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Induced pluripotent stem cells as an innovative model for therapy personalization of inflammatory bowel disease

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ABSTRACT

Crohn's disease (CD) is a chronic relapsing inflammatory bowel disease that may affect any part of the gastrointestinal tract but most commonly the ileum and the colon. The inflammation extends through the entire thickness of the bowel wall from the mucosa to the serosa. Thiopurines are drugs commonly used in active CD even if some adverse effects are reported. In particular, we focused on the study of thiopurine-induced pancreatitis (TIP), a severe and idiosyncratic adverse reaction that affects around 3-5% of CD patients treated with azathioprine, that leads to therapy interruption and could require ad hoc therapy with significative associated costs. Molecular mechanism of TIP is currently unknown and no fully validated biomarker is available to assist clinicians in preventing this adverse event. The hypothesis of TIP mechanism is that patients develop an idiosyncratic reaction affecting pancreatic exocrine cells caused by genetic predisposition, differences in thiopurine biotransformation, cytotoxicity or predisposition to immune cells activation.

Induced pluripotent stem cells (iPSCs) are stem cells obtained reprogramming adult somatic cells using a specific set of reprogramming factors. iPSCs mantain the donor genetic heritage and have become a powerful technique to model diseases and drug adverse effects in a personalized way. iPSCs can differentiate under adequate stimuli into almost every somatic lineage, representing an innovative model to study mechanisms of adverse drug reactions in individual patients' tissues not easily obtainable from human probands.

At IRCCS Burlo Garofolo (Trieste, Italy) three pediatric CD patients that developed TIP and three CD controls that did not developed TIP after azathioprine treatment were enrolled. Peripheral blood was collected and sent to Prof. Gilliani's group at Brescia University (Brescia, Italy) where peripheral blood mononuclear cells were isolated and reprogrammed using Sendai virus. CD iPSCs were differentiated in pancreatic exocrine cells using the protocol developed by Prof. Sasaki (Shinshu University, Japan). Briefly, this 4 steps protocol allows to obtain patient-specific exocrine pancreatic cells adding different stimuli to the medium. Each differentiation stage presents characteristic genetic expression markers: *OCT4* is characteristic of undifferentiated cells (iPSCs), *FOXA2* and *SOX17* of definitive endoderm (stage I), *PDX1* of pancreatic progenitors (stage III) and amylase, in particular its pancreatic isoforms *AMY2A* and *AMY2B* of pancreatic exocrine cells (stage IV).

Differentiation efficiency was analyzed by PCR-real time and immunofluorescence techniques.

The sensitivity to thiopurines of TIP (cases) and no-TIP (controls) CD patient-specific iPSCs and differentiated cells was investigated by MTT assay exposing cells to azathioprine, mercaptopurine and thioguanine for 72 hours. Cytotoxicity results showed an interesting higher sensitivity of the TIP-iPSCs in comparison to the control no-TIP iPSCs after mercaptopurine ($p = 0.016$, two way ANOVA TIP vs no-TIP) and thioguanine ($p = 0.0012$, two way ANOVA TIP vs no-TIP) treatment. Also pancreatic progenitor cells of TIP patients resulted significantly more sensitive with respect to no-TIP cells after mercaptopurine ($p = 0.017$, two way ANOVA TIP vs no-TIP) and thioguanine exposure ($p = 0.014$, two way ANOVA TIP vs no-TIP).

All patients resulted wild type for *TPMT* polymorphisms letting us to conclude that the different sensitivity between no-TIP and TIP iPSCs and pancreatic progenitors was not related to *TPMT* genetic variants but to other mechanisms.

Thiopurine effect is strictly correlated to the proliferation rate because these drugs are cell cycle-specific agents that interfere with the formation of the new DNA strand during the S phase of the cell cycle to exert cytotoxicity. iPSCs resulted extremely sensitive to thiopurines in comparison to differentiated cells and to a panel of immortalized lines including the H6C7 ductal pancreatic line. Analysis of cell cycle showed an higher percentage of cells in the S phase in CD-iPSCs with respect to the H6C7 line but not to stage I definitive endoderm cells and stage III pancreatic progenitor cells. The higher percentage of cells in the S phase of both CD-iPSCs lines well explains their higher sensitivity to thiopurines with respect to H6C7. However, the lower sensitivity of differentiatied cells with respect to iPSCs cannot be explained on the basis of the different proliferation, due to their similar proliferation to iPSCs. CD-iPSCs of patients with and without TIP showed no differences in terms of proliferation.

In this thesis, an innovative patient-specific model to study TIP in patients with CD based on iPSCs and their differentiation to exocrine pancreatic cells has been developed. Our findings show that cells from patients with TIP are more sensitive to thiopurines in this *in vitro* model despite a similar distribution among the different phases of the cell cycle between TIP and no-TIP cells. Further studies are needed to elucidate the molecular mechanism at the basis of this difference. The *in vitro* model established has proven to be suitable for studying and investigating TIP predisposition in a personalized way in

pediatric CD patients and could be further developed to study other drugs causing pancreatitis in other diseases.

RIASSUNTO

La malattia di Crohn (MC) è una malattia infiammatoria cronica recidivante intestinale che può colpire ogni parte del tratto gastrointestinale, più frequentemente l'ileo e il colon. L'infiammazione si estende per l'intero spessore della parete intestinale dalla mucosa alla sierosa. Le tiopurine sono farmaci comunemente impiegati nel trattamento della MC. Durante il trattamento circa il 3-5% dei pazienti sviluppa pancreatite, una grave reazione avversa idiosincratica, che determina l'interruzione della terapia e può richiedere trattamenti ad hoc, con significativi costi sul piano personale e sociale. Il meccanismo molecolare della pancreatite indotta dalle tiopurine (TIP) non è noto e non sono disponibili biomarcatori utili ai clinici nella prevenzione di questo effetto avverso.

L'ipotesi principale alla base di questo progetto è che la TIP sia dovuta ad una reazione idiosincratica delle cellule pancreatiche esocrine del paziente ai metaboliti tiopurinici causata da determinanti genetiche collegate a differenze nella biotrasformazione, citotossicità o predisposizione all'attivazione immunitaria.

Le cellule staminali pluripotenti indotte o iPSCs possono essere generate mediante riprogrammazione di cellule somatiche adulte come fibroblasti o cellule mononucleate di sangue periferico grazie alla trasfezione di uno specifico set di geni associati a staminalità e pluripotenza. Le iPSCs, dopo opportuna stimolazione, sono in grado di differenziare in ogni tipo di cellula dell'organismo rappresentando un modello innovativo utile allo studio delle reazioni avverse da farmaci anche in tessuti non facilmente accessibili come il tessuto pancreatico.

Presso l'IRCCS Burlo Garofolo sono stati arruolati tre pazienti pediatrici con MC trattati con azatioprina che hanno sviluppato TIP come reazione avversa al trattamento e tre pazienti con MC trattati con azatioprina che non l'hanno sviluppata. I campioni di sangue sono stati inviati all'Università degli studi di Brescia per l'isolamento e la riprogrammazione delle cellule mononucleate di sangue periferico in iPSCs mediante tecnica con Sendai virus. Una volta stabilizzate e caratterizzate, sono poi state utilizzate per il lavoro di questa tesi e differenziate in cellule di pancreas esocrino utilizzando il protocollo sviluppato dal Prof. Sasaki (Università Shinshu, Giappone) per il differenziamento di cellule staminali embrionali. Brevemente, il protocollo prevede 4 steps e permette di ottenere cellule pancreatiche esocrine aggiungendo specifici stimoli al terreno di coltura. Ogni step di

differenziamento presenta dei marker genetici caratteristici: *OCT4* caratteristico di cellule indifferenziate (iPSCs), *FOXA2* e *SOX17* di endoderma definitivo (stage I), *PDX1* dei progenitori pancreatici (stage III) e l'amilasi, in particolare le sue due isoforme pancreatiche *AMY2A* e *AMY2B* caratteristiche di cellule pancreatiche esocrine mature (stage IV). L'efficienza di differenziamento è stata analizzata mediante analisi di real-time PCR e immunofluorescenza. La sensibilità alle tiopurine delle iPSCs paziente-specifiche e delle cellule differenziate è stata investigata mediante test dell'MTT. I risultati ottenuti indicano una sensibilità maggiore delle iPSCs dei pazienti con TIP rispetto alle iPSCs dei controlli senza TIP dopo esposizione a mercaptopurina ($p = 0.016$, ANOVA a due vie, TIP vs no-TIP) ed a tioguanina ($p = 0.0012$, ANOVA a due vie, TIP vs no-TIP). Anche i progenitori pancreatici dei pazienti con TIP sono risultati significativamente più sensibili successivamente a trattamento con mercaptopurina ($p = 0.017$, ANOVA a due vie, TIP vs no-TIP) e tioguanina ($p = 0.014$, ANOVA a due vie, TIP vs no-TIP).

L'analisi genetica di polimorfismi associati a sviluppo di pancreatite ha evidenziato che tutti i pazienti sono risultati wild type per i polimorfismi del gene *TPMT* permettendoci di concludere che le differenze di sensibilità rilevate tra no-TIP iPSCs e TIP-iPSCs sono imputabili ad altri meccanismi.

L'effetto delle tiopurine è strettamente collegato alla proliferazione cellulare essendo questi farmaci degli agenti ciclo-specifici che interferiscono con la formazione della nuova elica del DNA durante la fase S del ciclo cellulare al fine di svolgere la loro azione citotossica. Le iPSCs di pazienti sia con che senza TIP, sono risultate estremamente sensibili alle tiopurine rispetto alle cellule differenziate e ad un pannello di linee stabilizzate, incluso la linea pancreatica duttale H6C7. Inoltre, le cellule iPSCs hanno presentato un'alta percentuale di cellule in fase S rispetto alla linea H6C7, ma non alle cellule differenziate in fase I, endoderma definitivo e fase III, progenitori pancreatici. Tale osservazione spiega la maggiore sensibilità delle iPSCs rispetto alle H6C7, ma non rispetto alle cellule differenziate.

In questa tesi è stato sviluppato un modello innovativo per lo studio della TIP in pazienti con MC basato su iPSCs e il loro differenziamento in cellule di pancreas esocrino. Le analisi svolte hanno indicato una maggiore sensibilità alle tiopurine delle cellule dei pazienti con TIP nonostante una distribuzione simile tra le diverse fasi del ciclo cellulare tra le cellule di pazienti con e senza TIP. I meccanismi molecolari alla base di queste differenze rimangono dunque da chiarire mediante ulteriori studi.

Il modello *in vitro* creato è risultato adatto per lo studio della predisposizione alla TIP in modo personalizzato in pazienti con MC e può essere ulteriormente sviluppato per lo studio della pancreatite indotta da farmaci in altre malattie.

1. Introduction

1.1 Stem cells

Stem cells are able to self-renew and differentiate into several specialized cell types. Stem cells can be divided in totipotent, pluripotent, multipotent, or unipotent according on their differentiation potential (1).

- Totipotent stem cells are able to differentiate both into embryonic or extra-embryonic cells.
- Pluripotent stem cells are able to differentiate into any cells except for extra-embryonic cells (fetal membrane).
- Multipotent stem cells differentiate into several cell types but only derived within one particular lineage.
- Unipotent stem cells can differentiate into only one cell type.

Stem cells can be classified also on their origin and can be divided into three different groups: adult, embryonic and induced pluripotent stem cells (2).

1.2 Induced pluripotent stem cells

1.2.1 Reprogramming of somatic cells

In 2006 Takahashi and Yamanaka (3) introduced to the scientific world a new type of stem cells, the so-called induced pluripotent stem cells or iPSCs. The era of reprogramming started with the creation of mouse pluripotent stem cells obtained introducing the four transcription factors *OCT3/4* (Octamer binding transcription factor3/4), *SOX2* (Sex determining region Y), *MYC* (MYC Proto-Oncogene) and *KLF4* (Kruppel Like Factor-4) in mouse fibroblasts. These factors are involved in forcing somatic cells to an embryonic-like state with characteristics highly similar to embryonic stem cells (ESCs). For instance, iPSCs have the capacity to differentiate into cells of all three germ layers and into any adult cell type as ESCs. However, it still remains controversial if iPSCs and ESCs can be described as equivalent cellular models (4).

In 2007 two different groups (5,6) successfully reprogrammed human somatic cells creating the possibility to avoid the ethically controversial use of ESCs. However, the reprogramming efficiency introducing the original four genes or a combination of *OCT3/4*, *SOX2*, *NANOG*, and *LIN28* was as low as around 1%. To reprogram somatic cells, both groups used integrative viral transduction technique that may lead to random viral transgene insertions with high risk of genetic, epigenetic and transcriptional abnormalities such as chromosomal aberration, accumulation of point mutations and aberrant methylation patterns. Integration of viral particles in cell genome increases the risk of expression of oncogenes leading to tumor formation (7,8). Limitations related to this reprogramming technique have been overcome during the following years. Indeed, since 2008, many other techniques have been used to obtain more safely and efficiently iPSCs. In particular, scientists focused on non-integrating methods in order to avoid the aforementioned limitations (9). The non-integrating methods provide the use of viral or non-viral vectors. In 2008, Stadfeld et al. (10) reprogrammed mouse fibroblasts and liver cells using the non-integrating adenovirus as vector of *OCT4*, *SOX2*, *KLF4*, and *MYC* genes avoiding their integration in the host genome. However, the efficiency was very low ranging from 0.001 to 0.0001% and 23% of cells were tetraploid, condition not seen in iPSCs obtained by integrating methods. During the following two years, these limitations were overcome by the introduction of the Sendai Virus (SeV) non-integrating vector. SeV is a negative sense single stranded RNA virus with the capacity to replicate in the cytoplasm without entering into the nucleus, therefore, appearing an excellent safe vehicle to generate iPSCs. Both human fibroblasts and blood cells (T cells) were successfully reprogrammed (11,12). Cells were obtained in around one month with 0.1% efficiency for blood cells and 1% for fibroblasts and the viral vector was easily removed by incubating cells for 5 days at 38-39 °C.

In addition to non-integrating viral vectors, there are several other methods based on free-integration technology. For instance, techniques based on direct delivery of episomal plasmids or minicircle vectors can be used, although with low efficiency with respect to viral reprogramming virus (13). Moreover, mRNA or miRNA transfection as well as proteins can be used to generate iPSCs without altering the DNA. However, obtaining iPSCs by these methods presents some limitations related to intense work efforts needed and low efficiency especially for miRNA and proteins vectors (14,15). All reprogramming methods described are reported in figure 1.

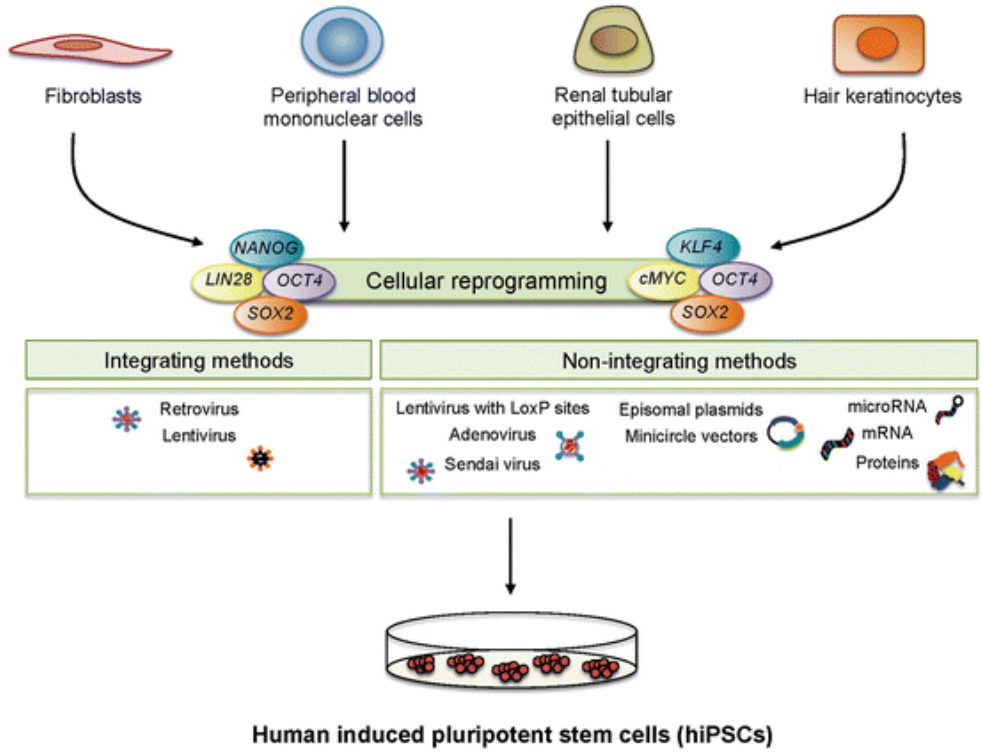

Figure 1. Different reprogramming methods to generate iPSCs starting from somatic cells. Integrating vs non-integrating methods (16).

1.2.2 iPSCs applications and personalized medicine

One of the most powerful property of iPSCs is the ability to differentiate, under an adequate stimulus, into any cell type (figure 2) preserving the donor individual genetic heritage, therefore providing a model genetically matched with a specific patient. The use of iPSCs gives the ground-breaking possibility to set up new personalized disease models in order to better and deeply study a wide variety of diseases as well as to establish more sensitive and personalized assays to better understand, and thus prevent, the development of adverse drug reactions (ADRs). Studying disease molecular mechanisms in a patient-specific manner and trying to find biomarkers useful for clinicians to prevent the development of ADRs are the key points of personalized therapy. The aforementioned applications of iPSCs fit well with the concept of personalized medicine, which tries to identify the best therapy for each patient at the right dose and at the right time, thus limiting the possibility of ADRs

development. Moreover, iPSCs can be used also during the drug screening process in order to assess the effects and the potential side effects of new compounds (17,18).
The use of IPSCs in clinical practice, mostly in regenerative medicine, has already began with significative results. (19–21).

1.2.3 Disease models

To prevent and treat human pathologies, it is fundamental to understand the underlying biological and molecular processes.(22). The most widely used approaches to achieve these goals are animal models, primary human cells and immortalized human cell lines. So far, concerning animal models for pharmacological studies, the most used are rats, mice, and guinea pigs. The mammalian genome is generally conserved across species (23) however, molecular mechanisms and genetic determinants are specific and distinctive for each species (22,24,25). Therefore, genetic manipulation to model diseases in animals frequently fails, obtaining pathologic conditions notably different from those in humans (26–28).
On the other hand, primary human cells have a limited lifespan; indeed, after a few days in culture, primary cells enter a permanent and irreversible proliferation arrest, referred to as replicative senescence (29,30).
To overcome these limitations, several cell immortalization techniques are available for obtaining immortalized cell lines able to keep the ability to divide and evade the normal cellular senescence. The most frequently used immortalization methods are forced expression of human telomerase, overexpression of viral oncogenes or a combination of both, as well as treatments with chemical agents such as benzopyrene or with radiation (31,32). However, immortalized cell lines lack some important functions with respect to primary cells. Moreover, the number of passages performed alters their phenotype and changes several properties with respect to the progenitor donors, which increase over time in culture (30,33). Overall, it would be useful to develop innovative and more patient-specific *in vitro* models. IPSCs are certainly a great tool in this regard for many reasons including, as mentioned above, the peculiar characteristic of matching the donor genetic background allowing to set up personalized disease models. Moreover, iPSCs are almost an inexhaustible source of cells avoiding the ethical problems related to ESCs and can differentiate, under specific stimuli, into any cells of the human body (figure 2).

After the discovery of iPSCs many researchers have dedicated their efforts to model a wide range of diseases using this technology. For instance, several diseases related to genetic disorders have been modeled especially in the neurological (34,35) and in the cardiac field (36,37) as well as important primary immunodeficiencies (38).

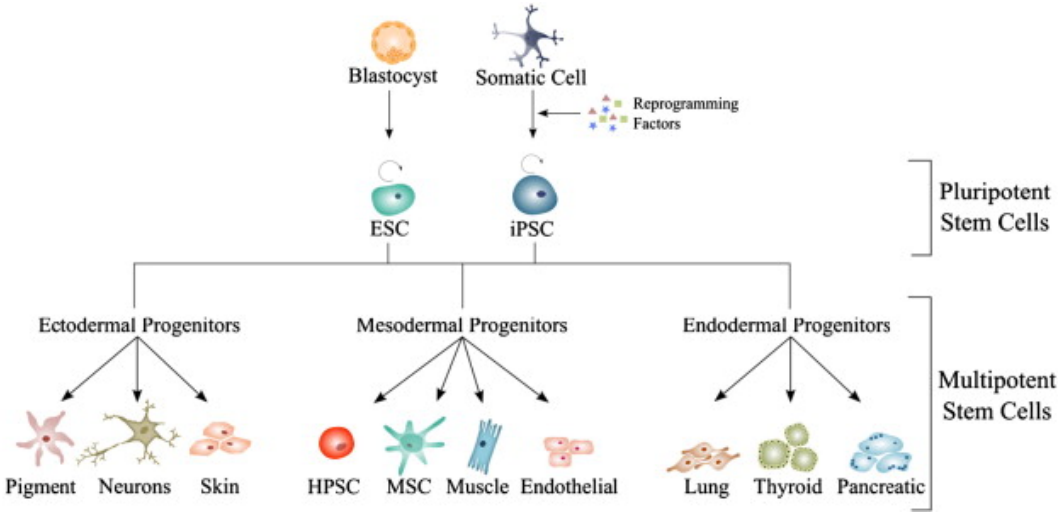

Figure 2. Differentiation potential of iPSCs and ESCs (39). Both cell types are able to differentiate into the three germ layers ectoderm, mesoderm and endoderm. From each germ layer it is possible to ideally generate cells characteristic of all human tissue except for extra-embryonic cells (fetal membrane).

