PARENTERAL NUTRITION AND BRANCHED CHAIN AMINO ACIDS: AN IN VIVO AND IN VITRO EVALUATION.

Thesis submitted for the degree of
Doctor of Philosophy

by

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PhD Course in Veterinary and Animal Science
Class XXX

UNIVERSITA' DEGLI STUDI DI MILANO

Academic Year 2016-2017

Abstract (English)

Parenteral nutrition (PN) offers the possibility to increase or to ensure nutrient intake, providing nutrients in sufficient doses to meet the veterinary and human patient's daily requirements. PN is a complex admixture based on amino acids, dextrose, lipids. Safety issues related to parenteral nutrition formulations have led to the development of guidelines for safe practices. The development of new formulation and the evaluation of the already used solution is the main research in PN practice. International guidelines are quite vague regarding the optimal doses of amino acid to administer to malnourished patients affected by liver desease. A decreased ratio of branched-chain amino acids (BCAA) to aromatic amino acids (AAA) is considered an important pathogenetic factor in hepatic disease. The PN solution are composed by different compound, where the BCAAs are always presents. Long-term oral supplementation with branched chain amino acids (BCAAs) as adjuvant during liver injuries, both in humans and animals, is still not totally clear. Aim of the present study was to determine how amino acids in PN and BCAAs on an in vitro study can preserve liver damage and function. First purpose of this study was to evaluate the use of partial parenteral nutrition (PPN) in dogs, using two different solutions: 11 dogs treated with a normal amino acidic concentration (Medium/Low Protein Level group, Freamine concentration $\leq$ 25%;1,9-2,2 gr/kg/die amino acids/proteins) as control group and 9 dogs with high-amino acid percentage (High Protein Level group, Freamine > 25%; 3,6-6 gr/kg/die amino acids/proteins) as cases. The evaluation of the two differents solutions is based on the liver damage during the therapy. Results reveal a not statistically significatives differencies for liver damage between High Protein Level group and Medium/Low Protein Level group. It means that an a hight amount of amino acids in PN could not affect and overload the hepatic metabolism, despite carrying out their more advantage effects on body lean mass.

The second purpose and study, which is the most importan for us, was to evaluate two differents in vitro models selected: A) hepatic rabbit cells plated in 2D monolayer and B) rabbit hepatic cells cultured onto 3D scaffolds, obtained from decellularized rabbit liver. Both A and B cells were treated with BCAAs. All cells adhered and proliferated, once plated. Moreover, the presence of hepatic progenitors (HPCs), were detected by immuno staining assay and mRNA expression. However, A cells were shown to produce albumin and FVII but quickly entered G0 and arrested growth by day 15. In contrast, cells in B, actively replicated and recellularized ECM rabbit liver, mimicking the original organ cyto-architecture, until day 21. Interestingly, under defined conditions, BCAA supplementation promoted vigorous growth of cell organoid and increased hepatocyte albumin and FVII production and expression. A selective amplification of self-renewing bi-potent HPCs was also observed. These results highlight the role of BCAAs in long term supplementation and support the use of 3D cultures as reliable in vitro models. In conclusion, these finding suggest that an high amino

acidic diet with a high BCAAs/AAA ratio coud be a valid therapeutic option in cachectic patients or patient with severe livere diseases.

Abstract (Italiano)

