experience with laboratory material.

Francisco Sequeira: Francisco not only provided us experienced knowledge about SensUs as he participated in last years' competition but also played an essential role in developing the app.

Raquel Rebordão: Raquel was also dedicated to the development of the app. Furthermore, she provided many contacts regarding health professionals and market research.

Vasco Silva: Vasco was a crucial part of the Management team while participating with his electrochemical knowledge in the BioTech team.

Teresa Marcelino: Teresa was committed to the Management team and had a huge part in the organization of our Instagram takeover.

6.2 - People who have given support

Several meetings with our supervisor and advisors occurred to develop this project in the best way possible. However, we would like to emphasize the dedication of Professors Ana Viana, Hugo Ferreira and Bruno Víctor. Professor Ana Viana was always available to discuss electrochemical principles and to help us find solutions to newly discovered obstacles. Professor Hugo Ferreira joined all of us together creating a diversified team and constantly supported our progresses while being particularly helpful in the management and entrepreneurship team. Professor Bruno Víctor was extremely accessible and essential to validate our molecular recognition strategy by helping us with biochemical simulations.

6.3 - Sponsors

TecLabs, the Faculty of Sciences' innovation center and incubator, was our biggest sponsor by providing us a workplace and a lot of crucial help in bureaucracy scenarios, both pre and post the COVID-19 situation.

PalmSens, the SensUs Partner we probably contacted the most, especially due to the device we describe from the very beginning of this document - Sensit Smart. Everyone at PalmSens was incredibly valuable and helpful and we are deeply thankful for everything.

Metrohm Dropsens was very important for us, as their staff helped us in the choosing of the best electrode for our electrochemical biosensor.

Huawei, another SensUs Partner, was a really nice opportunity for us because of the Premium Subscription we had in mind that would involve a Fit Band for cardiac and sleep monitoring. In spite of only having talked by email, they were extremely open to discuss every topic we questioned and wanted to know more about.

Future Diagnostics, which is another SensUs Partner, helped us about the IVDR CE Marking process, essential to the a more realistic view of our market entrance.

7 - Final Remarks

This year's competition was very challenging for all teams, particularly due to COVID-19. The global pandemic brought some crucial changes to the SensUs Event, starting on the impossibility of going to the labs, for testing and creating our biosensor, to the sad news about the cancellation of the presential event. Nevertheless, our team stayed motivated on doing the best we could, and we scheduled online meetings with doctors, patients, and a lot of specialists in different areas, in order to create the best conceptual biosensor and the best business plan. Our group of nine motivated students learnt a lot, and we can say that we achieved our goal of creating a creative and original idea that could take a successful pathway into the market.

As a whole and considering the unprecedented situation that the world has been facing due to Covid-19 pandemic, we believe that now more than ever, it makes sense to invest in a self-monitoring virtual solution, reducing the number of patients at the hospital, which may be a prosperous scenario for the transmission of the virus. For the future, we plan to test our idea in the labs, since no biosensor is created without practical experiments. With this competition we were able to contact doctors and patients for whom epilepsy is part of their daily lives, which strongly motivated us to bring our idea to the real world, in order to change these peoples' lives.

Finally, we would like to thank the SensUs organization for giving us the opportunity to participate in this amazing event and evolve as future professionals in different ways. The learning experience that we went through during these months was amazing. All of us are very proud of being part of the LxUs team, and nothing of this would have been possible without the fantastic support of our professors, Prof. Hugo, Prof. Ana, and Prof. Bruno, to whom we are very grateful.

8 - References

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[6] - Mirceski, Valentin & Skrzypek, Sławomira & Stojanov, Leon. (2018). Square-wave voltammetry. ChemTexts. 4. 10.1007/s40828-018-0073-0.

[7] – TecLabs Centro de Inovação, www.teclabs.pt

9 – Appendix

Table 1 [1] These values are based on various studies ["epilepsiforeningen.dk/epilepsi/epilepsi-i-tal/"; "epilepsie-niedersachsen.de/wissenswertes/aktuelle-daten-zur-epilepsie-und-zum-behandlungsstand.html"; "lakartidningen.se/klinik-och-vetenskap-1/artiklar-1/temaartikel/2018/05/epilepsins-orsaker-forekomst-och-prognos/"; "theepilepsynetwork.com/facts-and-statistics/"]; [2] PT values are taken from interviews with neurologists and the rest is from the same studies stated previously; [3] This is a fixed average percentage (80,10%).