1.2.4 Adverse drug reactions

The management and prevention of drug-induced toxicity in each patient treated with a certain drug is a key aspect of therapy personalization. Even today, adverse drug reactions (ADRs) are a severe issue of a wide range of drugs leading in some case to hospitalization, disability or even death. ADRs represent a major clinical problem, that has to be carefully managed by physicians which most times do not have reliable biomarkers to prevent them. Moreover, ADRs are related to high costs for pharmaceutical industries in terms of drug withdrawal from the market (40), but also in terms of clinical and economical efforts needed to resolve the side effect for the whole health care system (41). iPSCs can be a great tool to better understand, and thus prevent, drug-induced side effects, in particular with respect to animal models and immortalized cells. Moreover, ADRs are known to be related to the individual genetic patients' background, leading to a wide range of toxicities of different severity (42). Extremely helpful patient-specific assays can be developed using iPSCs

technology, given the matching of the donor genetic background. Indeed, by using iPSCs, it seems reasonable to precisely mimic the patient susceptibility to a specific drug, setting up powerful assays useful to identify predictive biomarkers. Moreover, it is important to consider the impact of ADRs on special populations such as pediatric patients that may respond differently to drugs with respect to adults and are prone to develop different patterns of ADRs, leading, in some cases, to more severe consequences such as permanent disability or even death (43,44). However, regarding the pediatric world, more efforts are still needed, even if some studies are already available (45–47). iPSCs may represent a great tool to model children sensitivity to new, but also old, drugs, shedding light on mechanisms of toxicity, resolving in part the problem of the lack of data directly obtained on pediatric patients. Indeed, with a simple blood sample it is possible to generate iPSCs by reprogramming peripheral blood mononuclear cells (PBMCs). Then, patient-specific stem cells obtained can be differentiated into the somatic cells of interest.

Several ADRs have been modeled during the past years using patient-specific iPSCs in order to study drug adverse effects in a more personalized way. So far, ADRs modeled with iPSCs include gastrointestinal toxicity, nephrotoxicity, cardiotoxicity and neurotoxicity (48).

1.2.5 Drug screening

Drug discovery is the first step for the generation of a new drug and the application of iPSCs technology in this early stage of drug development process represents an innovative strategy that could be used to solve drug attrition rate. Indeed, this could reduce costs for industries, through the development of more precise and predictive toxicity tests (49). The conventional drug discovery process takes several years and the majority of new molecules discovered never reach the market, also due to the lack of personalized drug toxicity models. This is partially caused by the use of animal models that do not faithfully reproduce human diseases (50) (figure 3). Instead, introducing iPSCs in the drug development process, alongside animal studies, could partially reduce this problem since with these cells it is possible to mimic in a specific manner the patient reactions to drugs representing a much better predictive tool.

To identify *in vitro* new pharmacological agents, the high-throughput screening (HTS) approach is frequently used on large libraries of molecules (51). It would be interesting to apply the HTS approach to iPSCs technology. For instance, libraries of iPSCs could be a very

useful approach to screen the different sensitivity of patients to new drugs (17,18,52), but also to discover specific lead compounds for the development of new therapeutic agents. However, in general HTS requires large number of cells, which may be difficult to obtain given the yield of differentiated cells of current protocols. Even if HTS performed with iPSCs is still needing optimization, it has several advantages in terms of: resemblance of *in vitro* pathogenic conditions, patient-specific cells and an extensive and unlimited cell-derived resource due to self-renewal and pluripotency.

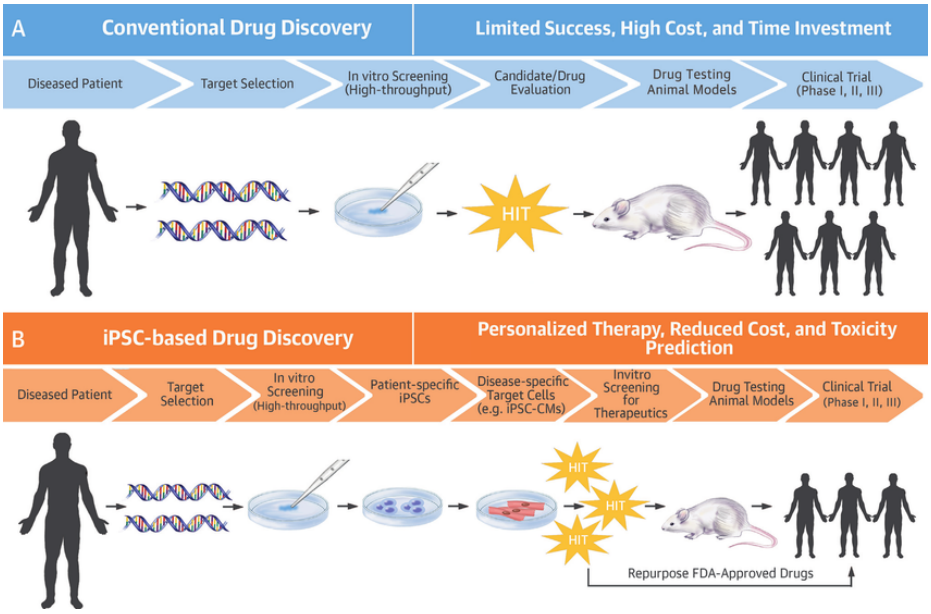

Figure 3. Conventional *vs* iPSCs-based drug discovery process (50). iPSCs allow to set up more personalized pre-clinical studies reducing costs for industries (i.e. drug withdrawal from the market for safety reasons). Introducing iPSCs into the drug discovery process, alongside the animal tests, could increase the efficacy of the drug pre-clinical stage.

1.2.6 Regenerative medicine

One of the powerful applications of iPSCs is regenerative medicine where, differentiated cells, obtained from patient-specific iPSCs, can be transplanted to the site of injury or degeneration (53). Indeed, using these cells it is possible to generate tissues characteristic of the patient avoiding, for example, the serious problem of immune reactions that can occur after non-autologous tissue transplants. The generation of tissues is possible thanks to the self-renewal property of iPSCs, which allows obtaining an almost inexhaustible source of patient-specific cells that have the capacity to differentiate into almost any cell of the human

body. It is therefore reasonable to perform an ideally and effectively autologous transplant avoiding the limitations of allografts such as tissue rejection and the lack of available donors. For instance, several studies have been carried out to assess the feasibility of autologous transplant of iPSCs-derived cardiomyocyte in case of heart failure in animal models (54,55). In detail, researchers have demonstrated functional recovery after iPSCs-derived cardiomyocytes transplantation via cell sheets (56,57) or needle injection (58) in porcine or rat heart failure models. However, there are still some limitations such as cell delivery route that can have a strong impact on cell survival after transplantation as well as the prevention of tumor formation caused by remaining undifferentiated cells (54). So far, many scientists are trying to overcome the aforementioned limitations. For example, recently Mandai et al. (19) transplanted a sheet of retinal pigment epithelial cells differentiated from iPSCs in a patient with neovascular age-related macular degeneration. However, although the transplanted tissue remained intact after one year since the surgery, no improvements in visual acuity were identified.

Beside the self-renewal property of iPSCs, which answers to the need of a number of cells sufficient to carry out transplants, these cells can be a powerful tool when used in gene therapy using gene correction technology. Indeed, it would be often necessary to correct genetic defects related to specific diseases with the purpose of transplanting functional cells to the patient as therapy or to use cells differentiated from iPSCs of healthy donors. In the past years, several successes on mouse models of cell sickle anemia, muscular dystrophy and Parkinson's disease have been already achieved (20). Many efforts have been done to translate results obtained in animal models to humans. For instance, of great importance to concretely exploit the potential of iPSCs in regenerative medicine, is to focus on the creation of an iPSCs-bank of healthy donors. In order to reduce the risk of immune reactions, patient and donors have to be matched for the human leukocyte antigen (HLA) profiles. Indeed, it is not reasonable to think to generate iPSCs from the single patient because this would be too expensive and too long while an iPSCs bank would be the best option. In this regard, the CiRA association in Kyoto started to create an iPSCs bank from Japanese healthy donors in 2013 and they estimate to cover the entire population in terms of HLA profiles in 2022 (59).

One concrete example of using healthy donor's iPSCs to treat disease in a clinical trial has been conducted in 2018 by Prof. Yun Takahashi (21), in Japan. In particular, Takahashi and his team conducted the first human clinical trial to treat Parkinson's disease using

dopaminergic neurons generated from iPSCs previously obtained from the peripheral blood cells of an HLA-homozygous healthy individual.

1.2.7 Personalized medicine

The focus of therapy personalization is to find the most appropriate treatment and dose for the patient avoiding the occurrence of ADRs. iPSCs technology well fits this purpose preserving the donor genetic heritage and leading to the possibility to create patient-specific models. So far, iPSCs have been used in several studies for therapy personalization and to study ADRs, especially in adults (60–63). However, regarding the pediatric field, more efforts are still needed, even if some works are already available (45–47). The purpose of using iPSCs for therapy personalization could be a real possibility for the next future. Indeed, with a simple blood sample or skin biopsy it is possible to reprogram PBMCs or fibroblasts into iPSCs, that can be subsequently differentiated into the somatic cells of interest (figure 4).

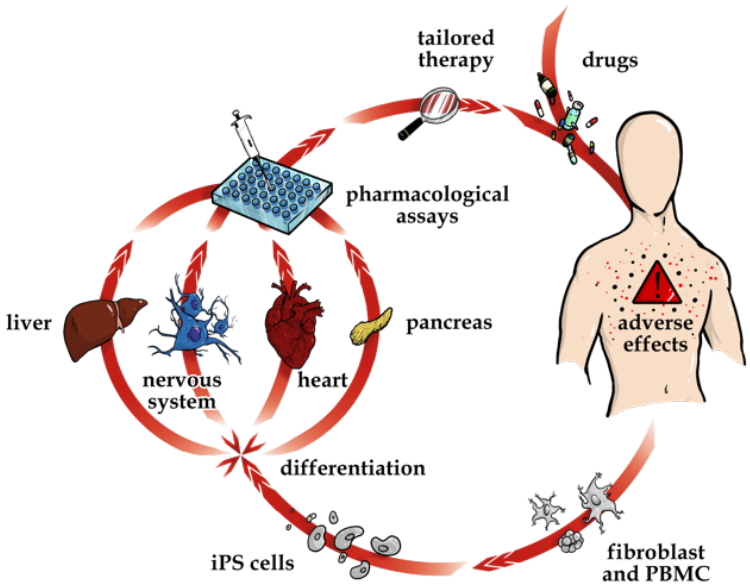

Figure 4. Therapy personalization using patient-specific iPSCs (48).

1.2.8 Differentiation of iPSCs

iPSCs are pluripotent cells that behave like ESCs and that can differentiate into almost all cells of the human body. Since the discovery of this technology in 2006, researchers have worked to develop several protocols to generate somatic cells characterized by the genetic heritage of the patient's. In particular, iPSCs can differentiate in the three germ layers mesoderm, ectoderm or endoderm (figure 5). Then, the germinal cells can be differentiated using adequate stimuli into a wide range of cell types such as cardiomyocytes (64), pancreatic cells (65,66), neurons (67), etc. Each differentiation protocol is specific for the desired cell type and, so far, in literature there are thousands of them. However, one issue related to this technology is the variable specificity and the different properties of the differentiated cells generated. In other words, differentiated cells obtained could be not characteristic of a single cell subtype but an heterogenous population of cells, due to uncomplete or aspecific differentiation (21,68–70). Indeed, it would be necessary to purify the generated cells in order to eliminate residual undifferentiated iPSCs or other types of cells randomly differentiated. To overcome this issue, cells can be purified using different strategies such as genetic markers or the expression of specific antigens. Cells obtained could be then used for the aforementioned applications such as regenerative medicine, cell therapy, the study of ADRs, for disease modelling or as a tool for drug screening.

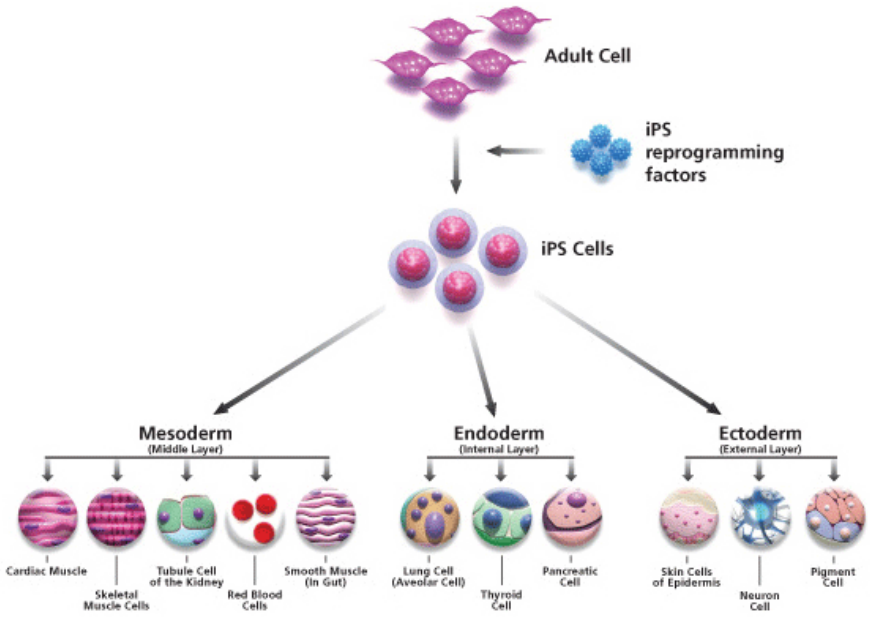

Figure 5. Differentiation potential of iPSCs. After iPSCs generation it is possible to stimulate cells to differentiate into mesoderm, endoderm or ectoderm. Then, each germ layer in turns leads to all cells of the human body (71).

1.2.9 Differentiation of iPSCs into exocrine pancreatic cells

In this project, Crohn's disease-patients iPSCs were differentiated into pancreatic exocrine cells using a protocol set up by Prof. K. Sasaki at Shinshu University (Matsumoto, Japan) on human embryonic stem cells (figure 6) (66). This technique is one of the cores in this project. The molecular mechanisms that regulate pancreatic acinar cell development remain unknown. So far, works on iPSCs exocrine pancreatic differentiation are limited (72,73) and studies has principally focused on the differentiation to endocrine rather than exocrine cells. Differentiating pancreatic exocrine cells from iPSCs would be a useful tool for elucidating the underlying mechanisms of pancreatic diseases or ADRs. The Japanese study (66) highlights that the pancreatic exocrine differentiation is highly promoted by a first stimulation of the definitive endoderm cells with fibroblast growth factor 7 (FGF7), known to be involved in pancreatic exocrine cells formation, a subsequent exposure to a combination of nicotinamide (NA), glucagon-like peptide-1 (GLP) and noggin and a final exposure to FGF7, GLP and retinoic acid. FGF7 is a critical factor for exocrine differentiation activating epithelial cell proliferation but repressing the development of the pancreatic epithelium into endocrine cells. FGF7 increased the number of amylase positive cells in a significant way. Moreover, it was confirmed that retinoic acid is a potent inducer of PDX1-positive progenitor cells as already highlighted by other works (74). However, so far, the individual role of the others molecules employed and their mechanisms in acinar pancreatic development still have to be clarified.

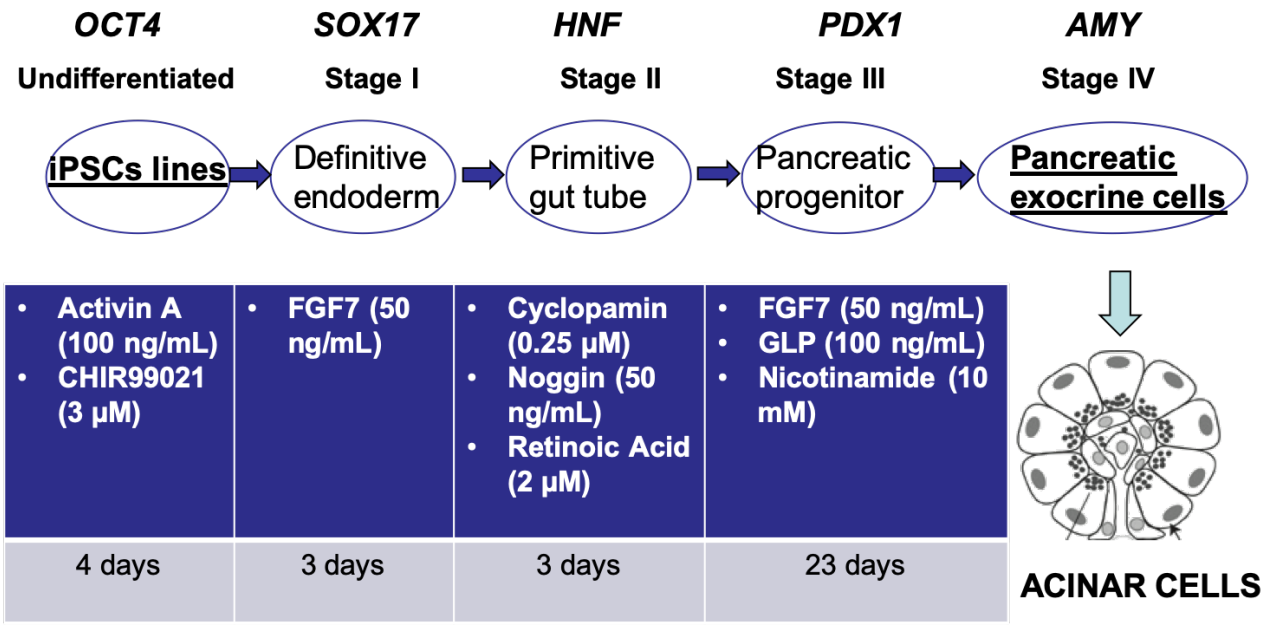

Figure 6. Differentiation protocol of CD patient specific iPSCs into pancreatic exocrine cells (see Material and Methods section). FGF7: fibroblast growth factor 7, GLP: glucagon-like peptide-1.

1.2.10 Limits of iPSCs

The first problem that can be addressed regarding iPSCs technology is the low efficiency in reprogramming. This issue is principally related to viral vectors, which deliver the reprogramming transcription factors by integration in the host cell genome, leading to chromosomal instability and tumorigenesis from insertional mutagenesis. However, during the past years, new safer and non-integrative reprogramming methods have been established, reaching better efficiency and avoiding the already mentioned issues (75).

Epigenetic memory and clonal variability are two other interesting points to discuss regarding iPSCs limits. iPSCs can present an epigenetic memory of the parent somatic cells that can influence the differentiation propensity and therefore the study outcomes. This peculiarity of iPSCs may predispose them to differentiate more readily into their parental cells than others and can be useful for example in cell replacement therapy. Kim et al. (76) observed in iPSCs a residual DNA methylation pattern of parent cells, which increase their propensity to differentiate along lineages related to the donor tissue, while restricting alternative cell fates. The epigenome of the parent cells was reset by authors by serial differentiations and reprogramming phases, or by treatment of iPSCs with chromatin-modifying drugs. These approaches could help researchers in increasing the differentiation

efficiency of iPSCs, limiting the problem related to epigenetic memory. The epigenetic memory of iPSCs can be affected also by the number of passages performed. Indeed, an increasing in the number of passages performed is related with the loss of epigenetic memory (77). Probably, the loss of parent cells epigenetic pattern is based on a slow replication-dependent process. However, further studies have to be performed to consolidate this hypothesis as indicated by authors and, to date, the origins of epigenetic loss remain to be determined.

Among iPSCs limits, the intra-variability of clones of the same patient is important to mention. This aspect was discussed by Thatava et al. (78) analyzing the variations in terms of pancreatic beta-cells differentiation of three iPSCs clone lines of three patients suffering from type 1 diabetes. Interestingly, a notable intra-patient variation, comparable to interpatient one, was identified by authors leading to conclude the necessity for a comprehensive fingerprinting of multiple patient-specific clones to obtain a representative pool of cells useable for biomedical applications such as ADRs studies. Also Yokobashi and colleagues (79) found some differences in germ-cells differentiation of iPSCs clones related to the female derived clones that where less efficient in terms of differentiation potential. To study ADRs, the clonal variability is a key point to keep in mind in order to set up useful standardized tools. Therefore, before the development of a model useful to predict patients' sensitivity, different clones should be first genetically checked to exclude for instance chromosomic aberrations, alterations in differentiation efficiency and variability in DNA methylation profiles. Also, the sensitivity to the drugs of interest should be analyzed in the different clones from the same patients, to exclude a variability in the response (80).

1.3 Crohn's disease

Inflammatory bowel diseases (IBD), encompassing Crohn's disease (CD) and ulcerative colitis, are chronic relapsing inflammatory disorders. CD can affect any part of the gastrointestinal tract but most commonly the ileum, colon or both with transmural inflammation while ulcerative colitis affects only the mucosa layer of the colon.

The annual incidence of CD is continuing to raise with the majority of cases reported in northern Europe, the United Kingdom and North America (81). Around 20-25% of CD

patients are younger than 20 years old. Although the incidence in children is lower than that in adults, it is also increasing with a clearly increment in children over 10 years of age (82,83). Although the etiology of CD remains unclear, it has been suggested that inflammation develops from a complex interaction between genetic predisposition, environmental risk factors and immune dysregulation to intestinal microbiota (81).

CD mostly occurs between young adulthood and the diagnosis is typically based on clinical signs and symptoms combined with laboratory tests, imaging and endoscopic techniques (84). Patients affected by CD may complain nausea, abdominal pain, diarrhea, weight loss and fever. The most typical pathological findings include discontinuous, transmural inflammation involving the whole thickness of the bowel wall, and a histological infiltrate of lymphocytes, plasma cells and granulocytes, with crypt atrophy and abscesses (84,85).

Treatments aim at reducing symptoms and at normalizing the biochemical alterations; however, mucosal healing has nowadays become the therapeutic goal in clinical practice, because of it is associated with a reduced risk of surgery, hospitalization and disease relapse. So far, corticosteroids in adults and enteral nutrition in children have been considered the standard treatment to induce the remission of the inflammatory process while thiopurines, in particular azathioprine, are the first-line therapy for preventing disease relapse in patients who achieved remission.