La nutrizione parenterale (PN) offre la possibilità di aumentare o di assicurare l'assunzione di nutrienti, fornendo sostanze nutritive in dosi sufficienti a soddisfare le esigenze quotidiane del paziente in medicina e in medicina veterinaria. La PN è una miscela complessa composta da aminoacidi, glucosio e lipidi. Le criticità di sicurezza relative alla composizione delle formulazioni parenterali hanno portato allo sviluppo di linee guida per una pratica clinica sicura. Infatti, lo sviluppo e lo studio delle diverse soluzioni argomento principale delle ricerche pubblicate in questo ultimo decennio. Purtroppo, però, le linee guida internazionali sono abbastanza generaliste riguardo le dosi ottimali e qualitative della porzione aminoacidica somministrata in pazienti malnutriti e affetti da malattie epatiche. Un ridotto rapporto tra amminoacidi a catena ramificata (BCAA) e aminoacidi aromatici (AAA) è considerato un importante fattore patogenetico durante patologie che affliggono il fegato o in caso di grave cachessia. Per codesto motivo, le soluzioni utilizzate e commercializzate i BCAA sono sempre presenti. L'integrazione orale a lungo termine con BCAA come adiuvante durante affezioni epatiche, sia negli esseri umani che negli animali, non è ancora del tutto chiara. Il primo scopo del mio progetto di dottorato è stato quello di valutare l'uso della nutrizione parenterale parziale (PPN) in cani ospedalizzati, utilizzando due differenti soluzioni: 11 cani trattati con una normale concentrazione aminoacidica (gruppo Medium / Low Protein Level, concentrazione di Freamine ≤ 25%, 1, 9-2,2 gr / kg / die aminoacidi / proteine) e 9 cani con percentuale aminoacidica alta (gruppo High Protein Level, Freamine> 25%, 3,6-6 gr / kg / die aminoacidi / proteine). La valutazione è stata basata sull'individuazione di un eventuale danno epatico durante la terapia nutrizionale. I risultati hanno rivelato una differenza tra il gruppo High Protein Level e il gruppo Medium / Low Protein Level non statisticamente significativa per danno epatico. Questo risultato ci permette di valuare un probalbile utilizzo di una PN ad alto contenuto aminoacidico migliorando le condizioni di cachessia o perdità muscolare senza sovraccaricare il metabolismo epatico. Il secondo scopo di questa tesi è stato la valutazione di concentrazioni differenti di BCAA in due diversi modelli in vitro: A) cellule epatiche di coniglio coltivate in un monostrato 2D e B) cellule epatiche di coniglio coltivate su scaffold decellularizzato. Le cellule A hanno dimostrato di produrre albumina e fattore sette (FVII) ma sono entrate in G0 rapidamente e hanno arrestato la crescita entro il giorno 15. Le cellule B, invece, si sono replicate attivamente e hanno ricellularizzato l'ECM del fegato di coniglio. L'integrazione di BCAA ha promosso la crescita dell'organoide e hanno permesso aumento di produzione di albumina di FVII. È stata osservata, inoltre, un'amplificazione selettiva delle HPC.

Questi risultati hanno evidenziano il ruolo dei BCAA nell'integrazione a lungo termine come sostengno di colture 3D e il loro particolare ruolo protettivo in corso di patologie epatiche.

Acknowledgements

I wish to thank all those who helped me and had role in my work.

A special thank to Professor Valentino Bontempo, Professor Fabio Acocella and Dr. Stefano Brizzola those have made this possible.

My greatest thanks to Professor Fulvio Gandolfi and Professor Tiziala AL Brevini for their invaluable support (both human and scientific) and availability throughout all these years.

Last but not least many thanks to Dr.ssa Nose for her emotional support.

TABLE OF CONTENETS

1. Chapter: Introduction 9

2. Chapter: Hypothesis and aim 15
HYPOTHESIS 16
AIMS 16

3. Chapter : Partial Parenteral Nutrition in Malnourished Dogs:impact on liver damage 17
INTRODUCTION 18
AIM OF THE RESEARCH 29
MATERIALS AND METHODS 30
PATIENTS 30
PERIPHERAL VENOUS ACCESS PREPARATION 32
PARENTERAL NUTRITION FORMULATION 32
PATIENT MONITORING 34
MONITORING OF LIVER RESPONSE TO PPN 34
MONITORING FOR COMPLICATIONS 34
STATISTICAL ANALYSIS 35
RESULTS 35
STATISTICAL ANALYSIS 42
DISCUSSION 43
CONCLUSION 46