	PT	DK + DE + SE	USA
# New Patients (per year)[1]	5500	47250	200000
# New Pediatric Patients (0-18y)[2]	1100	12125	26000
# Taking VPA[3]	881	9811	20826

Table 2 The Bottom-Up approach for sales forecast with the characterization of each different stage.

Sales Objective	Activity
From "Lead" to "Prospect"	The client has seen our ad, clicked on it (and read the initial page).
From "Prospect" to "Qualified"	The client has filled our form with their information to ask for more information and/or a quote.
From "Qualified" to "Developed"	The client has accepted the quote and asked more questions about the product or for a trial.
From "Developed" to "Commited"	The client has bought it.

Table 3 PT's lead-to-sale forecast using initially the value represented in Table 1. The number of opportunities in the "Committed" stage will be the one to use for further calculus.

Portugal		
Stage	Number of opportunities per year	Conversion Rate
Leads	881	70%
Prospects	617	50%
Qualified	309	35%
Developed	108	60%
Commited	65	N/A

Table 4 DK+DE+SE's lead-to-sale forecast using initially the value represented in Table 1. The number of opportunities in the "Committed" stage will be the one to use for further calculus.

Denmark, Sweden and Germany		
Stage	Number of opportunities per year	Conversion Rate
Leads	9811	70%
Prospects	6868	50%
Qualified	3434	35%
Developed	1202	60%
Commited	721	N/A

Table 5 USA's lead-to-sale forecast using initially the value represented in Table 1. The number of opportunities in the "Committed" stage will be the one to use for further calculus.

USA		
Stage	Number of opportunities per year	Conversion Rate
Leads	20826	70%
Prospects	14578	50%
Qualified	7289	35%
Developed	2551	60%
Commited	1531	N/A

Table 6 Distribution and description of activities belonging to the whole process of sales with the hours of effective work needed for each activity.

HUMAN RESOURCES EFFECTIVE HOURS & DISTRIBUTION OF ACTIVITY			
Activity	Steps	Number of Hours of Effective Work (p/ SPE or other factor)	Responsible
Sensor Assembly (p/SPE)	Film formation on the surface of the gold screen printed electrode (the electrode is immersed in the N-hydroxysuccinimide activated ester of lipoic acid solution)	0,25	Lab Technician
	Modification of STAT3 with ferrocene		
	Immobilization of STAT3 (with ferrocene) in the film previously formed on the surface of the gold screen printed electrode		
	Addition of a solution with free STAT3 and HDAC3		
	Addition of a solution with Src Kinase		
Website/App Development	Gathering Needs/Creating a plan	240	Website/App Developer
	Getting started on the website Design - Mockups	480	
	The Main Development Phase	1200	
	The Final Review	168	
Website/App Maintenance	Subcontracting - fixed price every month		
Quality Control	Checking that every batch SPE and Sensit Smart is working well	0,083	Quality Control
Data Analysts	Collecting Data & Data Mining	-	Data Analyst
	Creating Models		
	Evaluation of Patterns		
Technical Support	Receiving contacts from patients Receiving contacts from neurologists	0,1 (every call/email)	Technical Support
Sales and Partnerships	Contacting with different stakeholders	-	Sales Manager

Table 7 Description of the number of workers needed in each sector of activity from 2022 to 2028. The number of potential clients will be explained further on. [1] During 2020 the number of workers is still the number of team members. Beyond that will always be the number of needed workers only; [2] The number of SPE needed considering every patient makes a measurement per month during one year; [3] One Data Analyst for every 500 clients; [4] One Sales Manager for every 10 000 clients; [5] This will be always an Engineer (Biomedical) and it is a constant number; [6] This will always be an Engineer (Chemical) and it is a constant number; [7] During the first 3 years the marketing sector will be held by the Product/Project Management and then Marketing will have one worker for every 10 000 clients; [8] Each Neurologist will monitor 20 patients every month during a whole year.