However, thiopurines can cause severe side effects such as bone marrow suppression and pancreatitis leading to therapy interruption. Aminosalicylates, such as mesalazine or sulfasalazine, are used in mild colonic CD with superficial lesions (86). In addition to the conventional treatments, recent studies have described a 50% remission at 6 months in patients with refractory CD on low-dose thalidomide (50–100 mg/d) (87). CD is currently treated also with biologic drugs such as infliximab, adalimumab and certolizumab which are anti-tumor necrosis factor (anti-TNF) agents that are effective in inducing and maintaining remission. However, despite the high efficacy of anti-TNF agent, around 40% of patients show no clinical benefit or a loss of response over time (86). Surgery is usually indicated in case of failure of medical management, complication, or malignancy.

1.3.1 Crohn's disease in the pediatric population

As already mentioned, pediatric CD accounts for 20-25% of all the cases. About 80% of these patients are diagnosed in adolescence (i.e., 10-18 years old children) and the incidence is

continuously increasing (88). CD in children, adolescents and adults is similar; however, some clinical and pathological aspects can be different. For instance, one obvious difference with respect to adults relies on the disease consequences on growth and puberty (89). The treatment is the same in adults and pediatric patients for maintaining remission including immunomodulators and biologics while is different for inducing remission preferring enteral nutrition for pediatric patients rather than corticosteroids in adults. Children and adolescents better respond to immunomodulators in terms of remission maintenance (82,90). Moreover, children and adults present different ADRs. The reason relies mainly the variation in the pharmacokinetic and pharmacodynamic profiles between these two populations. However, for the pediatric group, ADRs have not been studied so thoroughly as in adults. The clinical and research experience drug safety in children is limited and more studies are needed. One of the reasons for the lack of these data is that, for ethical reasons, the effects of most drugs have been analyzed in clinical trials only in adults, resulting in a limited knowledge on children response. In order to avoid the well-known ethical limitation of pediatric trials, to increase the comprehension of drug response in children and set up safer and personalized treatments it is possible to appeal to innovative technologies, such as iPSCs (91).

1.4 Thiopurines

The thiopurines azathioprine, mercaptopurine and thioguanine are immunosuppressant drugs used to treat several pathologic conditions such as acute lymphoblastic leukemia, autoimmune disorders, including CD, and to prevent rejection after organ transplantation. These drugs are inactive pro-drugs that need an extensive hepatic metabolism mediated by several enzymes to be activated into thioguanine nucleotides (TGNs) (figure 7). So far, the precise mechanism of thiopurine pharmacological activity is still unclear however, the principal actions by which these drugs carry out their cytotoxicity can be defined as molecular mimicry (92). Azathioprine is rapidly and almost totally transformed in mercaptopurine and S-methyl-4-nitro-5-thioimidazole via an enzymatic and a non-enzymatic reaction. In particular, the enzymatic reaction is carried out by the glutathione-S-transferase enzymes (GSTs) while the non-enzymatic one by a spontaneous interaction with the reduced form of glutathione (93). The main enzymes involved in the initial metabolism of mercaptopurine and thioguanine are hypoxanthine-guanine phosphoribosyl

transferase (HGPRT), thiopurine methyltransferase (TPMT), aldehyde oxidase (AO) and xanthine oxidase (XO). Metabolites obtained after AO, TPMT and XO metabolism of mercaptopurine have no significant cytotoxic action. Among these TPMT is an important enzyme involved in the cytotoxicity of thiopurine drugs because its activity is highly variable between individuals, partially due to the presence of common genetic polymorphisms (94,95).

Considering mercaptopurine activation, the drug is first converted by to thioinosine 5'-monophosphate (TIMP) by hypoxanthine phosphoribosyltransferase 1 (HPRT1) and then to thioxanthine monophosphate (TXMP) by inosine monophosphate dehydrogenase (IMPDH). TXMP is converted in thioguanosine monophosphate (TGMP) by guanosine monophosphate synthase (GMPS). TGMP is then transformed in thioguanosine diphosphate (TGDP), thioguanosine triphosphate (TGTP) and in deoxyguanosine di- and tri-phosphate (dTGDP, dTGTP). The precursor metabolite TIMP acts as substrate of TPMT, besides converting mercaptopurine to inactive methyl-mercaptopurine (me-MP), biotransforms TIMP into the cytotoxic methyl-TIMP (me-TIMP) metabolite, a strong inhibitor of *de novo* purine synthesis related to cytotoxicity effect of azathioprine.

Thioguanine, with respect to mercaptopurine, is directly converted in TGMP which in turn is converted in TGDP, TGTP and dTGDP, dTGTP (96). Mercaptopurine and thioguanine, after the extensive aforementioned metabolism, exert their cytotoxic effect by:

- Incorporating TGTP and dTGTP into RNA and DNA, respectively. This incorporation is higher in cells that replicate more quickly such as activated T lymphocytes where incorporated TGNs levels have been found to be higher (97). In particular, dTGTP incorporation inhibits the function of several enzymes involved in DNA replication and repair, and induces DNA damage such as single strand-breaks, DNA–protein cross-links and chromatid exchanges. Incorporation of TGNs into RNA leads to damage as well. This damage to RNA and DNA ultimately results in cytotoxicity and apoptosis (98).
- Interfering with the *de novo* purine synthesis by meTIMP action that inhibits phosphoribosyl pyrophosphate amidotransferase (PPAT), the first enzyme involved in this pathway of purine synthesis (99).
- Inhibiting the activity of Rac1, an important antiapoptotic G-protein in activated T lymphocytes, by TGTP (100).

- For azathioprine, reducing the intracellular glutathione level used as substrate of GSTs in azathioprine transformation to mercaptopurine (101).

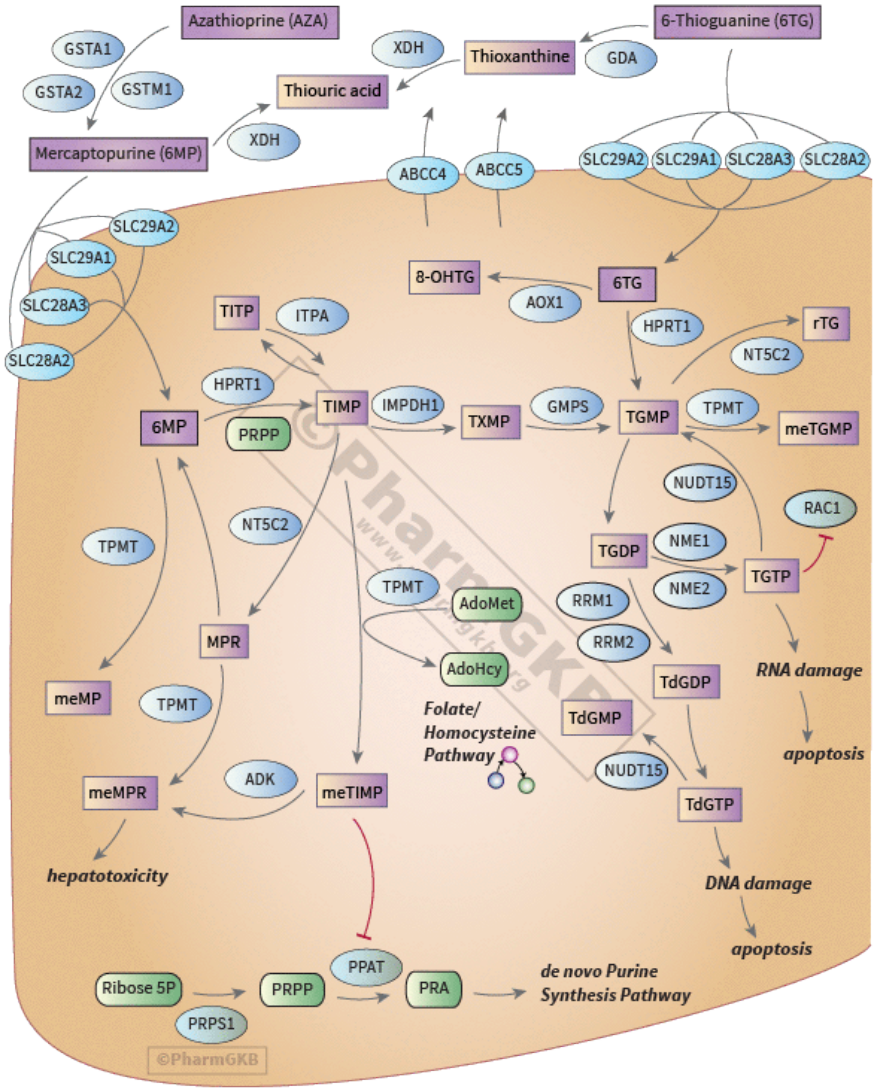

Figure 7. Thiopurine pathway (102). ABCC4, multidrug resistance-associated protein 4; ABCC5, multidrug resistance-associated protein 5; ADA, adenosine deaminase; ADK, adenosine kinase; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosyl-methionine; AHCY, S-adenosylhomocysteine hydrolase; AOX1, aldehyde oxidase 1; GAR, glycinamide ribotide; GART, phosphoribosylglycinamide formyltransferase; GMPS, guanosine monophosphate synthetase; GSTA1, glutathione-S-transferase A1; GSTA2, glutathione-S-transferase A2; GSTM1, glutathione-S-transferase M1; HPRT1, hypoxanthine guanine phosphoribosyltransferase 1; IMPDH1, inosine monophosphate dehydrogenase type 1; ITPA, inosine triphosphatase pyrophosphatase; MeMP, methylmercaptopurine; 6-meMPR, 6-methylmercaptopurine riboside; MeTGMP, methyl-thioguanosine monophosphate; MeTIMP, methyl-thioinosine monophosphate; MPR, 6-mercaptopurine riboside; NT5E, nucleotidase, ecto-5-prime; 8-OHTG, 8-hydroxythioguanine; PPAT, phosphoribosyl pyrophosphate amidotransferase; PRA, 5-phosphoribosylamine; PRPP, 5-phospho-D-ribose-1-pyrophosphate; PRPS1, phosphoribosyl pyrophosphate synthetase 1; RAC1, ras-related C3 botulinum toxin

substrate 1; SLC28A2, solute carrier family 28, member 2; SLC28A3, solute carrier family 28, member 3; SLC29A1, solute carrier family 29, member 1; SLC29A2, solute carrier family 29, member 2; TdGDP, 6-thio-deoxy-guanosine diphosphate; TdGTP, 6-thio-deoxy-guanosine triphosphate; TGDP, 6-thio-guanosine diphosphate; TGMP, thioguanosine monophosphate; TGTP, 6-thio-guanosine triphosphate; TIMP, thioinosine monophosphate; TITP, thioinosine triphosphate; TPMT, thiopurine S-methyltransferase; TXMP, thioxanthosine monophosphate; XDH, xanthine dehydrogenase.

1.4.1 Thiopurine-induced adverse effects

Thiopurines are drugs undergoing an extensive metabolism to became active and exert their cytotoxic effect in the most rapidly dividing cells such as activated T cell lymphocytes where azathioprine also downregulates genes involved in T cell immunity. The most critical and severe side effects related to thiopurine treatment are: bone marrow toxicity, hepatotoxicity and pancreatitis. Some of these thiopurine-induced adverse effects are strictly linked to their extensive metabolism that can be highly altered by different polymorphisms present in patients. For instance, the analysis of alterations in TPMT activity, enzyme coded by the *TPMT* gene fundamental in thiopurine inactivation, assists clinicians to determine the patient tolerance to thiopurine treatment and adapt the therapy to the individual sensitivity. These alterations can be caused by rare or common polymorphisms depending also on patient ethnicity such as TPMT*3A in Caucasian and TPMT *3C in Afro-American, Japanese and Chinese population. Patients with a complete deficiency of TPMT can develop a severe and potentially fatal myelosuppression while ultrahigh TPMT individuals present poor clinical response with low quantity of TGNs incorporation (103). However, around 50-70% of patients with normal TPMT activity develop leukopenia and hematotoxicity after thiopurine treatment; alterations in the nudix hydrolase 15 (NUDT15) emerged as a determinant factor in these side effects (103,104). Moreover, it is a strongly consolidated clinical option that patients taking thiopurines should be regularly monitored for intracellular TGNs and their methylated derivatives levels to reduce the risk of toxicity (105,106).

Inosine triphosphate pyrophosphatase (ITPA) enzyme is involved in thiopurine inactivation as well. Overall, in normal cells ITP is formed by the phosphorylation of IMP by ITPA that converts ITP back to IMP, avoiding its accumulation. This condition is benign in the absence of thiopurine therapy. However, ITPA variants in patients treated with thiopurines results in an accumulation of the potentially toxic thioinosine triphosphate

(TITP). In particular, ITPA converts TITP into the putatively less active TIMP. Moreover, low activity of the ITPA enzyme can increase the TGNs levels and the risk of hematological toxicities in acute lymphoblastic leukemia patients (107,108).

The GSTs family is also related to the development of thiopurine-adverse effects, especially for azathioprine treatment. Indeed, patients with polymorphisms in *GST* gene associated with reduced enzymatic activity present less ADRs such as neutropenia (109) and lymphopenia (110).

Hepatotoxicity is another severe side effect of thiopurine treatment related in particular to the Me-TIMP levels. Indeed, several clinical studies highlight the correlation between high levels of these toxic metabolites and the occurrence of hepatotoxicity in patients (111). Also in this case, to avoid the development of toxicity, thiopurine metabolite levels can be monitored with subsequent dose adjustment.

Pancreatitis can occur after thiopurine treatment as well and is not related to the dose of the drug used. The underlying mechanisms of this ADRs is unknown and treatment interruption is required to resolve it (94).

1.4.2 Thiopurine-induced pancreatitis

Acute pancreatitis (AP) is an inflammatory condition caused by pancreatic acinar injury, which spills proteases creating necrosis of pancreas parenchyma. The incidence of AP is increasing over time, ranging from 15 to 40 cases per 100,000 per year in European studies (112) and reaching an estimated annual incidence of 13 per 100,000 children (113). AP can occur for different reasons including gallstones, alcohol (114) and, less frequently, drugs (115). The majority of cases are mild to moderate but severe episodes can also occur. Severe cases may lead to death, while mild ones to patients' hospitalization. Even if drug induced-pancreatitis is relatively rare it is a serious problem both for the patient and the health system. Around 0.1 – 2 % of drugs are related to the development of this ADR, in particular asparaginase (116), nilotinib (117) and pazopanib (118), and cases can be mainly divided into mild and severe. An increased incidence of AP has been recorded in patients with IBD compared to the general population. This higher incidence can be mainly caused both by cholelithiasis, due to the higher risk of developing gallstones in CD patients than in general population, or by medications used (119,120). The drugs most related to AP development

in IBD patients are thiopurines (azathioprine and mercaptopurine). CD patients are the most predisposed to develop AP within the IBD population, the incidence is reported to be 3 – 5% of CD patients treated with thiopurine drugs in comparison to an incidence of 1 – 2% in patients with ulcerative colitis (112,121). The higher incidence in CD patients suggests that molecular mechanisms involved in CD, such as innate immunity, may also contribute to thiopurine induced-pancreatitis (TIP) pathogenesis. However, TIP is an idiosyncratic adverse reaction and mechanisms determining the predisposition to develop it are unknown. Understanding the reasons behind TIP development is important for patients that otherwise have to stop the treatment and be hospitalized until the symptoms are resolved. Mechanisms that may be proposed can be principally divided into three different groups: *genetic predisposition* (122,123), *alteration in thiopurine biotransformation* (91) and *abnormalities in innate or adaptative immunity* (124).

Genetic predisposition

As mentioned in the previous chapter, *TPMT* and *ITPA* variants are related to the development of severe adverse effects in patients. However, these important genes in thiopurine inactivation seem not responsible for pancreatitis predisposition (94,95). With the purpose to find genetic determinants to predict TIP in IBD patients, two different genome-wide association studies were recently performed. Both research groups found a strong association between the Class II HLA gene region polymorphism (rs2647087) and TIP. In particular, in the study performed by Heap and colleagues (122) the GWAS analysis was performed on cases and controls with IBD and then findings were validated in an independent cohort matched for drug exposure. In the first set of patients the authors identified a strong association of rs2647087 within the class II HLA region and development of TIP (odds ratio 2.59, 95% confidence interval 2.07–3.26, $p = 2 \times 10^{-16}$) and validated in the second cohort. Fine mapping of the HLA region further characterized the association with the HLA-DQA1*02:01–HLA-DRB1*07:01 haplotype. This study showed that patients heterozygous at rs2647087 have a 9% risk of developing pancreatitis after thiopurine administration, while the homozygous have a 17% risk. Also Wilson et al. (123) corroborate this association between the class II HLA region (at rs2647087). The risk of pancreatitis during azathioprine-therapy was highly predictable and genotype dependent: 0.53% for wild type (A/A), 4.25% (OR = 4.19, 95% CI 1.02- 36.45, p = 0.044) for heterozygous (A/C),

and 14.63% (OR = 15.83, 95% CI 3.80-145.26, p = 0.0001) for homozygous variant (C/C) patients. Overall, both studies concluded that the rs2647087 SNP is an important marker of azathioprine-induced pancreatitis risk.

Thiopurine biotransformation hypothesis

TIP in IBD patients could be related to a direct toxicity against pancreatic exocrine cells and/or to an accumulation of toxic thiopurine metabolites.
The incidence of TIP is higher in CD patients treated with azathioprine with respect to those treated with mercaptopurine (91). This different predisposition after treatment suggests a particular contribution of azathioprine in TIP development. Azathioprine is a prodrug that has to be converted in mercaptopurine. This conversion is mainly spontaneous, however the GST enzyme in part catalyzes this reaction using as substrate the antioxidant glutathione (125) leading to a significant increase of reactive oxygen species (ROS). The depletion of glutathione and the higher concentration of ROS characteristic of azathioprine may induce pancreatitis at higher level with respect to mercaptopurine.

Innate or adaptative immunity hypothesis

The incidence of TIP in CD patients is higher with respect to other conditions (e.g. ulcerative colitis, autoimmune hepatitis) leading to assume that the CD characteristic altered immune system may play a role in TIP development. TIP generally occurs within 30 days after thiopurine treatment. Also this latency let to hypothesize that TIP could be mediated by immunological reactions rather than by an accumulation of toxic metabolites that usually generate toxicity after several months of drug usage (91).

2. Aim of the research

Crohn's disease (CD) is an inflammatory condition characterized by granulomas, lesions and transmural inflammation. The inflammation can affect each part of the gastrointestinal tract but particularly the ileum and colon tracts are the most involved. No cure is available for CD so far, however several treatments are effective, including thiopurines, immunosuppressant drugs used mainly during maintenance of remission. Despite the efficacy of thiopurines, 3 to 5% of CD patients can develop pancreatitis, with a frequency that is higher in comparison to patients affected by other conditions. Pancreatitis is a severe idiosyncratic adverse effect generally occurring within 30 - 90 days after the start of treatment requiring an immediate interruption of the thiopurine therapy and in the most severe cases to patients hospitalization. So far, no biomarkers are available to help clinicians to prevent the development of pancreatitis also due to the inaccessibility of the pancreatic tissue for pharmacological analysis.

Induced pluripotent stem cells or iPSCs are stem cells obtained reprogramming somatic cells using the four Yamanaka's factors *OCT4*, *SOX2*, *KLF4* and *MYC*. iPSCs have the ability to differentiate under adequate stimuli into any cell type. Using iPSCs, it is possible to obtain cells otherwise hardly accessible from human probands. Several interesting studies have been already performed in liver, central nervous system, cardiac cells and ADRs. However, no study has been performed on pancreatic tissue and drug induced pancreatitis, so far.

The specific hypothesis is that TIP is due to an idiosyncratic reaction of patients' pancreatic exocrine cells caused by thiopurines sensitivity, manifesting as genetically determined differences in biotransformation, cytotoxicity or predisposition to immune cells activation.

The principal aim of this PhD research was to set up a personalized *in vitro* model to study TIP predisposition in pediatric CD patients treated with azathioprine differentiating patient-specific iPSCs into pancreatic exocrine cells.

The main purposes were:

(1) to establish CD patient-derived iPSCs starting from PBMCs

(2) to differentiate patient-derived iPSCs to amylase-producing mature exocrine pancreatic cells

(3) to perform pharmacological assays on thiopurine sensitivity of CD patient-specific iPSCs and differentiated patients' pancreatic cells.

The results of this research could be useful for studying mechanisms underlying TIP predisposition in CD patients using patient-specific pancreatic exocrine cells. The developed *in vitro* model could be also useful for discovering biomarkers important for clinicians in the prevention of this adverse effect.

3. Material and methods

3.1 iPSCs generation

CD patient-specific iPSCs and the healthy BJ-iPSCs were generated by Prof. Giliani's group at "A. Nocivelli" Institute of Molecular Medicine (Brescia, Italy) using the Cyto-Tune-iPS 2.0 Sendai Reprogramming Kit (ThermoFisher), a non-integrative technology based on three different vectors to deliver, efficiently and safely, Yamanaka Factors (*OCT4*, *SOX2*, *KLF4*, and *MYC*) into somatic cells to induce reprogramming towards iPSCs. Sendai Virus is a negative sense single stranded RNA virus that can replicate in the cytoplasm without entering in the nucleus until cells do not dilute and eventually eliminate thanks to replication. SeV vector is safe for humans and is one of the most used reprogramming techniques to generate iPSCs because it is easy-to-use, safe and efficient.

A total of six patients, three with pancreatitis and three without, were enrolled. All patients are males with an average age at the time of blood sampling of 16 ± 2.9 and 14 ± 3.5 years for cases and controls, respectively. Briefly, to generate the CD patient-specific iPSCs, 10 mL of whole blood of CD patients with and without pancreatitis after azathioprine treatment were collected at IRCCS Burlo Garofolo (Trieste, Italy). Pancreatitis was defined as severe abdominal pain accompanied by serum amylase level more than twice the normal limit or asymptomatic increase of amylase above twice the normal limit. In particular, two patients had symptomatic pancreatitis and one patient had asymptomatic hyperamylasemia.

Appropriate blood collection tubes containing lithium heparin as anticoagulant were used for samples, which were processed within 24 hours at "A. Nocivelli" Institute. PBMCs were isolated and reprogrammed into iPSCs in around 30 days. After PBMCs reprogramming, iPSCs clones were picked from days 21 - 28, selected based on expression of pluripotency markers and then established in culture.