4. Chapter: Use of 2D and 3D rabbit hepatocyte cultures to investigate Branched-Chain Amino Acid effects in liver 48
INTRODUCTION 49
LIVER REGENERATION 52
AIM OF THE RESEARCH 58
MATERIAL AND METHODS 59
ANIMALS AND LIVER HARVESTED 59
LIVER DECELLULARIZATION 60
CELLS ISOLATION AND CELLS CULTURE 63
BCAA EXPERIMELTAL DESIGN 64
ANALYSIS 66
RESULTS 71
MORPHOLOGICAL OBSERVATION ON THE MONOLAYER HEPATOCYTES CULTURE. 71
EFFECTS OF THE BCAAS ADDICTION IN MONOLAYER HEPATOCYTES CULTURE 72
PERFUSION DECELLULARIZATION OF THE RABBIT 73
MACROSCOPICAL ORGANOIDS EVALUATION 74
FUNCTIONAL ASSAY 75
PCR 77
MORPHOLOGY AND QUANTIFICATION OF THE REPOPULATION OF THE ORGANOID ECM 78
DISCUSSION 82
CONCLUSION 86

5. Chapter: General Conclusion 88

6. Chapter: References 91
INTRODUCTION 92

HYPOTHESIS and AIM 96
PARTIAL PARENTERAL NUTRITION IN MALNOURISHED DOGS: IMPACT ON LIVER DAMAGE 96
USE OF 2D AND 3D RABBIT HEPATOCYTE CULTURES TO INVESTIGATE BRANCHED-CHAIN AMINO ACID EFFECTS IN LIVER 100
GENERAL CONCLUSION 109