		2020 [1]	2021	2022	2023	2024	2025	2026	2027	2028
	# Potential Clients	0	0	0	65	871	1961	4908	8263	12930
	# SPE Needed [2]				780	10446	23531	58895	99160	155159
# Workers Needed p/ Activity	Sensor Assembly	1	1	1	1	2	4	8	13	21
	Website/App Development	2	2	2	0	0	0	0	0	0
	Website/App Maintenance	0	0	0	-	-	-	-	-	-
	Quality Control	0	0	0	1	1	2	3	5	7
	Data Analysts [3]	0	0	0	1	1	1	1	2	2
	Technical Support	0	0	0	1	1	1	1	1	1
	Sales and Partnerships [4]	0	0	0	1	1	1	1	1	2
	Project/Product Management [5]	4	1	1	1	1	1	1	1	1
	Research & Development [6]	3	1	1	1	1	1	1	1	1
	Marketing [7]	0	0	0	1	1	1	1	1	2
# Workers Needed p/ Job	Biomedical Engineer	5	1	1	1	1	1	1	1	1
	Chemical Engineer	2	1	1	1	1	1	1	1	1
	Website/App Developer	2	2	2	-	-	-	-	-	-
	Quality Control	0	0	0	1	1	2	3	5	7
	Data Analysts	0	0	0	1	1	1	1	2	2
	Marketing	0	0	0	1	1	1	1	1	2
	Technical Support	0	0	0	1	1	1	1	1	1
	Sales Manager	0	0	0	1	1	1	1	1	2
	Lab Technician	1	1	1	1	2	4	8	13	21
	Neurologists [8]	0	0	0	1	4	9	21	35	54

Table 8 Source: "doutorfinancas.pt/simulador-salario-liquido-2020/" using a base salary (875; 875; 800; 875; 875; 630; 700; 800; 700) along with meal vouchers (5,00€ per meal during 22 days) and already counting on taxes imposed by the Portuguese Government.

Average wage per function	
Function	Salary €/month
Biomedical Engineer	1 192,81 €
Chemical Engineer	1 192,81 €
Website/App Developer	1 100,00 €
Quality Control	1 192,81 €
Data Analysts	1 192,81 €
Marketing	889,63 €
Technical Support	976,25 €
Sales Manager	1 100,00 €
Lab Technician	976,25 €

Table 9 Source: "jobandsalaryabroad.com/pt/portugal/portuguese-neurologist-portugal.html" for a neurologist salary in Portugal and considering, according to some interviews, that a neurologist sees an average of 200 patients per month. The value per patient per month was then multiplied by a factor of 3.

Average wage per function - Others	
Function	Salary €/patient /month
Neurologist	35,15 €

Table 10 [(1)] These yearly salaries are based on 12-month salary year plus a 13th month (vacation salary). There is also an additional yearly increase of 2%.

Job[(1)]	Annual Salary (€) 2022	Annual Salary (€) 2023	Annual Salary (€) 2024	Annual Salary (€) 2025	Annual Salary (€) 2026	Annual Salary (€) 2027	Annual Salary (€) 2028
Biomedical Engineer	15 267,97 €	15 573,33 €	15 884,79 €	16 202,49 €	16 526,54 €	16 857,07 €	17 194,21 €
Chemical Engineer	15 267,97 €	15 573,33 €	15 884,79 €	16 202,49 €	16 526,54 €	16 857,07 €	17 194,21 €
Web/App Developer	14 080,00 €	14 361,60 €	14 648,83 €	14 941,81 €	15 240,64 €	15 545,46 €	15 856,37 €
Quality Control	- €	15 267,97 €	15 573,33 €	15 884,79 €	16 202,49 €	16 526,54 €	16 857,07 €
Data Analysts	- €	15 267,97 €	15 573,33 €	15 884,79 €	16 202,49 €	16 526,54 €	16 857,07 €
Marketing	- €	11 387,26 €	11 615,01 €	11 847,31 €	12 084,26 €	12 325,94 €	12 572,46 €
Technical Support	- €	12 496,00 €	12 745,92 €	13 000,84 €	13 260,86 €	13 526,07 €	13 796,59 €
Sales Manager	- €	14 080,00 €	14 361,60 €	14 648,83 €	14 941,81 €	15 240,64 €	15 545,46 €
Lab Technician	12 496,00 €	12 745,92 €	13 000,84 €	13 260,86 €	13 526,07 €	13 796,59 €	14 072,53 €
Neurologist	8 434,80 €	-	-	-	-	-	-

Table 11 The costs involved in Human Resources from 2020 until 2028. [(1)] 2020 and 2021 no one will receive salary because it will only be R&D and the start of some clinical trials; [(2)] During 2022 everyone will receive 50% of their stipulated salary because we have not entered the market yet; [(3)] The Web/App maintenance will be done by subcontracting a team, thereby the fixed value every year.