The healthy BJ-iPSCs line was generated using the same above described technique, starting from the healthy fibroblast commercial line BJ.

Karyotype stability was analyzed and confirmed by the "A. Nocivelli" group in parental cells and iPSCs at different time point of culture using Q-banding at 450 bands resolution according to the International System for Human Cytogenetic Nomenclature.

The 253G1-iPSCs iPSCs healthy line was kindly provided by Prof. Sasaki's group from Shinshu University (Matsumoto, Japan), generated from adult dermal fibroblasts using the retroviral transduction technique by Prof. Yamanaka and colleagues (Tokyo University, Japan).

3.2 Cell cultures

All iPSCs were maintained in StemMACS iPS-Brew XF medium (Miltenyi Biotec) on diluted Matrigel (Corning, Life Sciences) coated plates (1:60 Matrigel-DMEM/F12 medium) in order to allow cell adhesion. Cell passage was performed after reaching 80% of confluence, determined by visual examination of the cultures. iPSCs were passed using a standard protocol, avoiding the complete break up of clusters formed (see 3.3 section). The normal healthy pancreatic ductal line H6C7 was maintained in Keratinocyte SFM medium (Invitrogen) and the cells were passed weekly when reaching 80 - 90% of confluence using 0.5% of Trypsin-EDTA.
Cell cultures were maintained according to standard procedures in a humidified incubator at 37°C and with 5% CO_2, and cell passage was performed twice a week.

3.3 Cluster passage protocol

iPSCs grow in clusters and the standard passage avoids the total break up of clusters formed. The standard protocol used for long-term iPSCs cultures provides the following steps:

1. Aspirate the exhausted medium
2. Wash two times with an appropriate volume of phosphate buffer saline (PBS)
3. Add an appropriate volume of Versene (ThermoFisher) dissociating reagent and incubate 2 minutes in a humidified incubator at 37°C and with 5% CO_2
4. Check visually the effective dissociation
5. Remove the Versene solution

6. Add an appropriate volume of StemMACS iPS-Brew XF medium and gently pipet two times the cells
7. Transfer 1/5 of the medium containing the floating colonies into a new Matrigel-coated plate
8. Expose cells to 10 mM of Y-27632 (Rock inhibitor, Miltenyi Biotec - final concentration 10 μM) for 24 hours in order to facilitate cells adhesion

3.4 Single cells passage protocol

To perform cytotoxicity assay it is necessary to dissociate at a single cell level the colonies formed, in order for cells to be plated at a definite density. The single cell protocol provides a longer incubation time (5-6 minutes) of cells with the Versene dissociation reagent. The suspension of cells and medium have to be gently pipetted more times with respect to the standard protocol in order to obtain a single cells suspension. Cells have to be exposed to 10 μM of Y-27632 for 24 hours as for the standard procedure.

3.5 Freezing and Thawing

A stock of patient-specific iPSCs was created freezing cells reaching 70-90% of confluence using the CryoStor® cryopreservation media (Sigma-Aldrich), an optimized preservation media containing 10% dimethyl sulfoxide (DMSO). CryoStor® is recommended for the preservation of extremely sensitive cell types including iPSCs. One mL of CryoStor® is rapidly and gently added to the pellet of cells previously obtained centrifuging cells at 400xg. Cells in the CryoStor® solution are stored in appropriate cryogenic vials in a criostep container for the first 24 hours at -80°C allowing a gradual freezing. The day after, the cells are moved in liquid nitrogen for long term storage.
The process to thaw iPSCs have to be quick but gentle. Before starting the process, pre-warm 9 mL of medium in a 37°C water bath. Then, quickly thaw the vial of interest containing cells in a 37°C water bath until only a small ice crystal are observable. Gently transfer cells to the warmed medium and centrifuge 5 minutes at 400xg. Aspirate the medium and gently resuspend cells in an adequate volume of fresh medium containing 10 mM of Y-27632.

Transfer the cell suspension to a pre-coated Matrigel plate. Incubate cells for 24 hours and replace the medium or proceed to cell passage if cells looks confluent.

3.6 Mycoplasma detection

Mycoplasma contamination remains a serious problem for cell cultures and can create several different effects on contaminated cell. Some examples of consequences of contamination are: alterations of proliferation, of cellular metabolism and cellular morphology. In addition to these problems, several studies have highlighted that mycoplasma contamination negatively influence the reprogramming of cells, being a critical reason for failure of iPSCs generation. Thus, before performing reprogramming, all cell lines used in this research have been tested for the 16S rRNA gene for Mycoplasma detection. Also iPSCs obtained were checked for contamination once thawed. To check the presence of Mycoplasma a polymerase chain reaction (PCR) method was used.

3.7 Pancreatic exocrine differentiation protocol

The protocol was developed by Prof. K. Sasaki (66) to differentiate human embryonic stem cells into pancreatic exocrine cells. This protocol is based on a 4 steps procedure (figure 8):

1) differentiation of iPSCs into definitive endoderm by activin A (100 ng/mL, Sigma-Aldrich) and CHIR99021 (3 μM, Sigma-Aldrich) for 4 days (stage I);
2) differentiation into primitive gut tube by fibroblast growth factor (FGF-7 50 ng/mL, Abnova) for 3 days (stage II);
3) differentiation into pancreatic progenitor cells by a combination of cyclopamine (0.25 μM, Sigma-Aldrich), noggin (50 ng/mL, Invitrogen) and all-trans retinoic acid (2 μM, Sigma-Aldrich) (stage III) for 3 days;
4) differentiation into pancreatic exocrine cells by a combination of FGF-7 (50 ng/mL, Abnova), glucagon-like peptide 1 (100 ng/mL, RayBiotech) and nicotinamide (10 mM, Sigma-Aldrich) (stage IV) for 14 or 23 days for the healthy iPSCs lines (253G1-iPSCs and BJ-iPSCs) or the patient-specific CD iPSCs, respectively.

Each stage is characterized by distinctive markers. In particular, *OCT4* is a pluripotent marker characteristic of undifferentiated iPSCs, *SOX17* and *FOXA2* of definitive endoderm cells (stage I), *HNF* factors of primitive gut tube cells (stage II), *PDX1* of pancreatic progenitors (stage III) and *α-amylase* and its pancreatic isoforms *AMY2A* and *AMY2B* of pancreatic exocrine cells (stage IV).

Cells were grown in RPMI 1640 medium added with 1% of penicillin-streptomycin during all the differentiation process. To differentiate CD patient specific iPSCs it was necessary to increase the number of days needed to generate exocrine pancreatic cells (stage IV) from 14 days, as indicated by the original protocol, to 23 days. The standard protocol was used for both 253G1-iPSCs and BJ-iPSCs lines differentiation.

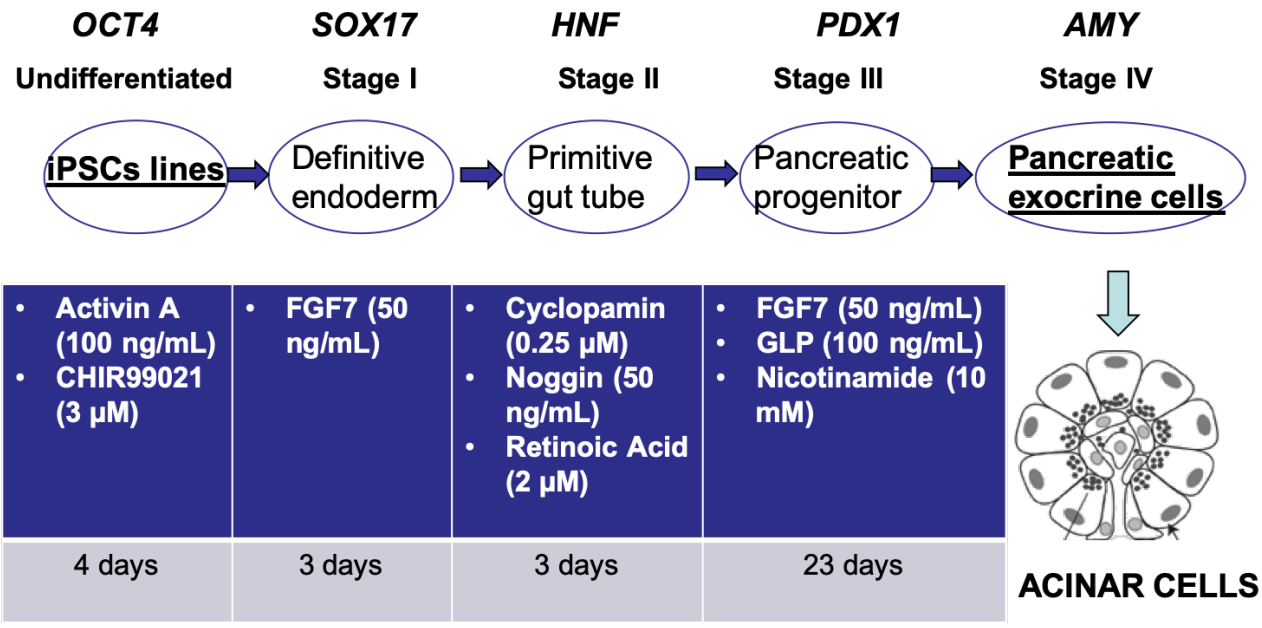

Figure 8. Protocol used to differentiate CD patient-specific iPSCs to pancreatic exocrine cells. Markers: *OCT4* (octamer-binding transcription factor 4), *SOX17* (SRY-box 17), *HNF* (Hepatocyte nuclear factors), *PDX1* (pancreatic and duodenal homeobox 1), *AMY* (*α-amylase*). Protocol used to differentiate the 253G1-iPSCs and the BJ-iPSCs healthy lines provides 14 days in the last step to obtain pancreatic exocrine cells.

3.8 Total RNA isolation

Total RNA of all iPSCs analyzed was extracted using the TRIzol® reagent (Thermo Scientific). TRIzol® reagent maintains the integrity of RNA due to the highly effective

inhibition of RNase activity while completely dissociates the nucleoprotein complex homogenizing cells. The samples were incubated with 1 mL of TRIzol® for 5 minutes at room temperature to dissociate the nucleoprotein complex. Chloroform (0,2 mL; Sigma-Aldrich) was added and after 3 minutes of incubation at room temperature, a centrifugation at 12,000 × g for 15 minutes at 4°C was performed. After centrifugation, the mixture separates into a lower red phenol-chloroform phase containing protein, an interphase containing DNA and a colorless upper aqueous phase containing RNA. The upper phase was then transferred into a new RNase-free tube to proceed with the RNA isolation procedure. After precipitation with 500 µL of 100% isopropanol (Sigma-Aldrich) and a wash step with 1 mL of 75% ethanol (Sigma-Aldrich), the RNA pellet was resuspended in 20 µL RNase-free water (Gibco-Life Technologies) and incubated in a water bath at 55–60 °C for 15 minutes. Then, the RNA concentration and purity were evaluated by a Nano Drop instrument (NanoDrop 2000, EuroClone®). Quantity of initial TRIzol® and following reagents were halved if the number of cells was < 1 x 10^6.

RNA of differentiated cells was extracted using the column PureLink™ Viral RNA/DNA Mini Kit (Invitrogen, ThermoFisher Scientific), which allows a more efficient and a highly pure RNA isolation even from a limited number of cells, such as in the case of differentiated samples. One hundred % ethanol (0,1 mL) was added to the aqueous phase, (previously obtained after TRIzol® incubation and isopropanol step) and the mixture was added to the column PureLink™ and a centrifugation at 12,000 × g for 1 minute at room temperature was performed. After centrifugation, the liquid filtered was discarded while the RNA was retained by the filter. After the two washes, the spin column was placed in another clean Wash Tube. Any residual Wash Buffer was removed by centrifuging the spin column at the maximum speed for 1 minute. The spin column was placed in a clean Recovery Tube and 10–50 µL sterile RNase-free water was added to resuspend the isolated RNA. The RNA sample was incubated for 1 minute at room temperature and then analyzed by a Nano Drop instrument (NanoDrop 2000, EuroClone®) for determining concentration and purity.

3.9 Reverse transcription

Reverse transcription is a process that converts RNA to single-stranded complementary DNA (cDNA) using a primer to the 3' end of the RNA template and serves as a starting point for the polymerase chain reaction (PCR). The reverse transcription was performed

using the High Capacity RNA to-cDNA Kit (Applied Biosystem) with up to 1 µg of total RNA per 20 µL of reaction containing 10 µL of 2 x RT Buffer, 1 µL of 20x RT Enzyme Mix. Reverse transcription was performed in a thermal cycler (Applied Biosystems 2720 Thermofischer Scientific). The thermal protocol provides a first incubation of samples to start the reaction at 37°C for 60 minutes and a stop of the reaction by heating to 95°C for 5 minutes and a final hold step at 4°C. The cDNA obtained is ready for use in real-time PCR applications or long-term storage in freezer.

3.10 Quantitative real-time PCR

The real-time PCR is a development of the PCR techniques that enables reliable detection and measurement of products generated during each cycle of PCR process. The amplification of the RNA sequence of interest can be obtained by a process consisting of 30-40 thermal cycles of heating and cooling.

The real-time PCR process can be generally divided into three steps:

-Initial denaturation.
At the start of real-time PCR, the temperature is raised to ensure that all complex double stranded cDNA molecules are separated into single strands for amplification.

-Cycling: denaturation, annealing and extension.
During denaturation the temperature is increased to 95°C and all double stranded cDNA are converted into single stranded cDNA.
During the annealing phase, the temperature is lowered to approximately 5°C below the melting temperature (TM) of the primers (often 45–60°C) to promote primer binding to the template. The primers are designed to bind the sequence of interest and the region of sequence that lies between them is referred to the amplicon. In general, the annealing temperature may be estimated to be 5 °C lower than the melting temperature of the primer-template DNA duplex. In the extension step, the temperature is increased to 72°C, which is optimum for DNA polymerase activity to allow the extension from the 3′ of each primer to the end of the amplicon.

-Repeat of cycling.

The denaturation, annealing and extension steps are repeated cyclically resulting in exponential amplification of the amplicon.

For quantifying the expression level of genes analyzed in this thesis, the fluorescent dye SYBR-Green, that intercalates in the PCR products at each thermal cycle, was used. In particular, the KiCqStart® SYBR® Green qPCR ReadyMix™ kit (Sigma-Aldrich) (126) was used. Both predesigned (KiCqStart® SYBR® Green Primers, Sigma-Aldrich) and custom primers were used. Primer sequences are reported in tables 1-3. The thermal cycler used was the CFX96 real-time system-C1000 (Bio-Rad Laboratories).

Expression levels of the target gene have to be normalized using an endogenous reference gene, the housekeeping gene. Beta-actin was used as normalizer and expression levels were reported as $2^{-\Delta Ct}$ (127,128). The results are provided as the mean and standard error of up to three replicates.

Gene	Primer	Sequence 5'->3'	T melting (°C)	Product size (bp)
ACTB	Forward	CGCCGCCAGCTCACCATG	86.5	120
	Reverse	CACGATGGAGGGGAAGACGC		
SOX2	Forward	CCCAGCAGACTTCACATGT	84.5	151
	Reverse	CCTCCCATTTCCCTCGTTTT		
OCT4	Forward	CCTCACTTCACTGCACTGTA	82.5	164
	Reverse	CAGGTTTTCTTTCCCTAGCT		
MYC	Forward	TGCCTCAAATTGGACTTTGG	73.5	192
	Reverse	GATTGAAATTCTGTGTAACTGC		

Table 1. Custom designed primers (Sigma-Aldrich) for real-time PCR analysis of stemness genes (*ACTB* beta-actin, *SOX2* SRY-box 2, *OCT4* POU class 5 homeobox 1, *MYC* proto-oncogene).

Gene	Primer	Sequence 5'->3'	T melting (°C)	Product size (bp)
	Forward	GGCGCAGCAGAATCCAGA		

SOX17	Reverse	CCACGACTTGCCCAGCAT	80.5	60
FOXA2	Forward	GGGAGCGGTGAAGATGGA	82.5	89
	Reverse	TCATGTTGCTCACGGAGGAGTA		
PDX1	Forward	AAAACGTAGTGATTGGAGG	86.0	122
	Reverse	CCAGACCTTGAAAAGAAGAC		
AMY2A	Forward	ACCTTTCATTTACCAGGAGG	79.0	148
	Reverse	GTAAGACATCTTCTCTCCATTC		
AMY2B	Forward	CTACAATGATGCTACTCAGG	79.5	181
	Reverse	AATTGCCTTTATGTCTCCAG		
α-AMYLASE	Forward	CTGACAACTTCAAAGCAAA	79.5	358
	Reverse	TACAGCATCCACATAAATACGA		

Table 2. Predesigned primers (Sigma-Aldrich) for real-time PCR analysis of differentiation markers. *SOX17* (SRY-box 17), *FOXA2* (Forkhead Box A2), *HNF* (Hepatocyte nuclear factors), *PDX1* (pancreatic and duodenal homeobox 1), *AMY2A* and *AMY2B* (pancreatic isoforms of *α-amylase*).

Gene	Primer	Sequence 5'->3'	T melting (°C)	Product size (bp)
HPRT1	Forward	ATAAGCCAGAGTTTGTTGG	77.0	179
	Reverse	ATAGGACTCCAGATGTTTCC		

Table 3. Predesigned primers (Sigma-Aldrich) for real-time PCR analysis of *HPRT1* (hypoxanthine phosphoribosyltransferase 1), gene coding for an important enzyme in thiopurine activation pathway.

3.11 Immunofluorescence assay

To confirm the successful differentiation of iPSCs, immunofluorescence analysis was performed analyzing PDX1 expression, a marker specific of pancreatic progenitor cells and the *a-amylase* expression, a marker characteristic of pancreatic exocrine cells. Undifferentiated iPSCs and pancreatic exocrine cells (stage IV) were analyzed. Cells were cultured and differentiated in 24-well plate on Matrigel-coated glasses. Cells were washed with 500 μL of PBS, fixed with 4% of paraformaldehyde in PBS (pH 7.4) for 30 min, permeabilized with 0.1% Triton X-100 in PBS and then treated with 1.5% normal donkey serum to block non-specific staining. After 30 min, the following diluted primary antibodies

were added and incubated overnight with the cells at 4 °C using the goat anti-PDX1 (1:200; R&D Systems) and rabbit anti-α-amylase (1:500; Sigma-Aldrich) antibodies. After the overnight incubation, cells were washed three times with PBS and incubated with a donkey anti-goat-PerCP 678 (1:1000 in PBS; Santa Cruz Biotechnology) or a donkey anti-rabbit 520 (1:1000 in PBS; Sigma-Aldrich) secondary antibodies together with 4,6-diamidino-2-phenylindole dihydrochloride for nuclei staining (DAPI; 1:1000 in PBS). The specimens were observed by a ZEISS Axio Observer Z1 or the Nikon Eclipse E800.

3.12 DNA extraction and pharmacogenomic analysis

Total genomic DNA was isolated from patient-specific CD iPSCs using a commercial kit (Gene Elute Blood Genomic DNA Kit Sigma-Aldrich, Milan, Italy) (129) according to the manufacturer's protocol.

TaqMan® SNP genotyping assays (Applied Biosystems, USA) were used to characterize the SNPs of interest: TPMT rs1142345, rs1800460 and rs1800462 and HLA-DQA1-HLA-DRB1 rs2647087 (table 4). The TaqMan® technique requires the use of an oligonucleotide probe containing a fluorescent reporter dye on the 5' end and a quencher dye on the 3' end and a pair of unlabeled primers. We used the 6-carboxyfluorescein (FAM) as fluorescent dye on 5' end for mutant allele while the 2'-chloro-7'phenyl-1,4-dichloro-6-carboxy-fluorescein dye (VIC) for the wild type allele. Samples genotyping was repeated twice.

Gene	SNP	Position	KIT-TaqMan
TPMT	rs1142345	chr6:18130687	C_____19567_20
TPMT	rs1800460	chr6:18138997	C__30634116_20
TPMT	rs1800462	chr6: 18143724	C__12091552_30
HLA-DQA1-HLA-DRB1	rs2647087	chr6:32681049-32681049	C__16052296_10

Table 4. TaqMan® probes used in real time PCR

3.13 Viability assay

Sulforhodamine B assay

Cells were seeded in 96-well Matrigel-coated plate at different concentrations and grown at 37°C in a humidified 5% CO_2 environment for 96 hours in order to determinate the optimal number of cells to be seeded for cytotoxicity assays. Cell concentrations analyzed were: $1.0x10^3$, $5.0x10^3$, $1.0x10^4$, $2.0x10^4$, $5.0x10^4$, $1.0x10^5$ cells per well. Cells were maintained for 24 hours in medium containing 10 μM of Y-27632 (Rock inhibitor, Miltenyi Biotec) in order to facilitate cells adhesion. After 96 hours of incubation, cells were washed two times with PBS and 12.5 μL (corresponding to ¼ ratio with respect to the initial volume of medium used to seed cells – 100 μL) of a cold 50% solution of trichloro acetic acid in water was added and the plate incubated at 4°C for one hour. The plate was washed two times with distilled water and then allowed to air-dry at room temperature. Fifty μL per well of sulforhodamine B solution were added at room temperature for 1 hour. After the incubation, three quickly rinses with 200 μL 1% (vol/vol) acetic acid were performed in order to remove unbound dye. The dye was subsequently solubilized by adding 200 μL of 10 mM tris(hydroxymethyl)aminomethane (Tris) base solution. The absorbance was read at 510 nm in a microplate reader (Bio Tek Instruments).

MTT assay

Cells were seeded in 96-well Matrigel-coated plates at approximately 1.0×10^4 per well. After a 24 hours incubation with 10 μM of Y-27632 (Rock inhibitor, Miltenyi Biotec) used to facilitate cells adhesion, different concentrations of azathioprine, mercaptopurine and thioguanine were dissolved in the culture medium and added to each well. Then, treated cells were incubated for 72 hours at 37°C in humidified atmosphere (5% CO_2) and in the last 4 hours of treatment a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added. The colorimetric MTT assay was performed to assess the metabolic activity of cells treated as described above. Ten μL stock MTT (5 mg/mL) were added to each well containing 100 μL of medium in order to obtain a final concentration of 0.5 mg/mL. Cells were lysed with dimethyl sulfoxide. Absorbance was measured at a double wavelength of 540 nm and 630 nm using a microplate reader (Automated Microplate

Reader EL311, BIO-TEK® Instruments, Vermont, USA). All measurements were done in three or four replicates, and at least three independent experiments were carried out.