7. Chapter:PhD Candidate activities 111

List of Abbrevation

AN: artificial nutrition

PCN: protein-caloric malnutrition

PN: parenteral nutrition

TPN: total parenteral nutrition

PPN: partial parenteral nutrition

EN: enteral nutrition

PPN: partial parenteral nutrition

PCR: protein to calorie ratio

HP: high protein

PER: partial energy requirement

RER: resting energy requirement

BCAAs: Branched chain amino acids

AHF: acute hepatic failure

ER: energy requirement

BW: body weight

RS: refeeding sindrome

ASPEN: American Society of Parenteral and Enteral Nutrition

ESPEN: European Society of Parenteral and Enteral Nutrition

HPC: hepatic progenitor cells

ECM: extracellular matrix

ALB: albumin

FVII: factor VII

ALT: alanine amonitransferase

AST: aspartate aminotransferase

LDH: lactate dehydrogenase

TP: total proteins

1. Chapter: Introduction

The anabolic response to severe injury releases a high amount of amino acids from muscle[1] into the general circulation, endowing new protein synthesis at the sites of tissue injury to optimized immune function and regulate the inflammatory response. The cost of this rapid body protein remodeling is anabolic inefficiency: the net loss of muscle protein exceeds protein mass gain elsewhere and the balance of whole nitrogen becomes strongly negative[2–7]. During early starvation, hepatic glycogenolysis and gluconeogenesis are increased to maintain blood glucose levels. In humans, hepatic glycogen stores are depleted within 24 hours[8,9]. Dogs deplete hepatic glycogen stores more slowly than humans, showing peak depletion between the second and third day of fasting[9]. After glycogen depletion, skeletal and visceral body proteins are broken down to provide amino acid precursors for gluconeogenesis[10]. This results in nitrogen loss and negative net nitrogen balance[11]. If starvation continues for more than several days in humans, the metabolic rate decreases, decreasing energy requirements. There is enhanced oxidation of fat, decreased breakdown of protein, and the development of ketosis. In healthy fasting dogs, fat mobilization progresses more slowly, and ketosis is milder than in human beings[12]. Fasting dogs also have efficient peripheral utilization of ketones more than humans; the contribution of ketone bodies to energy requirements increases from 7 per cent after an overnight fast to 13% after 10 days of starvation. The decreased energy requirements and increased utilization of fat for energy help decrease the use of protein for gluconeogenesis, sparing body protein. There are, however, still obligate glucose requirements for the renal medulla, bone marrow, circulating blood cells, and central nervous system, where the energy supply is obtained from continued protein catabolism[12]. Plasma amino acid concentrations could provide insight into this process, but they vary widely in critical illness, being affected by the phase and intensity of the injury response; the patient's existing nutritional, metabolic, and hemodynamic status; and the characteristics of the nutritional therapy provided. When measured in previously normal or not cachectic patients after successful fluid resuscitation and before exogenous nutrient provision, plasma nonessential amino acid concentrations are usually low and essential amino acid concentrations are either normal or low[13–20]. But circulating amino acid concentrations show only a static view of what is a highly dynamic process. Amino acid concentrations could be increased by intense net muscle proteolysis or inadequate liver uptake and metabolism in the setting of liver hypoperfusion or metabolic failure[17, 21,22], or they could be reduced despite a high rate of release from muscle that is still insufficient to match the combined rates of central protein synthesis, gluconeogenesis, and immediate amino acid catabolism, all of which are accelerated in critical illness. We can't argue that dietary protein deficiency is a common condition in acutely hospitalized patients, and universal in critical illness. In fact, inattention to these nutritional deficiencies leads to the disease called protein-energy malnutrition, the cardinal features of which are generalized muscle atrophy and fat loss.

Modern hospitalized patients have adequate (or more than adequate) fat reserves to draw upon during temporary periods of hypocaloric nutrition so muscle loss is considered much more dangerous than fat loss. The decrease of lean body mass despite to fat reserve is a life-threatening, debilitating and a difficult to reverse status. In addition to the muscle atrophy caused by deficient dietary protein and energy provision, many diseases or their treatments increase dietary protein requirements well above normal by increasing body amino acid or protein loss (in wound exudates or fistulas, inflammatory diarrhea, and renal replacement therapy) or by pathologically increasing muscle protein catabolism, as occurs with high-dose glucocorticoid therapy and as part of the systemic inflammatory response to sepsis and major trauma. In the last forty years has been a consensus in the literature and in the studies about N balance that critical illness increases human protein requirements[23–31]. Unfortunately, defined and hard data can't confirm or refuse the conclusion about the benefits of protein calorie supplementation. The results have been very conflicting. Peptide bond formation is a dehydration reaction. For this reason, free amino acids contain less protein substrate, and less energy, than the proteins they create[32]. For example, 100 g of hydrated mixed amino acids does not provide 400 kcal and 100 g protein but only 340 kcal and 83 g of protein, which substrate is formed by isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine). Arginine is synthesized, but not always in a sufficient amount but it is considered conditionally essential. Another eight amino acids (alanine, asparagine, aspartate, glutamate, glutamine, glycine, proline—actually an imino acid—and serine) are nonessential because they are readily synthesized from widely available intracellular carbohydrate molecules and amine groups in the large, rapidly interconverting pool of free non-essential free amino acids (NEAAs), predominantly glutamine, glutamate and alanine. The hypothesis that continues to await rigorous testing is that prompt, high-protein (2–2.5 g/kg per day human, 3-6 g/kg per day dogs) hypocaloric nutrition may improve clinical outcomes in catabolic critical illness. Early protein provision should be increased to somewhere between 1.5 and 2.5 g protein/kg normalized dry body weight per day[23-25] in humans. Patients who have a faster and more massive protein loss are evaluated and studied starting from the loss of N through the renal filter and then in the urine. These patients are the ones who can mostly takes advantages from suitably protein provision[33]. In veterinary medicine there are no precise data about the maximum dosage of protein in the diet during states of cachexia, remembering that the normal daily doses are 3 g protein /kg for dogs and 6 g protein/kg for cat[34,35]. Therefore, it is important to understand how nutrients can reach the blood system and bring energy to the various biological systems. When the gastrointestinal tract is functional, enteral nutrition is the preferred method of nutritional supplementation because it preserves the integrity of the gut mucosal barrier and function and is easier and less expensive than other methods such as parenteral nutrition (PN)[36]. PN can be classify as: Total parenteral nutrition