Staff Costs	2020[(1)]	2021[(1)]	2022[(2)]	2023	2024	2025	2026	2027	2028
# Biomedical Engineer	5	1	1	1	1	1	1	1	1
Full burdened salary (€)	- €	- €	7 633,98 €	15 573,33 €	15 884,79 €	16 202,49 €	16 526,54 €	16 857,07 €	17 194,21 €
# Chemical Engineer	2	1	1	1	1	1	1	1	1
Full burdened salary (€)	- €	- €	7 633,98 €	15 573,33 €	15 884,79 €	16 202,49 €	16 526,54 €	16 857,07 €	17 194,21 €
# Web/App Developer[(3)]	2	2	2	-	-	-	-	-	-
Full burdened salary (€)	- €	- €	14 080,00 €	15 186,00 €	15 186,00 €	15 186,00 €	15 186,00 €	15 186,00 €	15 186,00 €
# Quality Control	0	0	0	1	1	2	3	5	7
Full burdened salary (€)	- €	- €	- €	15 267,97 €	15 573,33 €	31 769,59 €	48 607,47 €	82 632,70 €	117 999,49 €
# Data Analysts	0	0	0	1	1	1	1	2	2
Full burdened salary (€)	- €	- €	- €	15 267,97 €	15 573,33 €	15 884,79 €	16 202,49 €	33 053,08 €	33 714,14 €
# Marketing	0	0	0	1	1	1	1	1	2
Full burdened salary (€)	- €	- €	- €	11 387,26 €	11 615,01 €	11 847,31 €	12 084,26 €	12 325,94 €	25 144,92 €
# Technical Support	0	0	0	1	1	1	1	1	1
Full burdened salary (€)	- €	- €	- €	12 496,00 €	12 745,92 €	13 000,84 €	13 260,86 €	13 526,07 €	13 796,59 €
# Sales Manager	0	0	0	1	1	1	1	1	2
Full burdened salary (€)	- €	- €	- €	14 080,00 €	14 361,60 €	14 648,83 €	14 941,81 €	15 240,64 €	31 090,92 €
# Lab Technician	1	1	1	1	2	4	8	13	21
Full burdened salary (€)	- €	- €	6 248,00 €	12 745,92 €	26 001,68 €	53 043,42 €	108 208,58 €	179 355,72 €	295 523,04 €
# Neurologists	0	0	0	1	4	9	21	35	54
Full burdened salary (€)	- €	- €	- €	8 434,80 €	33 739,20 €	75 913,20 €	177 130,80 €	295 218,00 €	455 479,20 €
Full Costs	0,00 €	0,00 €	35 595,97 €	136 012,57 €	176 565,65 €	263 698,96 €	438 675,34 €	680 252,29 €	1 022 322,72 €

Table 12 Prediction of sales in three different markets, considering the different dates of entry. Important to notice that we are assuming that every patient maintains its subscription during the three years and that, every new year, the number of new patients that enroll in our subscription program behaves according to the percentage described in the last column. Regarding the Data Companies, we predicted a world of 100 companies eventually available for this type of business and we that we would initiate with 5% from that sample and increase every year by 10% in interest.