3.14 Proliferation assay

Cell proliferation was determined by labeling metabolically active cells with [methyl-3H] thymidine. Cells were seeded on a 96-well Matrigel coated plates at 1.0×10^4 cells/well, and after 91 hours of incubation, were pulsed with [methyl-3H] thymidine (2.5 µCi/mL), and incubated 5 hours more. Cells were then washed with PBS, collected, and the radioactivity of the samples was determined by a liquid scintillation analyzer (Wallac 1450 Microbeta liquid scintillation counter, PerkinElmer, Milan, Italy). Raw count per minute (CPM) data were analyzed and compared to the no-stem lymphoblastoid line CCRF-CEM.

3.15 Cell cycle analysis

Cell cycle of patient-specific CD-iPSCs, stage I, stage III differentiated cells and H6C7 was analyzed using the flow-cytometry propidium iodide cellular uptake assay. For CD-iPSC and H6C7 samples, cells were cultured for 72 hours and harvested before reaching the confluence. Differentiated cells were collected at the end of the days necessary to differentiate them as indicated in the differentiation protocol.
Cells (2.0×10^6) were fixed in 70% ethanol on ice, washed twice with PBS, and allowed to stay in PBS for 1 hour at 4°C. Cells were stained overnight with 2 mL of a PBS/EDTA 0.5 mM solution containing 200 µL of propidium iodide (0.1 mg/mL) and 25 µL of 1 mg/mL RNase (Sigma-Aldrich). Samples were analyzed by the flow cytometer CYTOMICSTM FC500, Beckman Coulter Inc. Fullerton, CA. All flow cytometric measurements were carried out on storage data as list mode files and were analyzed with the FCS Express V3.

3.16 Statistical analysis

Results are presented as mean ± SE from up to three independent experiments, and nonlinear regression of concentration−response data was performed for computing EC50 values using GraphPad Prism version 7.00. Two-way ANOVA with Bonferroni post-test and t-test were used for the analysis of thiopurine cytotoxic effects of TIP and no-TIP CD patients' cells. T-test was used to analyze gene expression. P-values < 0.05 were considered statistically significant.

4. Results

4.1 Pancreatic exocrine differentiation

4.1.1 PCR real-time analysis

Differentiation of 253G1-iPSCs and BJ-iPSCs control lines

Preliminary differentiation experiments using the protocol developed for differentiating human embryonic stem cells (66) were performed on the 253G1-iPSCs and the BJ-iPSCs control lines derived from healthy donors. Differentiation markers were analyzed by real-time PCR. Experiments were initially performed using the 253G1-iPSCs line and mRNA levels of *OCT4*, *FOXA2*, *PDX1* and *α-amylase* were analyzed in undifferentiated cells, definitive endoderm cells (stage I of differentiation) and pancreatic progenitors (stage III of differentiation) in order to obtain preliminary qualitative data on iPSCs. *OCT4* is a pluripotency marker highly expressed in undifferentiated cells, *FOXA2* in endodermal cells (stage I), *PDX1* in pancreatic progenitors (stage III) and *α-amylase* in pancreatic exocrine cells (stage IV) (figure 8). Level of *OCT4* mRNA was higher in 253G1-iPSCs undifferentiated cells with respect to the other stages. *FOXA2* was highly expressed in definitive endoderm cells (stage I), being expressed around 500 fold than undifferentiated cells, *PDX1* and *α-amylase* in pancreatic progenitors (stage III), being expressed around 100 and 5 fold than undifferentiated cells, respectively (figure 9). The mRNA expression of key markers for pancreatic differentiation analyzed indicates that 253G1-iPSCs cells were differentiated into pancreatic progenitors, representing an important step in pancreatic development. Analysis of mRNA on stage IV cells was not performed due to technical reasons (unavailability of cells due to the length of the differentiation protocol).

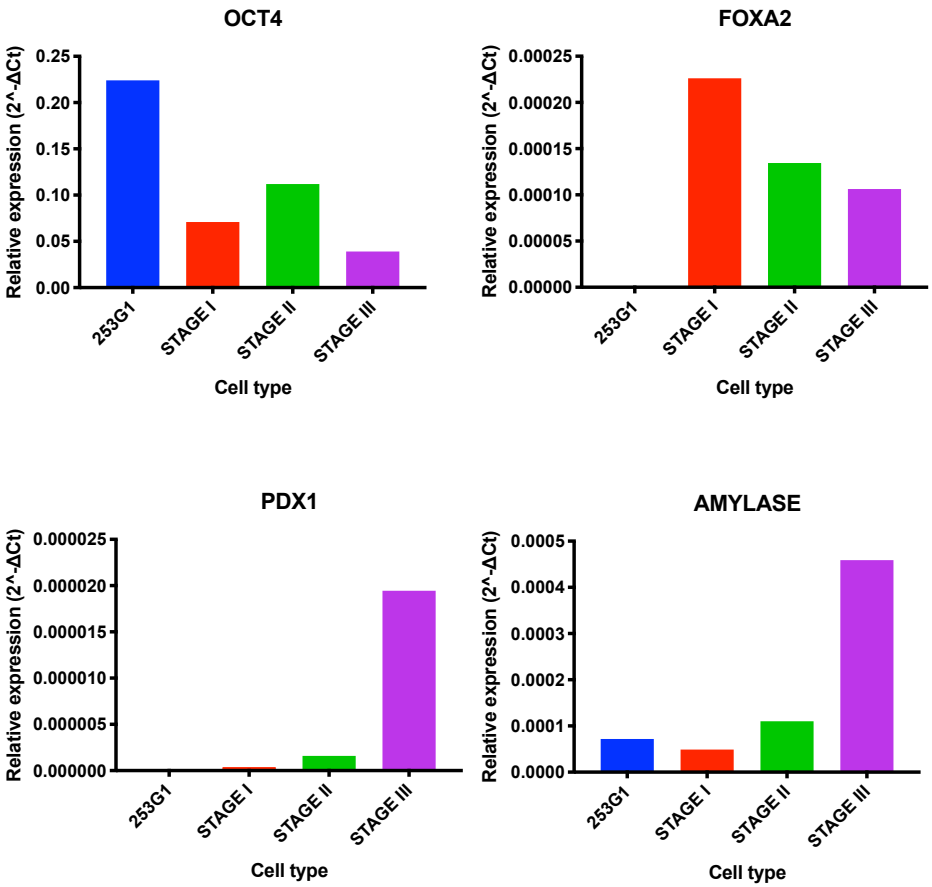

Figure 9. PCR-Real time analysis of differentiation marker levels during 253G1-iPSCs differentiation process to exocrine pancreatic cells. *OCT4* is a marker of pluripotent cells (253G1-iPSCs); *FOXA2* of endodermal cells (stage I); *PDX1* of pancreatic progenitor cells (stage III); *α-amylase* of pancreatic exocrine cells (stage IV). The data are reported as $2^{-\Delta Ct}$ values, using the housekeeping *β-actin* as reference. Data are obtained from a single differentiation experiment.

Analysis of key markers was performed also on the BJ-iPSCs healthy control line performing also quantitative evaluation (figure 10). *OCT4* was highly expressed in undifferentiated cells but also in stage I and stage III however a 4.5 fold decrease of its expression was found in the last stage of differentiation (stage IV). The endodermal markers *SOX17* and *FOXA2* were expressed 14.8 and 30.9 fold in stage I respectively compared to undifferentiated cells and stage III, however unexpectedly their expression increased again in the last stage of differentiation (stage IV). *PDX1*, marker of pancreatic progenitors (stage III), increased 539.2 fold in stage I and 972.6 fold in stage IV in comparison to undifferentiated cells. Surprisingly, *PDX1* was around half expressed in stage III with respect to stage I and stage IV.

The pancreatic exocrine marker *α-amylase* and its isoforms *AMY2A* and *AMY2B* showed an interesting trend. mRNA level of *α-amylase* was surprisingly high in undifferentiated cells with respect to the other stages in particular being expressed 1.8 fold with respect to stage IV. Despite this peculiarity, *α-amylase* level increased as expected in the IV stage of differentiation 1.9 fold compared to stage I and 3.0 fold with respect to stage III.

The pattern of *AMY2A* and *AMY2B* mRNA levels was interesting as well. In particular, for both isoforms the highest level was reached in stage I and not in the last stage (stage IV) as expected. Regarding *AMY2A*, the lowest level was found in stage III while, for *AMY2B* in stage IV. *AMY2A* level was expressed 1.7 fold higher in stage IV compared to undifferentiated cells while *AMY2B* was interestingly 3.8 fold higher expressed in undifferentiated cells with respect to stage IV.

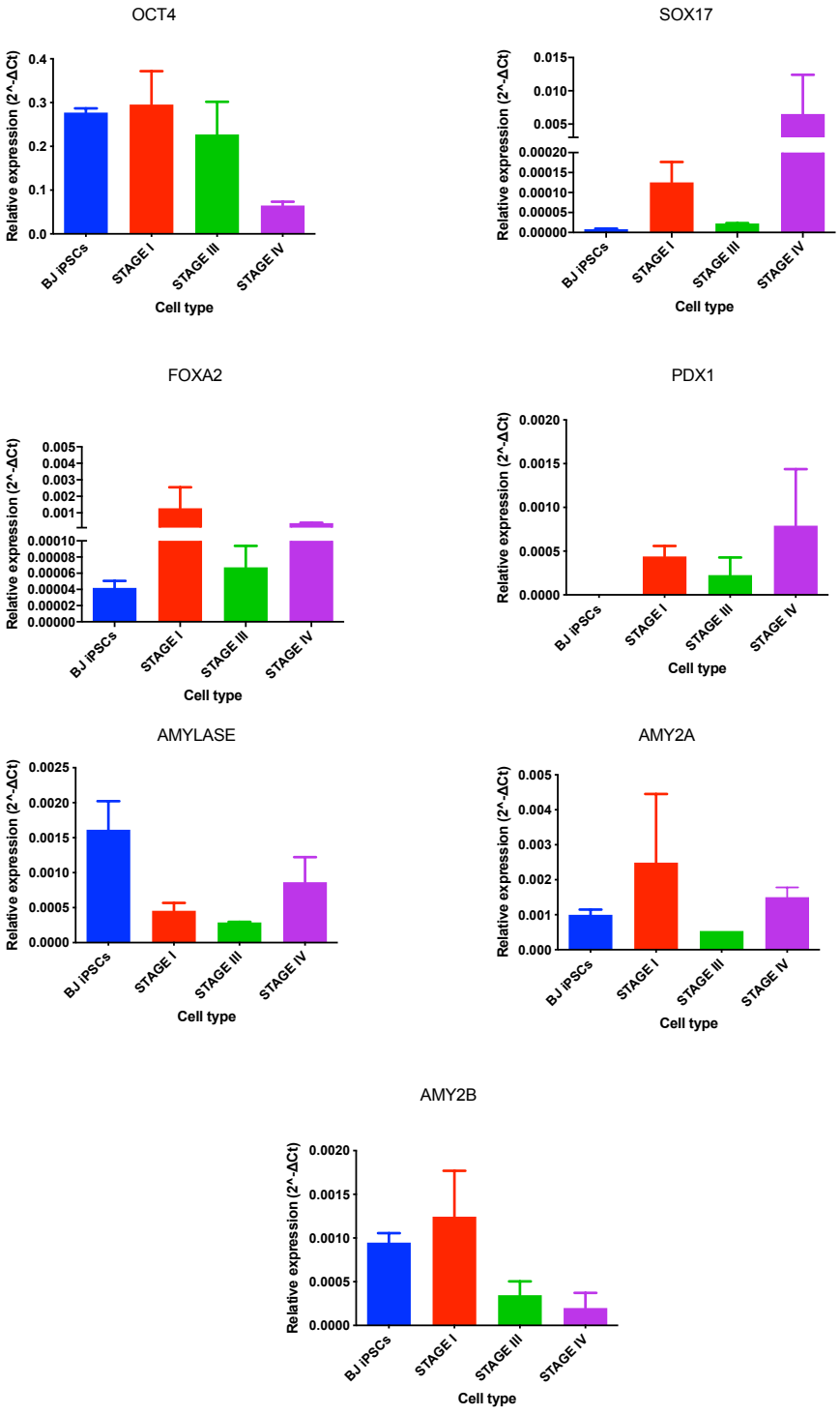

Figure 10. Real-time PCR analysis of differentiation marker levels during BJ-iPSCs differentiation process. *OCT4* is a marker of pluripotent cells (BJ-iPSCs); *SOX17* and *FOXA2* of endodermal cells (stage I); *PDX1* of pancreatic progenitor cells (stage III); *α-amylase*, *AMY2A* and *AMY2B of* pancreatic exocrine cells (stage IV). The data are reported as means ± SE of 3 independent experiments, evaluating $2^{-\Delta Ct}$ values, using the housekeeping *β-actin* as reference.

Differentiation of patient-specific CD-iPSCs

Once confirmed that it was possible to differentiate iPSCs using the protocol set up for embryonic stem cells, we focused on patient specific CD-iPSCs differentiation. iPSCs of one CD patient without and one with pancreatitis after thiopurine treatment were differentiated. Differentiation efficiency was analyzed by PCR-real time and results are reported in figure 10. The differentiation protocol successfully induced the different stages as revealed by real time PCR analysis: expression of the pluripotency marker *OCT4* was higher in iPSCs (iPSCs), *SOX17* in definitive endoderm (stage I), *PDX1* in pancreatic progenitors (stage III) and the two isoforms of *α-amylase* (*AMY2A*, *AMY2B*) in pancreatic exocrine cells (stage IV). In particular, *OCT4* was 193.3 and 87.2 fold higher in iPSCs (iPSCs) with respect to stage IV differentiated cells in no-TIP and TIP cells, respectively. The definitive endoderm marker *SOX17* resulted in no-TIP and TIP definitive endoderm cells (stage I) compared to undifferentiated cells 6783.5 and 624.3 fold higher, respectively. *PDX1* in pancreatic progenitors (stage III) was 1157.7 and 85.0 times higher compared to undifferentiated cells in no-TIP and TIP cells, respectively. *AMY2A* was 5.5 and 3.3 fold higher in pancreatic exocrine cells (stage IV) in comparison to undifferentiated cells in no-TIP and TIP cells, respectively and *AMY2B* 5.8 and 3.1 fold higher in pancreatic exocrine cells (stage IV) compared to undifferentiated cells in no-TIP and TIP cells, respectively (figure 11).

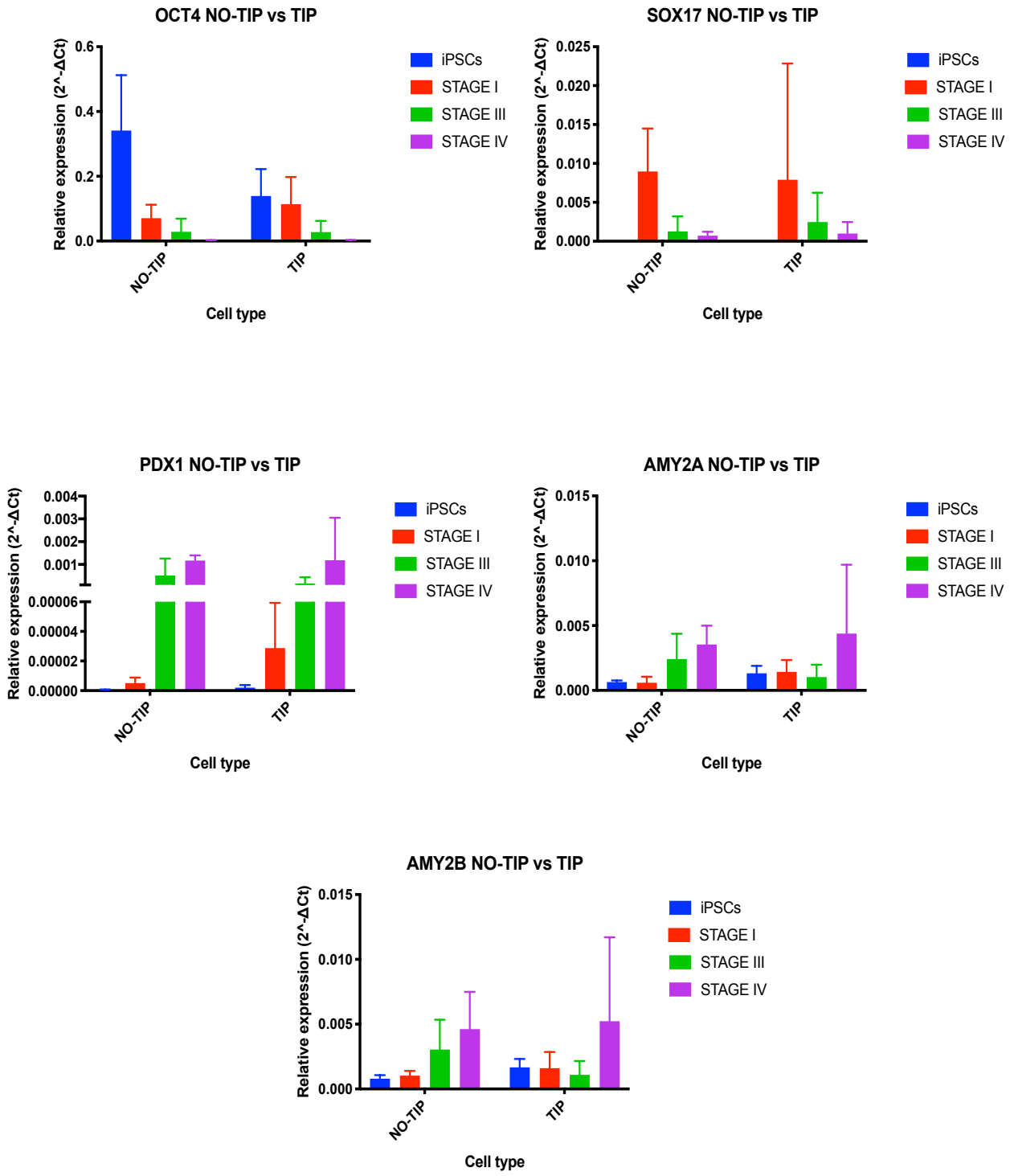

Figure 11. Real-time PCR time analysis of differentiation marker levels during CD-iPSCs differentiation process. *OCT4* is a marker of pluripotent cells (iPSCs); *SOX17* of endodermal (stage I); *PDX1* of pancreatic progenitor cells (stage III); *AMY2A* and *AMY2B* of pancreatic exocrine cells (stage IV). The data are reported as means ± SE of 3 independent experiments, evaluating $2^{-\Delta Ct}$ values, using the housekeeping *β-actin* as reference.

Amylase expression level in pancreatic cells differentiated from iPSCs and H6C7 ductal pancreatic cells

Expression levels of the two pancreatic isoforms *AMY2A* and *AMY2B* of *α-amylase*, key gene of pancreatic exocrine cells, were analyzed by real-time PCR. Both isoforms resulted equally expressed in the ductal pancreatic healthy H6C7 line compared to pancreatic progenitors (stage III), obtained differentiating CD-iPSCs of one patient without TIP, while mature pancreatic exocrine cells (stage IV) showed around double levels of both of isoforms (figure 12).

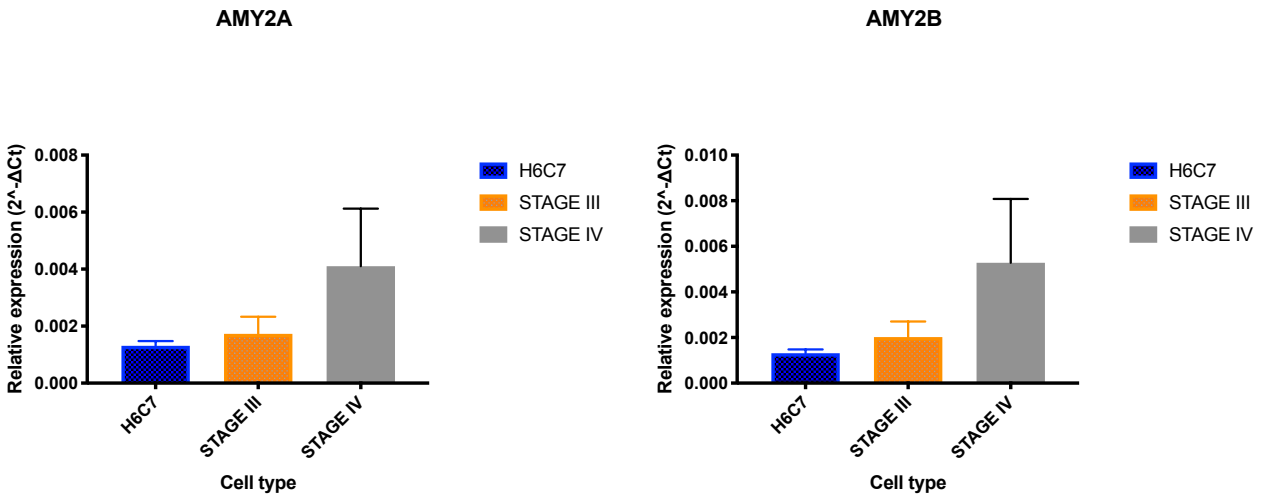

Figure 12. Real-time PCR results of *AMY2A* and *AMY2B* expression levels in the human ductal healthy pancreatic line H6C7, stage III and stage IV cells derived from iPSCs. The data are reported as means ± SE of 3 independent experiments, evaluating $2^{-\Delta Ct}$ values, using the housekeeping *β-actin* as reference.