(TPN) and Partial parenteral nutrition (PPN) (sometimes the term PPN is commonly use also to indicate peripheral parenteral nutrition). TPN is recommended when the gastrointestinal tract is not functional, or it is undesirable to use it, like severe malabsorption, prolonged ileus, and after some gastrointestinal surgeries[37]. The solutions used in TPN are usually a combination of glucose, amino acids and lipids. Owing to the high osmolality (greater than 800 mOsm litre–1) of TPN solutions it has been recommended that they be administered through a central vein to prevent peripheral vein thrombosis. Disadvantages of using TPN include cost, necessity (and difficulty) of accessing and maintaining a central venous catheter, risk of infection or central vein thrombus, and metabolic disturbances. These difficulties and disadvantages limit the use of TPN in veterinary practices. The goal of PPN is to spare breakdown of endogenous tissue and visceral proteins by providing an energy source such as glucose or lipids, and/or by providing amino acids that are catabolized for energy and used for protein synthesis without overload the cells metabolism of each single organ. In fasting human patients, infusion of 5 per cent glucose solution is proposed to decrease nitrogen loss because the solution partially fulfills energy requirements and reduces the amount of glucose that must be produced by gluconeogenesis[36]. Administration of 5 per cent glucose solutions has been proposed to be useful for protein sparing in veterinary patients and in humans[36]. but conversely, other authors consider it to have little protein sparing effect for veterinary patients due to the small amount of energy provided[37]. It has been known that administration of amino acids stimulates resting energy expenditure and hence thermogenesis[38], even during general anaesthesia[39] . In humans and rats, it has been shown that amino acid infusion causes nutrient- induced thermogenesis as a result of heat production in skeletal muscle and prevents reduction in metabolism and hypothermia during anaesthesia[39-41]. Moreover, studies using anaesthetized rats showed that administration of amino acids increases the plasma insulin concentration and the anabolic effect of insulin on muscle[42]. These findings suggest that amino acids facilitate heat generation and retention in the body. The metabolic and thermogenic effects of amino acid infusion have also been studied in dogs[43-44]. However, a clinically useful infusion protocol has yet to be established, because adequate dose and treatment practices for infusion of amino acids are unknown especially in animals. Talking about differences between veterinary and human medicine, over the years, the PN has been the subject of controversies regarding the use of standard or specific formulas for the individual patient. Many human hospital pharmacies purchase premixed ready-to-use PN products that are either dual-chamber (amino acids and dextrose, with or without electrolytes) or triple-chamber (amino acids, dextrose and lipid). Shortly prior to infusion, the internal membranes separating the chambers are broken, their contents intermixed and vitamins, trace minerals and additional electrolytes added[45,46]. These products are convenient and potentially cost-effective, but their fixed nutrient composition is a drawback. In