	Market Entry	After Market Entry (Years)					
	2023	2024	2025	2026	2027	2028	%
Portugal	37 700,00 €	86 710,00 €	150 423,00 €	195 549,90 €	254 214,87 €	330 479,33 €	
# Cumulative Monthly Subscribers - Basic	52	120	207	270	351	456	30%
Price per subscription	45,00 €	45,00 €	45,00 €	45,00 €	45,00 €	45,00 €	
Subtotal	28 080,00 €	64 584,00 €	112 039,20 €	145 650,96 €	189 346,25 €	246 150,12 €	
# Cumulative Monthly Subscribers - Premium	13	30	52	67	88	114	20%
Price 1st Month Subscription	80,00 €	80,00 €	80,00 €	80,00 €	80,00 €	80,00 €	
Price per subscription	60,00 €	60,00 €	60,00 €	60,00 €	60,00 €	60,00 €	
Subtotal	9 620,00 €	22 126,00 €	38 383,80 €	49 898,94 €	64 868,62 €	84 329,21 €	
Europe (wo/ Portugal)	0,00 €	460 719,00 €	1 082 797,80 €	1 926 194,76 €	2 613 215,47 €	3 558 353,03 €	
# Cumulative Monthly Subscribers - Basic	0	577	1384	2515	3521	4929	40%
Price per subscription	50,00 €	50,00 €	50,00 €	50,00 €	50,00 €	50,00 €	
Subtotal	0,00 €	346 080,00 €	830 592,00 €	1 508 908,80 €	2 112 472,32 €	2 957 461,25 €	
# Cumulative Monthly Subscribers - Premium	0	144	317	525	630	756	20%
Price 1st Month Subsctiption	80,00 €	80,00 €	80,00 €	80,00 €	80,00 €	80,00 €	
Price per subscription	65,00 €	65,00 €	65,00 €	65,00 €	65,00 €	65,00 €	
Subtotal	0,00 €	114 639,00 €	252 205,80 €	417 285,96 €	500 743,15 €	600 891,78 €	
United States of America	0,00 €	0,00 €	0,00 €	733 572,00 €	1 760 572,80 €	3 198 373,92 €	
# Cumulative Monthly Subscribers - Basic	0	0	0	918	2204	4004	40%
Price per subscription	50,00 €	50,00 €	50,00 €	50,00 €	50,00 €	50,00 €	
Subtotal	0,00 €	0,00 €	0,00 €	551 040,00 €	1 322 496,00 €	2 402 534,40 €	
# Cumulative Monthly Subscribers - Premium	0	0	0	230	551	1001	20%
Price 1st Month Subsctiption	80,00 €	80,00 €	80,00 €	80,00 €	80,00 €	80,00 €	
Price per subscription	65,00 €	65,00 €	65,00 €	65,00 €	65,00 €	65,00 €	
Subtotal	0,00 €	0,00 €	0,00 €	182 532,00 €	438 076,80 €	795 839,52 €	
Data Companies	19 500,00 €	287 265,00 €	711 810,33 €	1 806 788,98 €	3 225 772,89 €	5 440 308,94 €	
# Cumulative Monthly Subscribers - Business	5	6	6	7	7	8	
Price per monthly package of patient data	5,00 €	5,00 €	5,00 €	5,00 €	5,00 €	5,00 €	
Subtotal per company	3 900,00 €	52 230,00 €	117 654,60 €	271 493,46 €	440 649,26 €	675 600,77 €	
Total	57 200,00 €	834 694,00 €	1 945 031,13 €	4 662 105,64 €	7 853 776,04 €	12 527 515,22 €	

Table 13 An overall view of materials costs – includes secondary materials (involved mainly in preparation of SPE); subcomponents (secondary costs + packaging) and main component costs (Sensit Smart device + HUAWEI Band 4)

Secondary Materials Cost	
Materials	Price (€)
Gold electrode	2,8753
STAT 3	0,4
HDAC3	0,27
Kinase	1
H2SO4	0,0002
H2O2	0,00109
Alumina slurry	
KOH	0,0000065
Ethanol	0,00016
Lip-NHS	0,0033
Tris-HCL	0,0004
NaCl	0,000000015
Glicerol	0,00055
Nitrogen	
Ethanolamine	0,00125
Mercaptohexanoic acid	0,0003
Total	4,552556515

Cost of each subcomponent	
Components	Price (€)
Electrode	4,552556515
Packaging	0,071428571
Total	4,623985086

Cost for each main component	
Order Units	Price (€)
50	446,00 €
100	379,00 €
500	292,00 €
1000	235,00 €
5000	200,00 €
HUAWEI Band 4	20,00 €

Table 14 Annual costs regarding the main components distributed according to the number of new patients every year. Important to notice that the Sensit Smart behave in a lending type of business, which means that every patient that ends the subscription gives its device to another new patient that have just entered the subscription.