Improving differentiation efficiency

In order to increase CD-iPSCs differentiation, we prolonged the exposure time to the stimuli necessary to obtain pancreatic exocrine cells up to 23 days. To this purpose, we analyzed the expression level of *PDX1*, *AMY2A* and *AMY2B* after 14 days, the standard exposure-time indicated by the protocol, and 23 days of stimulation (figure 13). Results showed a 2.0 and a 3.9 fold expression of *AMY2A* in cells stimulated for 23 days in comparison to those stimulated for 14 days in no-TIP and TIP patients, respectively. *AMY2B* was 2.6 fold expressed in cells after 23 days of stimulation in comparison to 14 days for no-TIP cells while 4.6 fold for TIP cells. *PDX1* in cells stimulated for 23 days was expressed 2.0 fold and 15.8

fold with respect to 14 days of exposure with the stimuli in no-TIP and TIP cells, respectively. However, even if an increment was detected, the statistical analysis did not highlight significant differences (two-way ANOVA, $p > 0.05$).

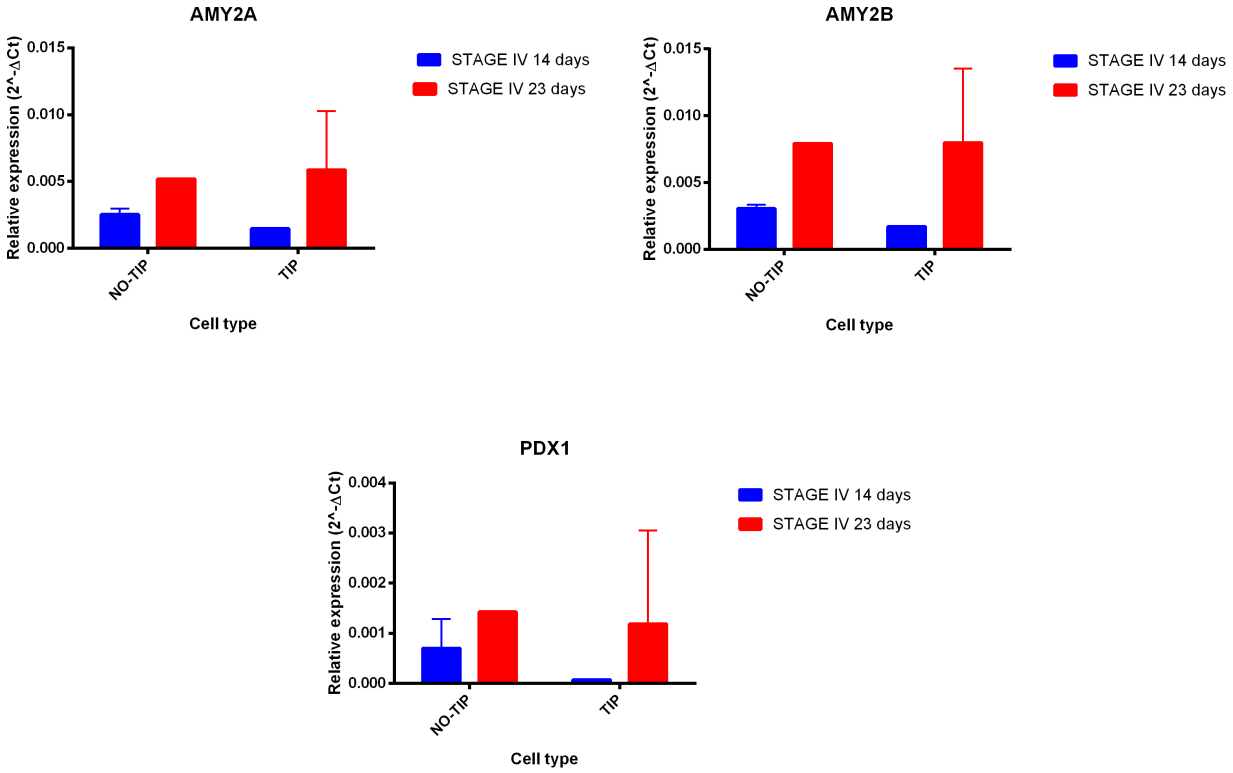

Figure 13. Real-time PCR results about *PDX1*, *AMY2A* and *AMY2B* levels after 14 or 23 days of differentiation of pancreatic progenitors (stage III) obtained from iPSCs, with stage IV stimuli. The data are reported as means ± SE of 3 independent experiments, evaluating $2^{-\Delta Ct}$ values, using the housekeeping *β-actin* as reference.

4.2 Immunofluorescence assay

Differentiation efficiency of 253G1-iPSCs cells was analyzed also by immunofluorescence in terms of PDX1 and α-amylase protein expression. In particular, PDX1 is a nuclear marker while α-amylase is located into the cytoplasm.

Figure 14 shows the result obtained in undifferentiated and differentiated 253G1-iPSCs cells (stage IV).

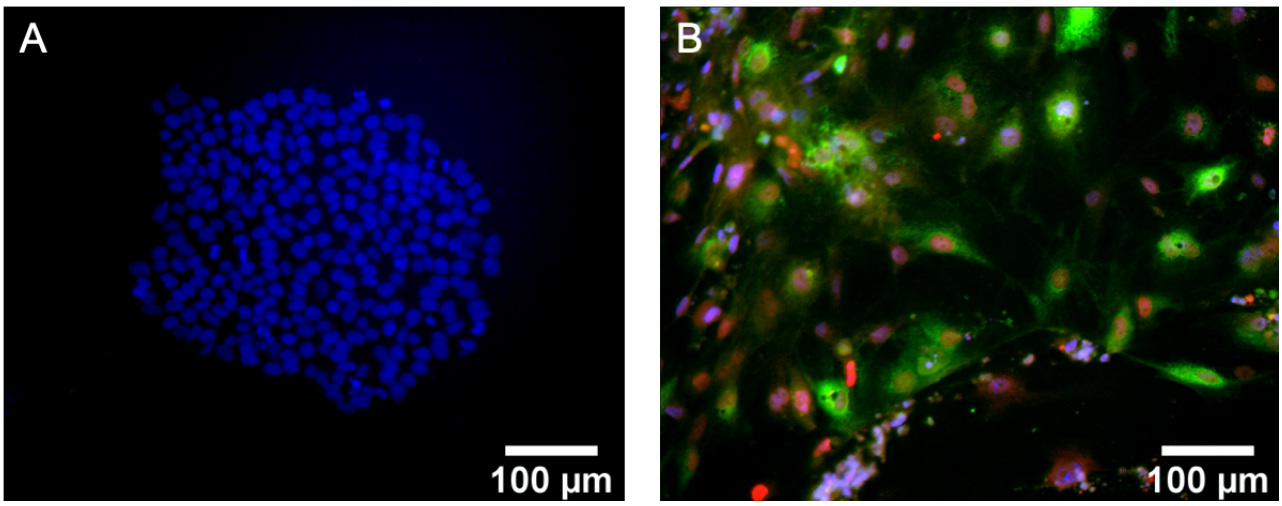

Figure 14. Immunofluorescence assay of 253G1-iPSCs undifferentiated cells (A) and stage IV differentiated cells (B) stained with PDX-1 (red), α-amylase (green) antibodies and DAPI for nuclei (blue). Images are representative of three analyses.

The expression of PDX1 and α-amylase protein markers were confirmed by immunofluorescence in CD-iPSCs and differentiated pancreatic exocrine cells of patients with and without TIP. As shown in figures 15 and 16, the undifferentiated CD-iPSCs were negative for both α-amylase and PDX1, while the differentiated pancreatic exocrine cells were positive for α-amylase (green) and PDX1 (red) even if only partially in the no-TIP exocrine cells (Figure 14).

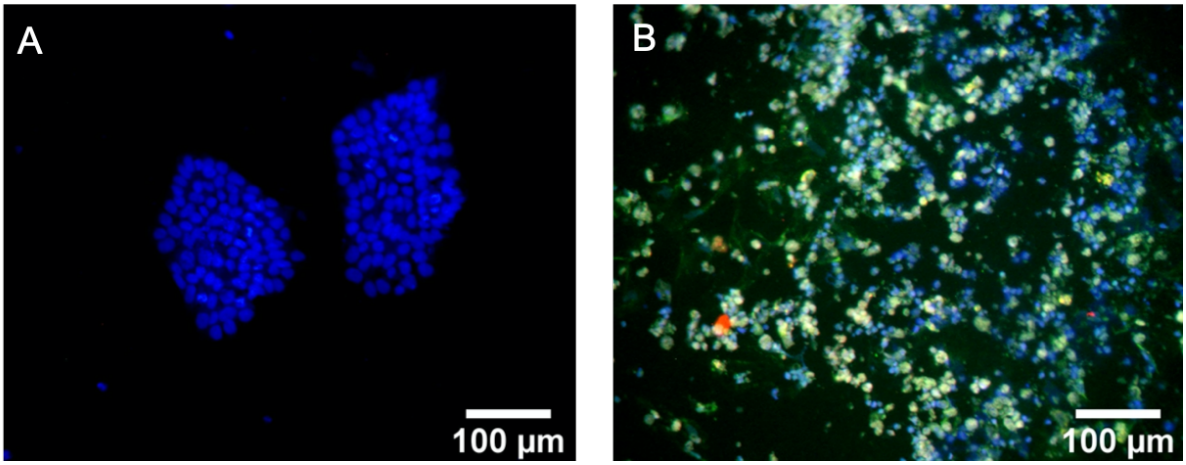

Figure 15. Immunofluorescence assay of no-TIP CD-iPSCs undifferentiated cells (A) and stage IV differentiated cells (B) stained with PDX-1 (red), α-amylase (green) antibodies and DAPI for nuclei (blue). Images are representative of three analyses.

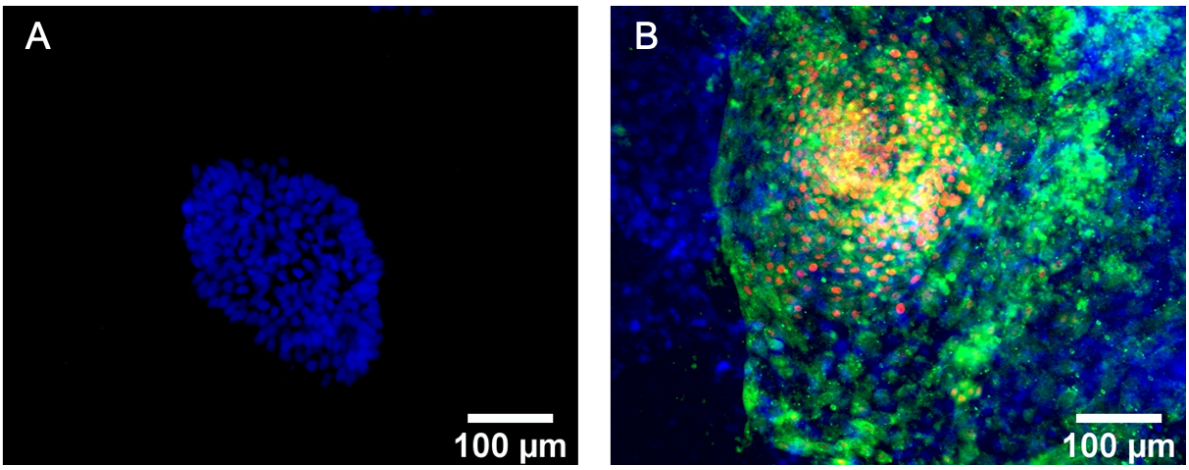

Figure 16. Immunofluorescence assay of TIP CD-iPSCs undifferentiated cells (A) and stage IV differentiated cells (B) stained with PDX-1 (red), α-amylase (green) antibodies and DAPI for nuclei (blue). Images are representative of three analyses.

Both pancreatic exocrine markers were analyzed also in the H6C7 pancreatic ductal cell line and results are reported in figure 17. Cells were positive for PDX1 (red) inside the nuclei and for α-amylase (green) in the cytoplasm.

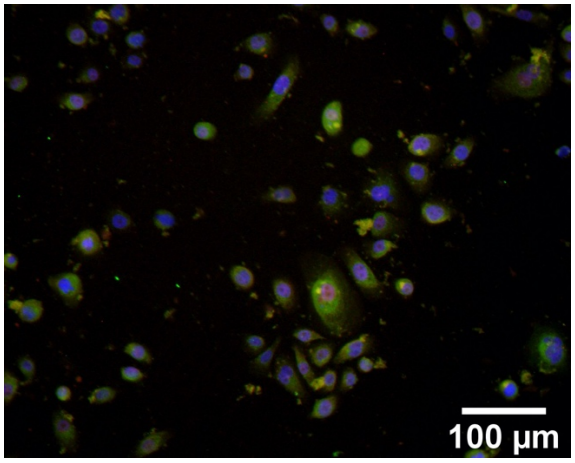

Figure 17. Immunofluorescence assay of the ductal pancreatic H6C7 line stained with PDX-1 (red), α-amylase (green) antibodies and DAPI for nuclei (blue). Images are representative of three analyses.

4.3 Cytotoxicity assays

As a first step, to investigate the feasibility of carrying out cytotoxicity assays to study thiopurine sensitivity of patient-specific CD iPSCs, preliminary experiments were carried out on the BJ-iPSCs healthy line and the 253G1-iPSCs to evaluate: i) the effect of single cell

passage on stemness properties; ii) the expression of the enzyme involved in thiopurine activation (HPRT1) and iii) cell density optimal to seed cells for the pharmacological assays.

4.3.1 Single cells and clusters

To perform cytotoxicity assay, it is necessary to obtain a single-cell suspension, in order for cells to be plated at a definite density. iPSCs grow in colonies formed by round shape clusters (figure 18). For long-term cultures the standard protocol avoids the complete break up of clusters formed, in order to reduce the probability of affecting cells' stemness properties and of genetic aberration (130).

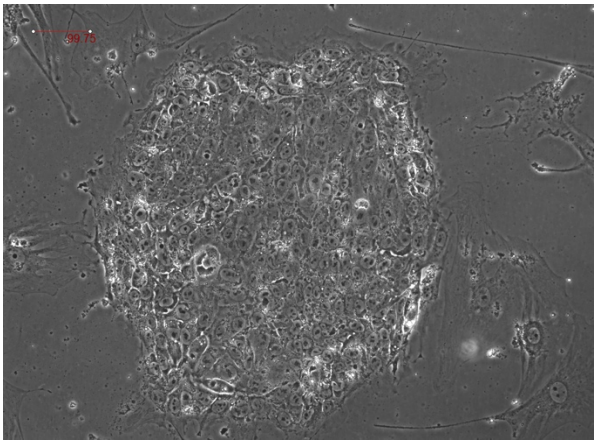

Figure 18. Morphology of a standard iPSCs colony three days after passage using the cluster dissociation protocol recommended for iPSCs.

Therefore, before carrying out cytotoxicity assays, we wondered if the single cell passage could affect stemness properties in terms of gene expression of pluripotency markers. To identify any alteration that could have occurred after single cell passage, necessary for the subsequent cytotoxicity assays, expression levels of *SOX2, OCT4* and *MYC* stemness genes were analyzed by real-time PCR in the BJ-iPSCs control line. Results were compared between cell cultures passed using the standard cluster dissociation protocol, after three days of culture, or single cell protocol (figure 19). Results obtained demonstrate that single cell passage, performed within the first passage, does not alter stemness gene expression supporting its use for cytotoxicity studies ($p > 0.05$, t-test).

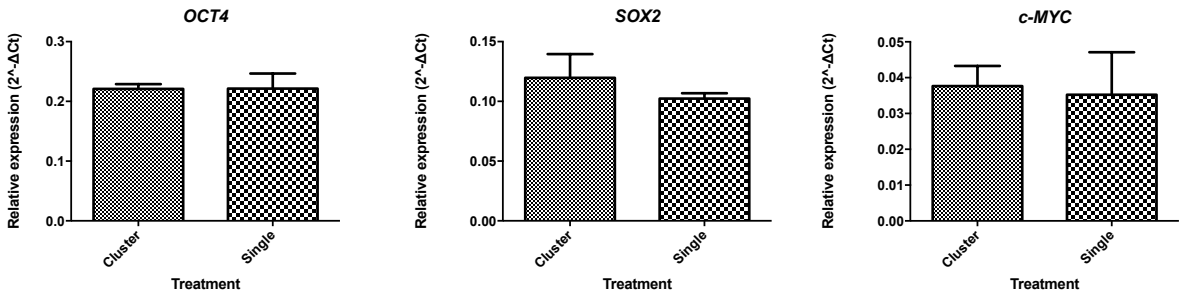

Figure 19. Real time PCR analysis of *SOX2*, *OCT4* and *MYC* stemness genes after single and cluster passage on BJ-iPSCs line. The data are reported as means ± SE of 3 independent experiments, evaluating $2^{-\Delta Ct}$ values, using *β-actin* as housekeeping. No statistically significant difference was observed (t-test).

4.3.2 *HPRT1* expression

HPRT1 is an important enzyme in thiopurine activation pathway. Expression of *HPRT1* was analyzed in BJ-iPSCs by real-time PCR assay and its expression compared with that of the lymphoblastoid thiopurine sensitive line CCRF-CEM. As shown in figure 20, *HPRT1* resulted expressed in BJ-iPSCs even if significantly lower ($p = 0.014$, t-test) compared to the CCRF-CEM line.

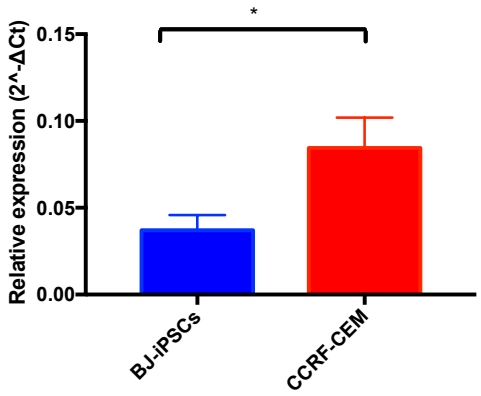

Figure 20. Real-time PCR results of *HPRT1* expression in the BJ-iPSCs healthy line compared to the lymphoblastoid thiopurine sensitive line CCRF-CEM. The data are reported as means ± SE of 3 independent experiments, evaluating $2^{-\Delta Ct}$ values, using *β-actin* as housekeeping reference. Statistical difference *, $p \leq 0.05$, BJ-iPSCs vs CCRF-CEM, t-test.

4.3.3 Cell density

BJ-iPSCs were seeded at different densities from 1,000 to 100,000 cells per well in 96-well plates. After 96 hours of incubation, MTT and SRB assays were performed. Results reported in figures 21-22 indicated 10,000 cell/well an appropriate number of cells to be seeded to perform cytotoxicity assay in terms of absorbance intensity.

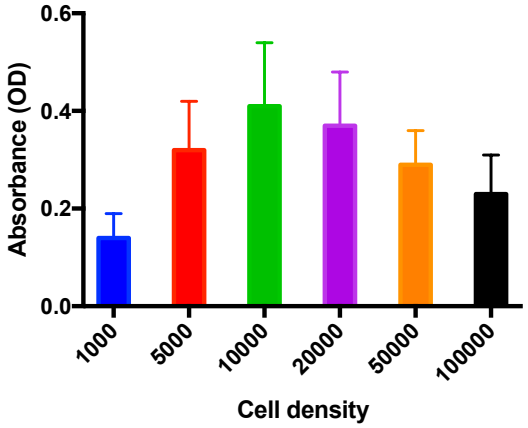

Figure 21. MTT results of BJ-iPSCs viability after 96 hours of culture. The data are reported as means ± SE of 3 independent experiments performed in triplicate.

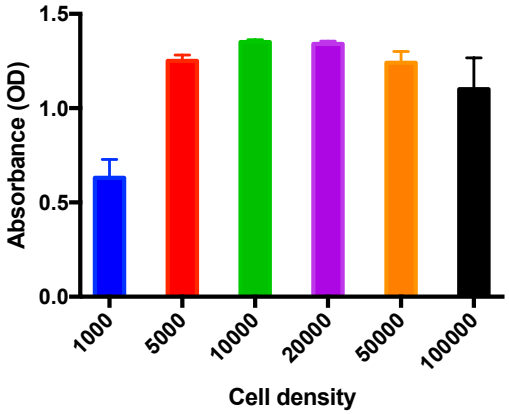

Figure 22. SRB results after 96 hours of culture of the healthy BJ-iPSCs line. The data are reported as means ± SE of 3 independent experiments performed in triplicate.

4.3.4 Sensitivity of iPSCs from healthy donors

Cytotoxicity effects of thiopurines were tested by MTT test initially on BJ-iPSCs exposing the cells for 72 hours to azathioprine, mercaptopurine and thioguanine. As shown in figure

23, BJ-iPSCs resulted highly sensitive to thiopurines, as shown by the low EC_{50} values calculated (table 5).

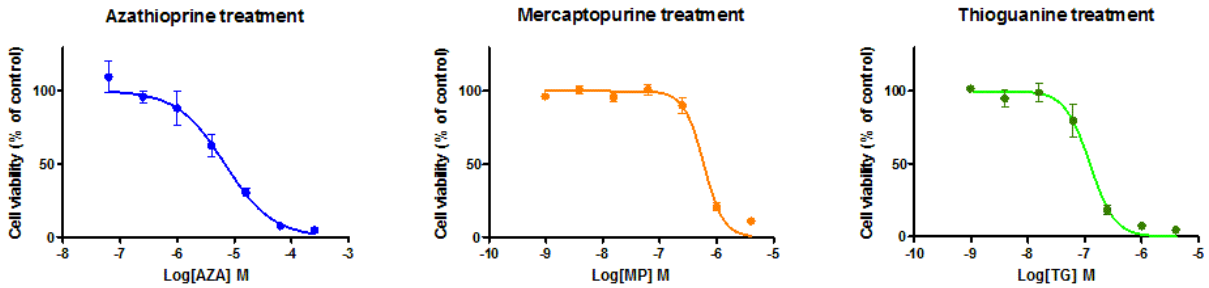

Figure 23. Cytotoxicity effects of azathioprine, mercaptopurine and thioguanine on BJ-iPSCs line. Cells were exposed for 72 hours to drugs and cytotoxicity effects were analyzed by MTT assay. The data are reported as means ± SE of 4 independent experiments performed in triplicate. O.D.% observed to untreated cells.