		2023	2024	2025	2026	2027	2028
PT	# New Monthly Subscribers - Basic	52	68	88	114	149	193
	# New Monthly Subscribers - Premium	13	17	22	29	37	48
DK + DE + SE	# New Monthly Subscribers - Basic	0	577	808	1131	1583	2216
	# New Monthly Subscribers - Premium	0	144	173	208	249	299
USA	# New Monthly Subscribers - Basic	0	0	0	918	1286	1800
	# New Monthly Subscribers - Premium	0	0	0	230	276	331
	# New Patients	65	806	1090	2629	3579	4887
	# Sensit Smart Needed	65	806	1090	2564	2773	3796
	Subtotal Annual Order Sensit Smart	28 990,00 €	235 352,00 €	256 150,00 €	602 535,54 €	651 735,68 €	892 169,62 €
	# HUAWEI Band 4 Needed	13	161	195	466	562	678
	Subtotal Annual Order Sensit Smart	260,00 €	3 222,00 €	3 900,20 €	9 316,18 €	11 236,54 €	13 558,10 €

Table 15 Financial planning including all the cost and revenues throughout the years. [1] According to Future Diagnostics, in average, a clinical trial would cost 150k and there is also an extra of 100k involving costs of document emission; [2] Source: A. G. da Cunha Ferreira, LDA. Consultoria & "upcounsel.com/how-much-does-a-patent-cost" – note that the patenting only begins when there is already a proof of concept; [3] During the first three years, there is no consideration of HUAWEI Band 4 and there the costs were made according to the number of patients we aim to use during those different stages (10, 20 and 100 respectively); [4] The Lab Space is considered to be 100€ for every Lab Technician; [5] In 2022 there is an average of 1,79% of costs in marketing considering your public (Table 3 in "Leads" Stage); after that it is always 2% of your sales according to the Google Ads

	2020	2021	2022	2023	2024	2025	2026	2027	2028
Project Phase	Proof of concept	Pilot study and beggining of clinical tria	Clinical trial and documentation emission	Market entry: PT	Market entry: EU	Currently selling on DK, DE, SE and PT	Market entry: USA	Currently selling on DK, DE, SE, PT and USA	Currently selling on DK, DE, SE, PT and USA
# of Clients	0	0	0	65	871	1961	4525	7344	11260
# of Monthly Subscriptions	0	0	0	780	10452	23532	54300	88128	135120
Costs									
Certification (CE and FDA)[1]	- €	(50 000,00) €	(200 000,00) €	- €	(150 000,00) €	(250 000,00) €	- €	- €	- €
Patenting[2]	- €	(2 258,47) €	(4 741,53) €	- €	- €	(8 000,00) €	- €	- €	- €
Materials[3]	(4 737,44) €	(9 474,88) €	(40 674,39) €	(32 596,71) €	(283 681,89) €	(364 961,62) €	(853 617,93) €	(1 059 238,23) €	(1 516 962,48) €
HR	- €	- €	(35 595,97) €	(136 012,57) €	(176 565,65) €	(263 698,96) €	(421 805,74) €	(599 663,42) €	(904 204,48) €
Lab Space[4]	- €	(100,00) €	(100,00) €	(100,00) €	(200,00) €	(400,00) €	(800,00) €	(1 200,00) €	(1 800,00) €
Marketing[5]	- €	- €	(274,00) €	(1 144,00) €	(16 693,88) €	(38 900,62) €	(93 242,11) €	(157 075,52) €	(250 550,30) €
Revenue streams									
Sales	- €	- €	- €	57 200,00 €	834 694,00 €	1 945 031,13 €	4 662 105,64 €	7 853 776,04 €	12 527 515,22 €
Financing[6]	455 872,52 €	- €	- €	- €	- €	- €	- €	- €	- €
Net income	451 135,08 €	(61 833,35) €	(281 385,89) €	(112 653,28) €	207 552,58 €	1 019 069,93 €	3 292 639,86 €	6 036 598,86 €	9 853 997,96 €

Table 16- Values needed to calculate payback period and break-even point. It is important to notice that the financing value presented in the last table it is not considered here.

	2020	2021	2022	2023	2024	2025	2026	2027	2028
Net Cash Flow	(4 737,44) €	(61 833,35) €	(281 385,89) €	(112 913,28) €	204 330,58 €	1 015 169,73 €	3 283 323,68 €	6 025 362,32 €	9 840 439,86 €
Cumulative Cash Flow	(4 737,44) €	(66 570,79) €	(347 956,68) €	(460 869,96) €	(256 539,38) €	758 630,35 €	4 041 954,03 €	10 067 316,35 €	19 907 756,21 €
Cummulative Cost	(4 737,44) €	(66 570,79) €	(347 956,68) €	(518 069,96) €	(1 148 433,38) €	(2 078 294,78) €	(3 457 076,74) €	(5 285 490,45) €	(7 972 565,81) €
Cumulative Revenues	456 132,52 €	456 132,52 €	456 132,52 €	513 332,52 €	1 348 026,52 €	3 293 057,65 €	7 955 163,29 €	15 808 939,32 €	28 336 454,54 €
Payback Period					x				
Break-even point					x				

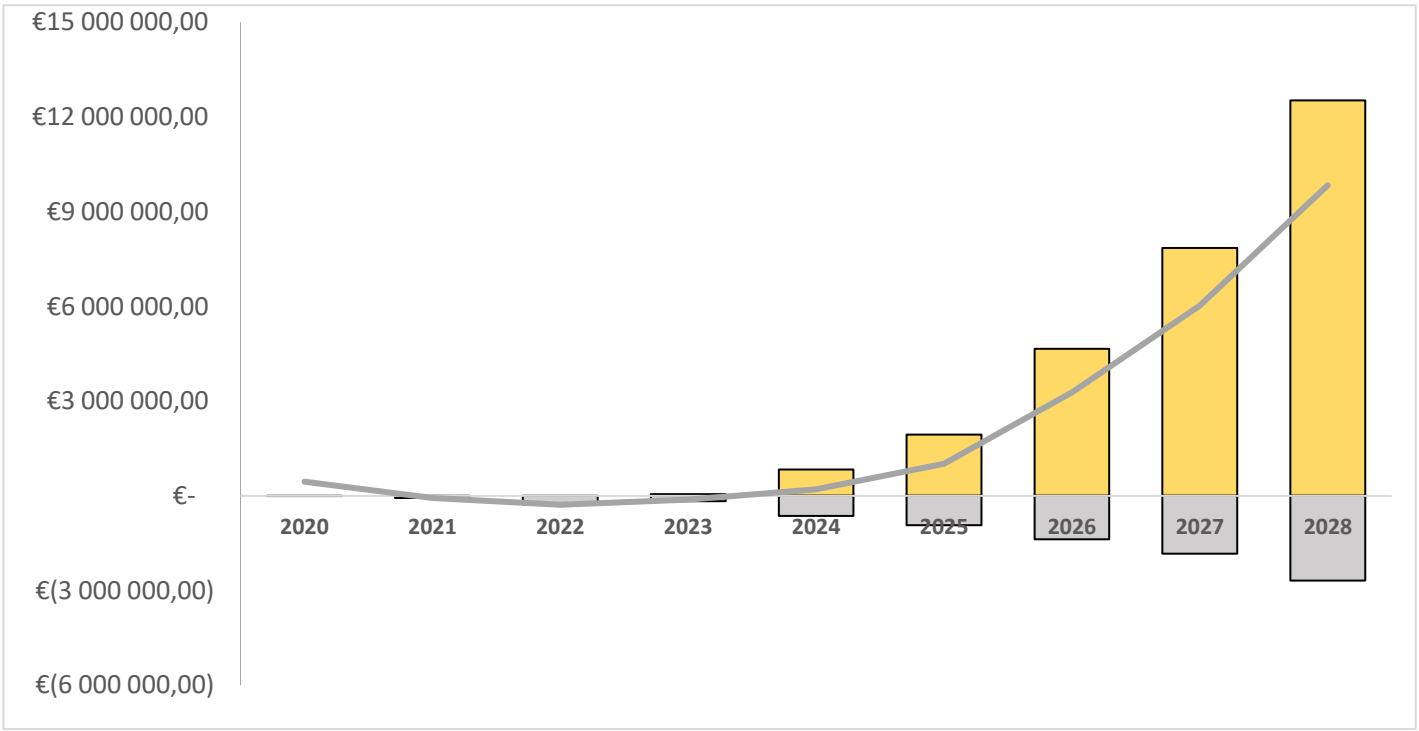

Figure 6- Graph with the financial perspective from 2020 until 2028, with an expected net income in that last year of ~10M€