BJ-iPSCs	EC_{50} [M]	95% C.I.
AZATHIOPRINE	6.84×10^{-6} M	4.69×10^{-6} M to 9.99×10^{-6} M
MERCAPTOPURINE	5.94×10^{-7} M	5.06×10^{-7} M to 6.97×10^{-7} M
THIOGUANINE	1.23×10^{-7} M	9.68×10^{-8} M to 1.56×10^{-7} M

Table 5. EC_{50} values and 95% confidence intervals (C.I.) in BJ-iPSCs healthy line after azathioprine, mercaptopurine and thioguanine 72 hours exposure. The data are reported as means of 4 independent experiments performed in triplicate and 95% C.I.

4.3.5 BJ-iPSCs proliferation

Cell proliferation of the BJ-iPSC healthy line was analyzed by the [methyl-3H] thymidine incorporation assay. Results were compared to the proliferation rate of stabilized leukemic cells (CCRF-CEM). As shown in figure 24, the BJ-iPSC line proliferates at a significantly higher rate in comparison to the stabilized CCRF-CEM cells, at all cell densities tested (2.5×10^3, 5.0×10^3, 1.0×10^4 cells/well) but not at the lowest density (1.0×10^3) ($p < 0.0001$, two-way ANOVA and Bonferroni's post test).

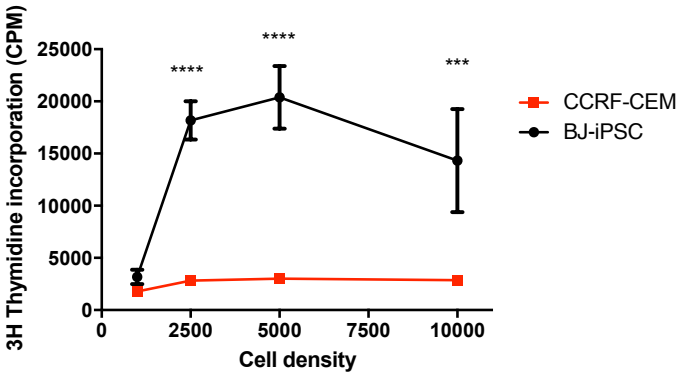

Figure 24. Cell proliferation of BJ-iPSC and CCRF-CEM lines evaluated by the 3H-thymidine incorporation assay. The data are reported as means ± SE of 3 independent experiments performed in triplicate. Statistical difference: ***, $p < 0.001$; ****, $p < 0.0001$ (BJ-iPSC vs CCRF-CEM, two-way ANOVA, and Bonferroni's post-test). 3 H-thymidine incorporation into DNA (counts per minute or CPM).

4.3.6 253G1-iPSCs sensitivity towards thiopurines drugs

Sensitivity of the 253G1-iPSCs line towards thiopurines was analyzed by MTT assay after 72 hours exposure to azathioprine, mercaptopurine and thioguanine drugs. As shown in figure 25, 253G1-iPSCs resulted highly sensitive to thiopurines. EC_{50} values and confidence intervals (C.I.) calculated are reported in table 5.

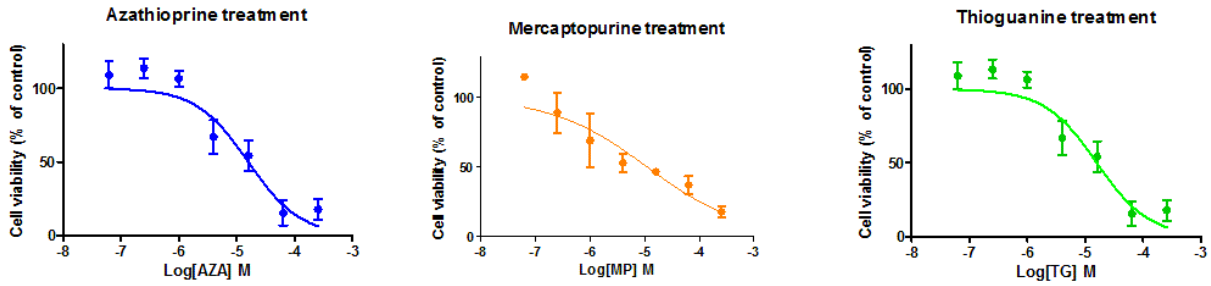

Figure 25. Cytotoxicity effects of azathioprine, mercaptopurine and thioguanine on 253G1-iPSCs healthy line. Cells were exposed for 72 hours to drugs and cytotoxicity effects were analyzed by MTT assay. The data are reported as means ± SE of 3 independent experiments performed in triplicate. O.D.% observed to untreated cells.

253G1-iPSCs	EC_{50} [M]	95% C.I.
AZATHIOPRINE	1.59×10^{-5} M	8.63×10^{-6} M to 2.91×10^{-5} M
MERCAPTOPURINE	1.12×10^{-5} M	4.45×10^{-6} M to 2.80×10^{-5} M
THIOGUANINE	1.59×10^{-5} M	8.63×10^{-6} M to 2.91×10^{-5} M

Table 6. EC_{50} values and 95% C.I. in 253G1-iPSCs cells after azathioprine, mercaptopurine and thioguanine 72 hours exposure. The data are reported as means of 3 independent experiments performed in triplicate and 95% C.I.

4.3.7 CD-iPSCs sensitivity towards thiopurines drugs

Sensitivity of CD-iPSCs towards thiopurines was analyzed on three lines obtained from three patients manifesting thiopurines-induced pancreatitis (TIP CD-iPSCs) and three control lines obtained from patients not manifesting thiopurines-induced pancreatitis (no-TIP CD-iPSCs) in triplicate. Cells were exposed to azathioprine, mercaptopurine and thioguanine for 72 hours and results obtained are reported in figure 26. The average EC_{50} values calculated for the three no-TIP CD-iPSCs considered as controls and for the three TIP CD-iPSCs representing the cases are reported in tables 7-8.

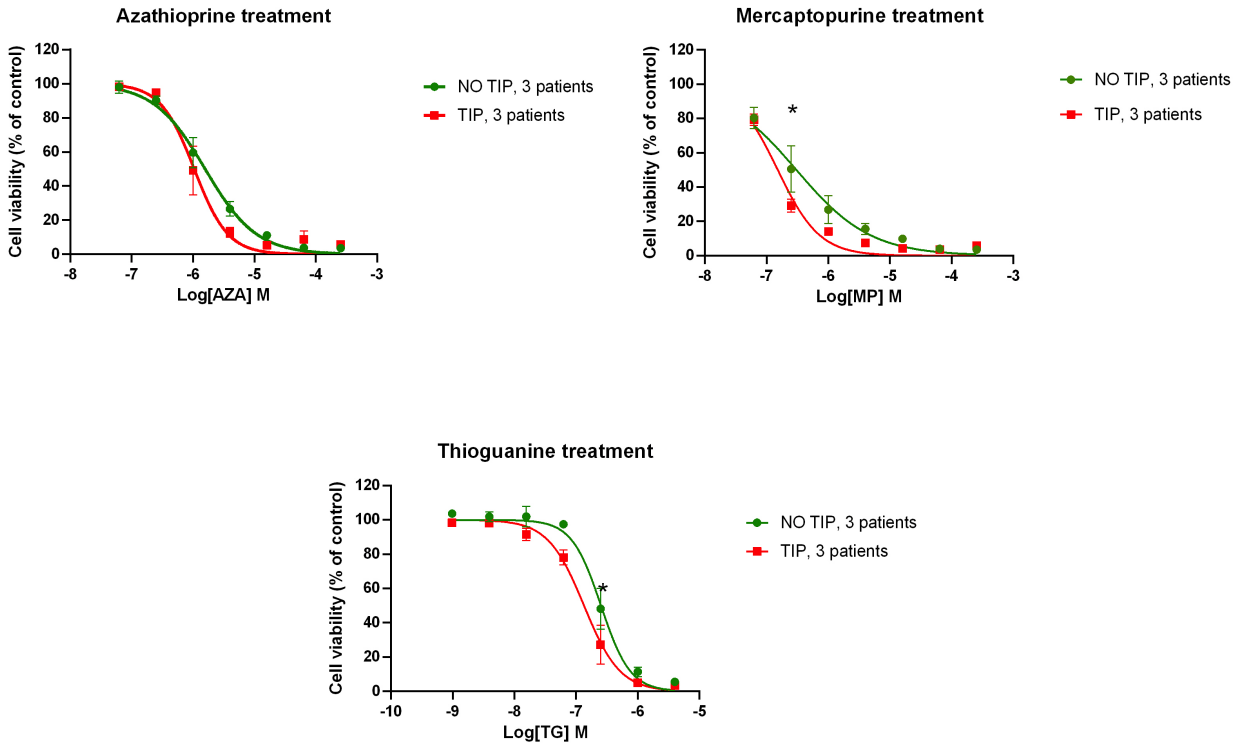

Figure 26. Cytotoxicity effects of azathioprine, mercaptopurine and thioguanine on no-TIP (green line) and TIP (red line) CD-iPSCs. Cells were exposed for 72 hours to drugs and cytotoxicity effects were analyzed by MTT assay. The data are reported as means ± SE of 3 independent experiments performed in triplicate for each patient (three TIP and three no-TIP). O.D.% observed to untreated cells.

No-TIP CD-iPSCs	**EC_{50} [M]**	**95% C.I.**
AZATHIOPRINE	1.58×10^{-6} M	1.26×10^{-6} M to 1.99×10^{-6} M
MERCAPTOPURINE	3.05×10^{-7} M	1.93×10^{-7} M to 4.71×10^{-7} M
THIOGUANINE	2.54×10^{-7} M	2.05×10^{-7} M to 3.21×10^{-7} M

Table 7. Average EC_{50} values and 95% C.I. calculated for the three no-TIP CD-iPSCs after azathioprine, mercaptopurine and thioguanine after 72 hours of exposure. The data are reported as means of 3 independent experiments performed in triplicate and 95% C.I.

TIP CD-iPSCs	**EC_{50} [M]**	**95% C.I.**
AZATHIOPRINE	1.05×10^{-6} M	7.96×10^{-7} M to 1.40×10^{-6} M
MERCAPTOPURINE	1.52×10^{-7} M	1.25×10^{-7} M to 1.85×10^{-7} M
THIOGUANINE	1.35×10^{-7} M	1.08×10^{-7} M to 1.67×10^{-7} M

Table 8. Average EC_{50} values and 95% C.I. calculated for the three TIP CD-iPSCs after azathioprine, mercaptopurine and thioguanine 72 hours exposure. The data are reported as means of 3 independent experiments performed in triplicate and 95% C.I.

Two-way ANOVA analysis of concentration-response curve showed an overall significative difference of sensitivity between cases (TIP CD-iPSCs) and controls (no-TIP CD-iPSCs) after mercaptopurine and thioguanine treatment. In particular, TIP CD-iPSCs resulted more sensitive to mercaptopurine ($p = 0.016$, two way ANOVA TIP vs no-TIP) and thioguanine ($p = 0.0012$, two way ANOVA TIP vs no-TIP) in comparison to no-TIP CD-iPSCs. In particular, the post-hoc test showed a significant difference for mercaptopurine and thioguanine at 2.5×10^{-7} M. The cell viability resulted 50% compared to untreated controls in no-TIP and 29% in TIP iPSCs after mercaptopurine exposure and 48% in no-TIP and 27%

in TIP iPSCs after thioguanine exposure. No significant difference was observed for azathioprine (figure 25).

4.3.8 Definitive endoderm cells sensitivity towards thiopurines drugs

Sensitivity of definitive endoderm cells towards thiopurines was analyzed in the three cases (TIP patients) in comparison to the three controls (no-TIP patients) in triplicate. Derived-stage I cells were exposed to azathioprine, mercaptopurine and thioguanine for 72 hours and results obtained are reported in figure 27. Average EC_{50} values calculated for the three no-TIP controls and the three TIP cases, differentiated to definitive endoderm cells are reported in tables 9-10.

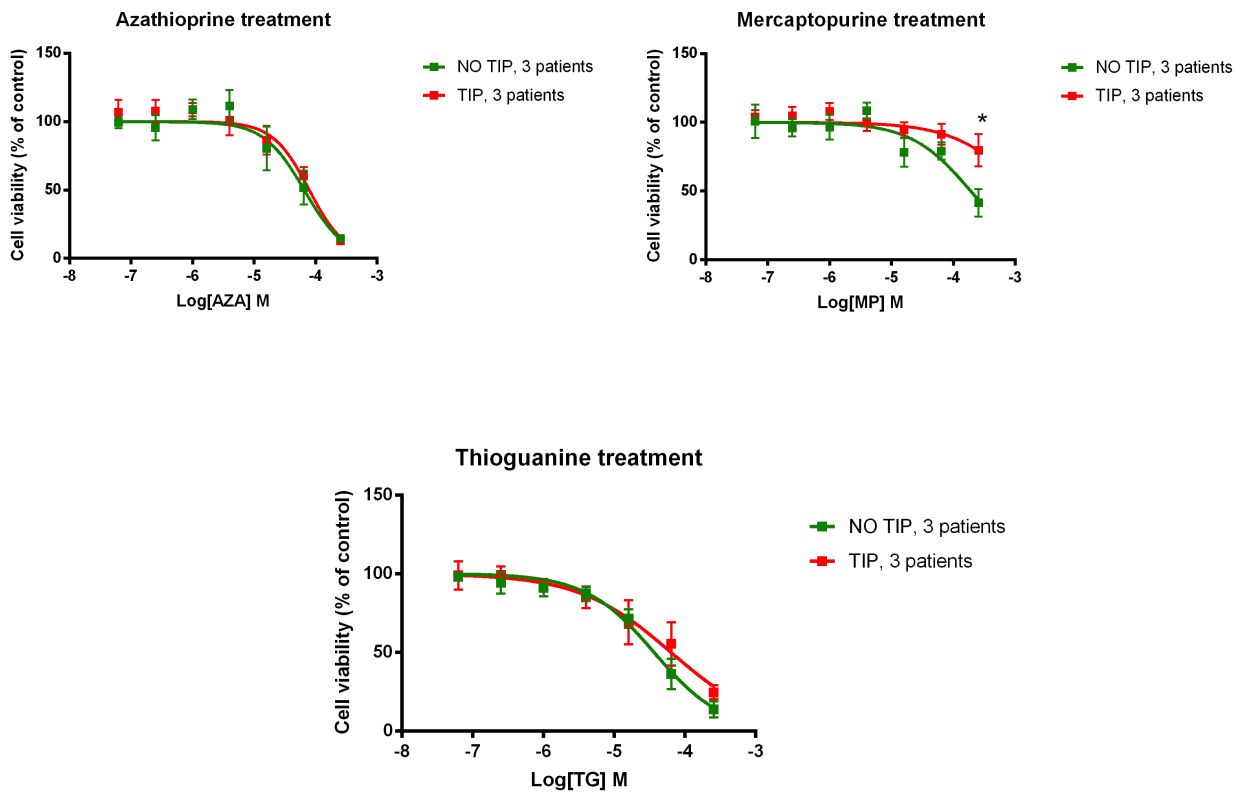

Figure 27. Cytotoxicity effects of azathioprine, mercaptopurine and thioguanine on no-TIP (green line) and TIP (red line) definitive endoderm cells. Cells were exposed for 72 hours to drugs and cytotoxicity effects were analyzed by MTT assay. The data are reported as means ± SE of 3 independent experiments performed in triplicate. O.D.% observed to untreated cells.

No-TIP definitive endoderm cells	EC_{50} [M]	95% C.I.
AZATHIOPRINE	6.52×10^{-5} M	3.90×10^{-5} M to 1.11×10^{-4} M
MERCAPTOPURINE	1.91×10^{-4} M	1.05×10^{-4} M to 5.57×10^{-4} M
THIOGUANINE	3.67×10^{-5} M	2.58×10^{-5} M to 5.21×10^{-5} M

Table 9. Average EC_{50} values and 95% C.I. calculated for the three no-TIP controls differentiated into definitive endoderm cells after azathioprine, mercaptopurine and thioguanine 72 hours exposure. The data are reported as means of 3 independent experiments performed in triplicate and 95% C.I.

TIP definitive endoderm cells	EC_{50} [M]	95% C.I.
AZATHIOPRINE	7.89×10^{-5} M	5.35×10^{-5} M to 1.16×10^{-4} M
MERCAPTOPURINE	1.42×10^{-3} M	-
THIOGUANINE	6.37×10^{-5} M	3.45×10^{-5} M to 1.30×10^{-4} M

Table 10. Average EC_{50} values and 95% C.I. calculated for the three TIP cases differentiated into definitive endoderm cells after azathioprine, mercaptopurine and thioguanine 72 hours exposure. The data are reported as means of 3 independent experiments performed in triplicate and 95% C.I.

The exposure of TIP and no-TIP cell lines differentiated into definitive endoderm cells to the thiopurine drugs tested did not produced any significant differences in terms of cytotoxic response except after mercaptopurine treatment. In particular, the two-way ANOVA analysis of concentration-response curve showed a significant higher cytotoxic effect ($p = 0.011$, two way ANOVA TIP vs no-TIP) in the no-TIP controls differentiated into definitive endoderm cells. The post-hoc test showed a significant difference for mercaptopurine at the highest concentration tested with an effect of 41% of viability in no-TIP cells in comparison to 80% in TIP cells with respect to untreated controls.

4.3.9 Pancreatic progenitor sensitivity towards thiopurines drugs

Sensitivity of pancreatic progenitor cells towards thiopurines was analyzed for the three cases (TIP patients) in comparison to the three controls (no-TIP patients) in triplicate. Derived-stage III cells were exposed to azathioprine, mercaptopurine and thioguanine for 72 hours and results obtained are reported in figure 28. Average EC_{50} values calculated for the three no-TIP controls and the three TIP cases, differentiated into pancreatic progenitor cells are reported in tables 11-12.

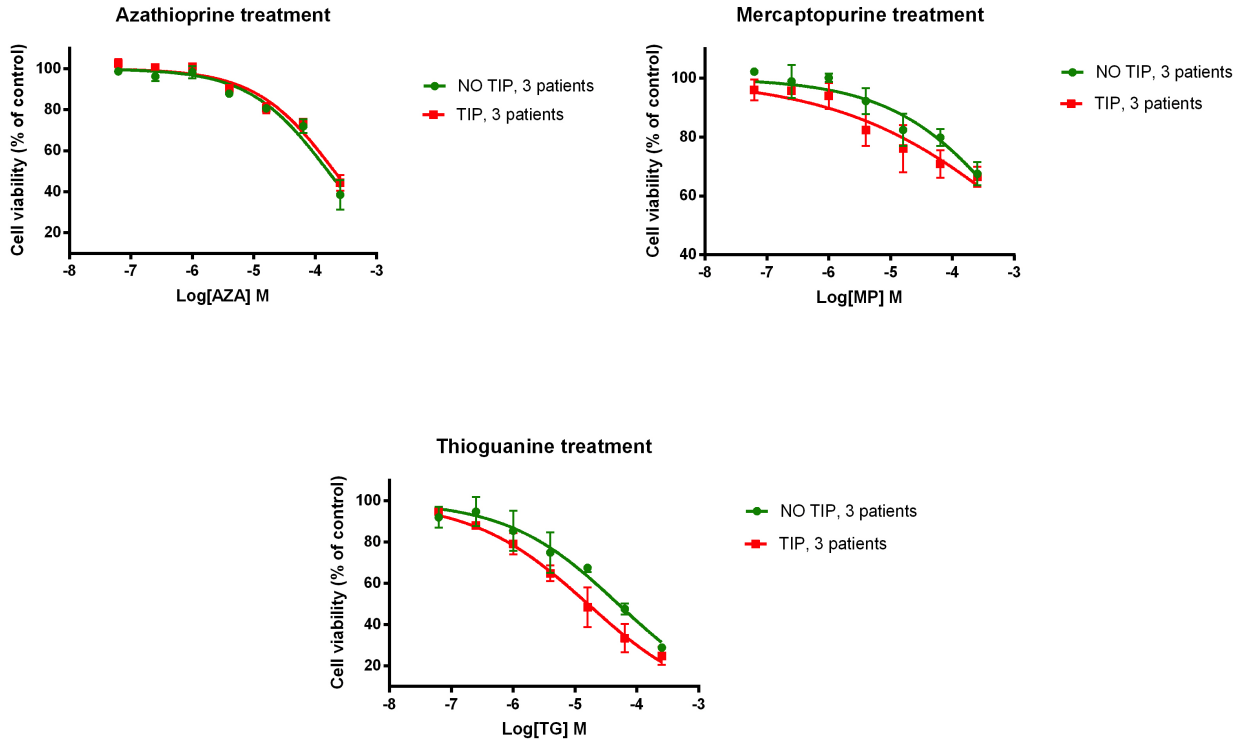

Figure 28. Cytotoxicity effects of azathioprine, mercaptopurine and thioguanine on no-TIP (green line) and TIP (red line) pancreatic progenitor cells. Cells were exposed for 72 hours to drugs and cytotoxicity effects were analyzed by MTT assay. The data are reported as means ± SE of 3 independent experiments performed in triplicate. O.D.% observed to untreated cells.

Pancreatic progenitor cells of TIP patients were slightly more sensitive to mercaptopurine and thioguanine in comparison to no-TIP patients. Two-way ANOVA analysis of concentration-response curve showed an overall significative difference of sensitivity for mercaptopurine and thioguanine (mercaptopurine $p = 0.017$; thioguanine $p = 0.014$, two way ANOVA TIP vs no-TIP patients). However, due to the limited number of samples

analyzed, the post-hoc test did not showed any significant differences. No differences were identified after azathioprine treatment.

No-TIP pancreatic progenitor cells	**EC_{50} [M]**	**95% C.I.**
AZATHIOPRINE	1.63×10^{-4} M	1.18×10^{-4} M to 2.44×10^{-4} M
MERCAPTOPURINE	> 2.56×10^{-4} M	-
THIOGUANINE	5.02×10^{-5} M	2.95×10^{-5} M to 9.37×10^{-5} M

Table 11. Average EC_{50} values and 95% C.I. calculated for the three no-TIP controls differentiated into pancreatic progenitor cells after azathioprine, mercaptopurine and thioguanine 72 hours exposure. The data are reported as means of 3 independent experiments performed in triplicate and 95% C.I.

TIP pancreatic progenitor cells	**EC_{50} [M]**	**95% C.I.**
AZATHIOPRINE	2.06×10^{-4} M	1.59×10^{-4} M to 2.85×10^{-4} M
MERCAPTOPURINE	> 2.56×10^{-4} M	-
THIOGUANINE	1.62×10^{-5} M	1.05×10^{-5} M to 2.58×10^{-5} M

Table 12. Average EC_{50} values and 95% C.I. calculated for the three TIP cases differentiated into pancreatic progenitor cells after azathioprine, mercaptopurine and thioguanine 72 hours exposure. The data are reported as means of 3 independent experiments performed in triplicate and 95% C.I.

4.3.10 H6C7 sensitivity

Sensitivity of the human ductal pancreatic H6C7 line was tested exposing cells to azathioprine, mercaptopurine and thioguanine for 72 hours. Cells resulted resistant to azathioprine and mercaptopurine in the range of concentrations tested (2.56×10^{-4} M – 6.25×10^{-8} M, EC_{50} > 2.56×10^{-4} M), but slightly sensitive to thioguanine (EC_{50} 6.77×10^{-4}M). Cytotoxicity results are showed in figure 29.

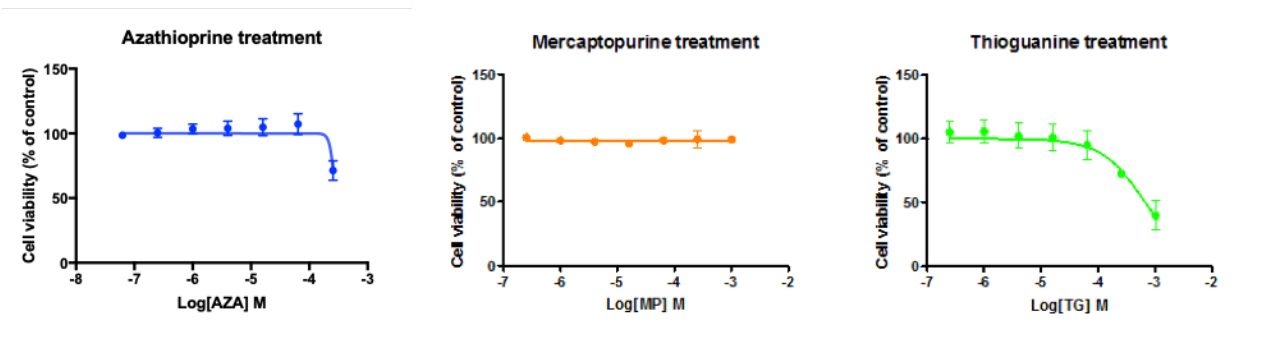

Figure 29. Cytotoxicity effects of azathioprine, mercaptopurine and thioguanine on H6C7 ductal pancreatic line. Cells were exposed for 72 hours to drugs and cytotoxicity effects were analyzed by MTT assay. The data are reported as means ± SE of 3 independent experiments performed in triplicate. O.D.% observed to untreated cells.

4.4 Cell cycle analysis

Cell cycle of patient-specific CD-iPSCs and of differentiated cells (definitive endoderm cells - stage I and pancreatic progenitor cells - stage III) was analyzed by measuring propidium iodide uptake. Both controls (no-TIP) and cases (TIP) CD-iPSCs (no-TIP 38.21% ± 0.30% vs TIP 35.62% ± 5.48%) as well as the corresponding definitive endoderm cells (no-TIP 37.34% ± 19.70 % vs TIP 38.59% ± 18.78%) and pancreatic progenitors (no-TIP 32.42% ± 4.33%) were characterized by high percentage of cells in the S phase compared to the H6C7 pancreatic ductal line (21.99% ± 2.29%). No differences in terms of percentage of cells in the S phase between no-TIP and TIP cells were identified.

	G0	S	G2/M
No-TIP iPSCs (mean ± SEM)	29.07% ± 1.82%	38.21% ± 0.30%	21.22% ± 1.38%
TIP iPSCs (mean ± SEM)	31.70%± 0.77%	35.62% ± 5.48%	23.88% ± 2.83%

	G0	S	G2/M
No-TIP definitive endoderm cells (mean ± SEM)	28.53% ± 11.46%	37.34% ± 19.70%	21.57% ± 1.14%
TIP definitive endoderm cells (mean ± SEM)	32.11% ± 10.72%	38.59% ± 18.78%	24.01% ± 5.97%

	G0	S	G2/M
No-TIP pancreatic progenitor cells - STAGE III	45.13% ± 6.67%	32.42% ± 4.33%	20.00% ± 2.45%

	G0	S	G2/M
H6C7 (mean ± SEM)	56.21% ± 8.0%	21.99% ± 2.29%	14.38% ± 1.72%

Table 13. Cell cycle analyses of CD-iPSCs, definitive endoderm cells, pancreatic progenitor cells and H6C7 pancreatic ductal cells. Results are reported as mean ± SEM of two or three replicates.

4.5 SNPs analysis

All patients resulted wild type for the TPMT polymorphisms rs1142345, rs1800460 and rs1800462 tested.

Analysis of the SNP rs2647087 in the Class II HLA gene region related with the predisposition of developing TIP in CD patients, revealed one no-TIP control wild type for the polymorphism (AA) and two no-TIP controls homozygous mutated (CC), while two patients with TIP resulted wild type for the polymorphism (AA) and one homozygous mutated (CC) (table 14).

	TIP	No-TIP
AA	2	1
AC	0	0
CC	1	2

Table 14. Genotyping analysis of rs2647087 SNP in CD-iPSCs of three no-TIP and three TIP patients.

5. Discussion

TIP is an idiosyncratic adverse effect that occurs in around 3-5% of CD patients. So far, mechanisms determining the predisposition to develop this adverse effect are unknown and no biomarker for clinicians for its prevention is available. Consequences of TIP lead to the interruption of thiopurine therapy and, in the most severe cases, to patients' hospitalization. To the best of our knowledge, no patient-specific *in vitro* model is available to study TIP. In this regard, iPSCs could be a great tool, given the peculiar ability of these cells to differentiate, under adequate stimuli, into almost any cell type. Differentiation of patient-specific iPSCs into pancreatic exocrine cells, tissue involved in TIP development, could reproduce patient-specific TIP sensitivity allowing to study *in vitro* predisposition to this adverse effect.

The expression of stemness and differentiation gene markers was evaluated by real-time PCR and immunofluorescence to confirm the successful generation of pancreatic exocrine cells. Preliminary analysis of iPSCs differentiation was carried out on the 253G1-iPSCs and the BJ-iPSCs control lines obtained reprogramming cells from healthy donors. Genes analyzed were *OCT4*, characteristic of undifferentiated cells, *SOX17* and *FOXA2* of definitive endoderm cells, *PDX1* of pancreatic progenitors, *α-amylase* and its isoforms *AMY2A* and *AMY2B* of pancreatic exocrine cells. For both lines, definitive endoderm cells (stage I) were efficiently induced by stimulating cells for 4 days with activin A and CHIR99021, primitive gut tube (stage II) by FGF7 for 3 days, pancreatic progenitors (stage III) by a combination of cyclopamine, noggin, retinoic acid for 3 days and pancreatic exocrine cells using FGF7, glucagon-like peptide 1 and nicotinamide for 14 days, successfully applying a protocol developed in hESCs (66).

Real-time PCR and immunofluorescence results about 253G1-iPSCs differentiation suggest an efficient generation of pancreatic progenitor cells demonstrating that the protocol initially set up for hESCs can be applied to induce pancreatic differentiation starting from iPSCs as well. Indeed, all gene markers analyzed confirmed the pancreatic development: *OCT4* was higher in the undifferentiated iPSCs, decreasing gradually during the differentiation, *FOXA2* was higher in definitive endoderm cells (stage I), while *PDX1* and *α-amylase* were higher in pancreatic progenitors (stage III), as expected.

The real-time PCR analysis of the key gene markers was performed for BJ-iPSCs as well. Differentiation confirmed that *OCT4* level was higher in undifferentiated iPSCs as

compared to differentiated cells. Interestingly, *SOX17* and *FOXA2* were higher in definitive endoderm cells (stage I), in comparison to undifferentiated iPSCs, but also in exocrine pancreatic cells (stage IV). The unexpected high level of *SOX17* and *FOXA2* markers in exocrine pancreatic cells could be probably due to the survival of some definitive endoderm cells during the differentiation process suggesting the presence of a mixture of cells, rather than a single cell type in the last stage of differentiation, as clearly reported in the literature (131). *PDX1* was higher in pancreatic progenitors and pancreatic exocrine cells with respect to iPSCs. Interestingly, the expression of *α-amylase* and its pancreatic isoforms *AMY2A* and *AMY2B* were higher in undifferentiated BJ-iPSCs in comparison to pancreatic exocrine cells and 253G1-iPSCs previously analyzed. However, this peculiarity did not interfere with the differentiation, given the first decrease of *α-amylase, AMY2A* and *AMY2B* levels and the subsequent increment in differentiated cells. However, overall mRNA results led us to assume that the BJ-iPSCs line was probably not the appropriate iPSCs line to generate pancreatic exocrine cells given their peculiar high basal expression of *α-amylase* and its isoforms in undifferentiated stage. To the best of our knowledge, studies based on the differentiation of different iPSC lines into pancreatic exocrine cells did not present high basal expression of amylase (66,72,73).

Differentiation data on patient-specific CD iPSCs confirmed results found for 253G1-iPSCs and BJ-iPSC lines, except for the *α-amylase* peculiarity typical of BJ-iPSCs. In particular, levels of gene markers were correctly expressed in all the differentiation stages: level of *OCT4* was higher in undifferentiated cells, *SOX17* in definitive endoderm cells, *PDX1* in pancreatic progenitors and *α-amylase* and its isoforms *AMY2A* and *AMY2B* in pancreatic exocrine cells.

In particular, the protocol applied aimed to generate pancreatic acinar cells producing the *α-amylase* enzyme peculiar of these cells. To confirm the acinar lineage, *AMY2A* and *AMY2B* mRNA levels of pancreatic progenitors and pancreatic exocrine cells were compared to those of the pancreatic ductal H6C7 line. The higher expression of both isoforms of *α-amylase* in differentiated cells suggested the acinar development.

Differentiation efficiency of all cell types (except for the BJ-iPSCs line, given its unsuitability for pancreatic cells differentiation) was confirmed by immunofluorescence analysis. Results observed on pancreatic exocrine cells obtained differentiating 253G1-iPSCs and CD-iPSCs

confirmed their successful differentiation, highlighting the expression of PDX1 transcription factor in the nuclei and of α-amylase enzyme in the cytoplasm.

Differentiation protocols to obtain a specific cell type from iPSCs are becoming more and more efficient reducing the presence of undifferentiated cells or other not desired cell types. Recently, advances in the production of pancreatic exocrine cells from iPSCs have been reported (72,73). Hohwieler and his group (72) reported the differentiation of about 30% acinar cells and 60% ductal cells while Ito et al. (73) generated around 40-60% and 15% of pancreatic acinar and ductal cells respectively, improving the efficiency in terms of acinar cells generation. These protocols were similar to our regarding the obtainment of definitive endoderm cells using Activin A and CHIR99021, while different for the following stages using other stimuli and using the 3D culture method. For example, Hohwieler and colleagues (72) used keratinocyte growth factor to obtain gut-tube endoderm cells, LDN-193189, PD0325901, retinoid acid and SANT-1 to generate pancreatic endoderm and a mixture of fibroblasts growth factor 10, indolactam V, SB431542 and glucose for pancreatic progenitors. Then, cells obtained were moved to a 3D Matrigel-based culture adding fibroblasts growth factor 2 and nicotinamide to the medium or in ultra-low-attachment plates with β-mercaptoethanol supplemented with fibroblasts growth factor 10, epidermal growth factor, CHIR99021 and phorbol myristate acetate. In our case, even though no direct analysis on efficacy differentiation and evaluation of the ductal subpopulation was carried out, a set of experiments were performed to increase the differentiation of pancreatic cells. With this aim, a fruitful approach was to extend the exposure time of pancreatic progenitors with differentiation stimuli needed to obtain mature pancreatic exocrine cells. Exposure time was increased from 14 to 23 days and experiments were performed using the CD-iPSCs. In particular, extending the stimulation up to 23 days the mRNA expression of *AMY2A*, *AMY2B* and *PDX1* increases around 2.0 - 15 times letting us to assume that probably the standard protocol could be ameliorated prolonging the last stage of differentiation. However, results about improving the efficiency of the differentiation protocol are still preliminary and should be replicated to confirm them. Further studies will also consider implementation of protocol changes according to differentiation procedure to exocrine pancreas recently published such as the use of 3D cultures. We will also implement the distinction between the acinar and ductal phenotypes by analyzing mRNA and protein expression of amylase and chymotrypsin C for acinar cells while SOX9 and cytokeratin 19 for ductal cells (72,73).To provide a patient-specific model to study TIP predisposition in

CD patients we analyzed the sensitivity of undifferentiated and differentiated cells to thiopurines with the purpose to define if cells may recapitulate the patient's peculiar sensitivity to these agents. The MTT assay, a gold standard for cytotoxicity analysis, requires a single cell suspension in order to plate a precise number of cells per well. However, iPSCs grow in colonies formed by round shape clusters and the standard passage for long-term cultures avoids the complete break up of clusters formed, in order to reduce the probability of affecting cells' stemness properties or genome stability. Therefore, before starting cytotoxicity assays, we wondered if the single cell passage may affect stemness properties in term of gene expression of pluripotency markers. Therefore, stemness gene expression of iPSCs cultured into single cell or in clusters was compared. In particular, the expression levels of *OCT4*, *SOX2*, and *MYC* stemness genes were analyzed. Results showed no significant differences in term of relative expression of gene levels after single and cluster passage suggesting that single cell passage performed within the first passage does not alter iPSCs stemness and confirming therefore that iPSCs can successfully be used for cytotoxicity studies. Even other authors successfully applied the MTT or similar cytotoxicity assays to iPSCs and differentiated cells (63,132,133) using successfully the single cells approach.

Cytotoxicity analysis of no-TIP and TIP CD patients iPSCs, definitive endoderm and pancreatic progenitors identified a slightly higher sensitivity to mercaptopurine and to thioguanine for TIP iPSCs and pancreatic progenitors cells in comparison to no-TIP controls. The data obtained suggest that our *in vitro* model could reproduce TIP predisposition using iPSCs and pancreatic progenitors. However, differences between no-TIP and TIP pancreatic progenitors cells were not as marked as in iPSCs. In particular, to confirm results obtained it would be important to extend the study to a larger cohort of patients and to perform sensitivity analysis of pancreatic exocrine cells, tissue actually involved in TIP development. Definitive endoderm cells did not show any differences except after mercaptopurine exposure, where no-TIP cells resulted more sensitive. This result is in contrast with those obtained in iPSCs and pancreatic progenitors, where cell models well reproduced TIP sensitivity. This observation suggests that definitive endoderm cells cannot be considered a representative cell model to study TIP as iPSCs and pancreatic progenitors. Indeed, while iPSCs reproduce faithfully genetic background of patients and pancreatic progenitors the tissue involved in TIP development, definitive endoderm cells are an in-between stage, common to several different somatic lineages in which different mechanism(s) of toxicity

may be displayed. Moreover, performing experiments on definitive endoderm cells we highlighted some difficulties in obtaining consistent replicates with respect to iPSCs and pancreatic progenitors. The observation that iPSCs and differentiated cells at the pancreatic precursor stage obtained from patients developing TIP are more sensitive to the direct cytotoxic effect of thiopurine lead to speculate that an intrinsic molecular factor, such as a genetic variant, could predispose to this adverse effect. Therefore the patient-specific model system we developed could be used also to identified this molecular factor that could then be validated as a biomarker (134,135). *TPMT* variants are correlated with thiopurine toxicity (103). Patients with a complete deficiency of *TPMT* can develop severe and potentially fatal myelosuppression while ultrahigh *TPMT* individuals present poor clinical response with a low quantity of TGNs incorporation (103). Most of the works in literature have not indicated a correlation between *TPMT* activity and TIP predisposition (94,95). The presence of *TPMT* variants could affect *in vitro* sensitivity to thiopurines becoming a cofounder for our analysis. Genotyping analysis revealed that all patients analyzed were wild type for *TPMT* confirming that sensitivity differences seen in iPSCs and pancreatic progenitors cannot be attributed to this gene. As reported in the literature (122,123), the rs2647087 SNP in the Class II HLA gene region is associated with the predisposition to develop TIP as adverse effect in CD patients. The risk correlated to this mutation was estimated at around 9.0% or 4.3% and 17.0% or 14.6%, depending on the study, for heterozygous and homozygous variant patients, respectively. Exploratory analysis of rs2647087 SNP was performed; however, given the limited number of patients, it was not possible to confirm what found in the literature.

Overall, iPSCs resulted extremely sensitive to thiopurine drugs, with EC50s in the sub micromolar range after 72 hours exposure, resulting around 100 times more sensitive in comparison to differentiated cells and to a panel of immortalized cell lines previously analyzed in our laboratory, including the ductal human pancreatic cell line H6C7. The higher cytotoxic effect of thiopurines in iPSCs with respect to the other cell types could be related to the different proliferation rate specific of iPSCs with respect to stable lines and differentiated cells. Indeed, thiopurine cytotoxic effects are strictly correlated to proliferation rate since these drugs are cell cycle-specific agents that interfere with the formation of the new DNA strand during the S phase to exert cytotoxicity (136). Analysis of cell cycle showed a significantly higher percentage of cells in the S phase of both no-TIP and TIP CD-iPSCs in comparison to stabilized cell lines such as H6C7. This difference in

percentage in cells in the DNA synthesis phase could well explain the difference in sensitivity to thiopurines observed. However, no significant differences in terms of distribution between the cell cycle phases were observed for CD-iPSCs and the differentiated cells, definitive endoderm cells and pancreatic progenitors. Therefore, the lower sensitivity of differentiated cells with respect to iPSCs cannot be explained on the basis of the different distribution in cell cycle, letting us to assume different mechanisms at the basis of the reduced sensitivity of the differentiated cells in comparison to iPSCs. No significant differences were identified between no-TIP and TIP cell cycle distribution, suggesting that sensitivity differences identified by the MTT assay also are not related to this cellular feature.

Taken together, the results obtained are encouraging, even though our patient-specific *in vitro* model still presents some limitations that have to be overcome. One problem is, for instance, the time necessary to generate pancreatic exocrine cells that is currently too long to allow TIP predisposition screening before thiopurine treatment and, as already discussed above, the importance of ameliorating the efficiency of the differentiation. Moreover, thiopurine drugs are pro-drugs that need to be activated by several enzymes inside the liver before exerting their cytotoxic effect. Thus, it is conceivable that thiopurine drugs do not directly arrive to the pancreatic tissue as themselves, but rather as metabolites. Therefore, to check and confirm sensitivity results using this personalized *in vitro* model, it would be important to expose cells to a mixture of thiopurine metabolites or of medium of immortalized human hepatocytes exposed to these drugs. It is also important to remind that TIP predisposition could be influenced by the contribution of immune cells that can attack the pancreatic tissue after thiopurine administration. This aspect has to be considered, modeled and studied as well (124,137).

Another important point to keep in mind is that to make this model useful when applied to the clinical practice, it would be important to collect blood samples of CD patients before thiopurine treatment start in order to generate iPSCs of patients and test thiopurine sensitivity before pancreatitis development. Indeed, in this way it would be possible to perform TIP risk screening before starting the treatment.

Despite all, the potentiality of this model based on differentiated patient-specific cells generated from iPSCs to study cytotoxicity of drugs is confirmed also by the literature. Recently, similarly to our purpose to study ADRs using iPSCs as a patient specific model, Choudhury et al. (132) set up an *in vitro* model to study idiosyncratic hepatotoxicity induced

by pazopanib using hepatocytes derived from patient-specific iPSCs who developed the adverse effect after treatment. Also Li and his group (138) developed a similar model to study hepatotoxicity caused by valproic acid in patients with Alpers-Huttenlocher syndrome who have higher risk to develop hepatotoxicity after treatment. Several other studies have been published regarding adverse effects on other organs and iPSCs such as neurotoxicity, nephrotoxicity and cardiotoxicity as we reviewed recently (80). However, to the best of our knowledge, studies based on adverse effects affecting the pancreatic tissue and iPSCs are still limited making this work innovative and an important basis for studying in a personalized way pancreatic drug-induced adverse effects.

Overall, the results obtained suggest that this model could be useful for identifying new biomarkers that could be used as predictors of TIP helping clinicians in the prevention of this adverse effect. Moreover, it is reasonable to think that the model established could be proposed as a predictive *in vitro* assay to screen patients' predisposition of TIP before starting the thiopurine treatment. However, as already discussed above, different aspects have to be improved before its application in the clinic such as the time needed to obtain pancreatic cells and the efficiency of the differentiation protocol.

Moreover, to confirm and consolidate the obtained data and to find significative differences in term of sensitivity, the cohort of patients enrolled have to be enlarged.

Another important aspect that has to be addressed for studying TIP predisposition could be attributed to the patients' immune cells that can contribute, through mechanisms not yet discovered, to the development of this adverse effect influencing the safety of the treatment. Considering these observations, the model should also recapitulate the patient-specific immune response in order to have a comprehensive overview of the problem.

6. Conclusion

In conclusion, this study demonstrates the possibility to differentiate patient-specific CD iPSCs into amylase producing pancreatic exocrine cells using a protocol initially developed for ESCs. The *in vitro* model established has proven to be suitable for studying and investigating TIP predisposition in a personalized way in pediatric CD patients.

Differences highlighted by the MTT assay in undifferentiated iPSCs and in pancreatic progenitor cells seem to recapitulate the patients' sensitivity with higher cytotoxic effects in TIP patients, in comparison to no-TIP controls, after thiopurines treatment. However, the reasons behind these sensitivity differences remain unclear and have to be further investigated.

In addition to the study of TIP, the model developed could be applied to investigate mechanisms of pancreatitis caused by other drugs. Indeed, several drugs such as asparaginase, nilotinib and pazopanib can cause pancreatitis as adverse effect. Therefore, the personalized *in vitro* model of human exocrine pancreatic tissue created in this study could be applied also to study sensitivity of other drugs leading to safer and more targeted therapies.

7. References

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