veterinary medicine and in some cases like for home PN the compositions can be selected and can meet the specific needs of each patient, mixing the three compound as amino acids, dextrose and lipid directly in a single bag or bottle. In this case, it is possible to meet a patient's protein requirement and avoid calorie overfeeding by selecting an appropriate fixed-composition product. The downside of this approach is the inconvenience of stocking the pharmacy with many different premixed products. In some situations—especially critical illness, in which 2.5 g/kg protein substrate per day may be required[47]. Diets high in protein pose a potential acid load to the liver, mainly as sulfates and phosphates. It was hypothesized that calcium and hence bone mass was lost in order to buffer this acid load. the protein-induced acid-load to the liver remains, e.g. as sulfuric acid from the oxidation of methionine and cysteine[48]. Rigorous data on the comparative efficacy of various formulations of parenteral nutrition, the optimal timing of administration, and the role of specific clinical factors (e.g. blood glucose control) on clinical outcomes with different parenteral nutrition formulations are lacking[49]. Standard amino acid solutions contain all nine essential amino acids in patterns and amounts designed to match or exceed their recommended dietary allowances, sufficient amine N from some NEAAs to support the synthesis of all them, and a generous amount of arginine[47]. The recommended normal adult protein requirement is 0.8 g/kg per day. To meet this requirement a parenteral amino acid solution must be infused at the rate of ~1.0 g/kg per day. Specialized amino acid solutions are available for patients with hepatic insufficiency (increased amounts of branched-chain amino acids and less methionine, phenylalanine and tryptophan), renal insufficiency (essential amino acids largely or only), and protein-catabolic critical illness (increased branched-chain amino acids). The properties of the branch-chain amino acids (valine, leucine, isoleucine) as energy substrates, substrates for gluconeogenesis, and modulators of muscle protein metabolism make the use of branch-chain amino acids-enriched solutions theoretically appropriate for the management of the metabolic alterations that occur in cachexia and sepsis[50]. The in vitro and in vivo animal data available have suggested a benefit for the branch-chain amino acid on protein synthesis and degradation in sepsis. Branch-chain amino acids-enriched solutions (45%) increase the rate of hepatic protein synthesis and acute-phase proteins in critically ill patients. These proteins may be important in host defense mechanisms against infection. Thus, the administration of solutions containing a high proportion of branch-chain amino acid may improve the chances of survival for such patients via this mechanism[51]. The use of high concentrations of various amino acids in the diet may perturb neoplastic cell growth and liver dysfunction. To date, the most effective amino acid for this purpose either in cell culture and in vivo has been arginine and branched-chain amino acids (BCAAs)[50]. In human medicine, certain liver neoplastic conditions and liver insufficiency are often accompanied by changes in metabolism resulting in cachexia. Studies have suggested that branched-chain amino acids

may help to preserve lean body mass in models of muscle atrophy[51–53]. More recent studies suggest that leucine has profound effects upregulating signaling events (i.e., mTOR activation), leading to protein synthesis in skeletal muscle[54]. The exact mechanisms for this upregulation remain elusive, but treatment with branched-chain amino acids (BCAAs) remains attractive as a therapeutic modality for ameliorating lean body mass in cachectic patients. BCAAs are beneficial during cachexia and hepatic failure determining their effects on neoplastic cell growth is important. Neoplastic cell lines (canine osteosarcoma, canine bronchoalveolar carcinoma, and Madine-Darby canine kidney cells) under the influence of high concentrations of BCAAs (leucine, isoleucine, valine) were examined for their ability to augment or diminish cell growth. Branched chain amino acids (BCAA) have been shown to affect gene expression, protein metabolism, apoptosis and regeneration of hepatocytes, and insulin resistance. They have also been shown to inhibit the proliferation of liver cancer cells in vitro and ameliorate the hepatic liver cells regeneration and, moreover, are essential for lymphocyte proliferation. All these features of BCAAs are studied and evaluated for a new nutrition strategy in different human pathologies. In veterinary medicine the use of the BCAAs as integration of a normal dietary plan seems to be a valid choice for the same benefit found in human clinical nutrition[55]. The mechanism by which BCAA specifically improves plasma albumin levels is not fully understood yet. The synthesis and secretion of albumin increased when rat primary hepatocytes were cultured with an appropriate Fischer's ratio (3.0). The addition of amino acids similar to the plasma composition was found to promote albumin synthesis in primary hepatocytes and in liver perfusion experiments[56-59] and the removal of amino acids decreased albumin synthesis[59]. The first scientist applies the single nourishment concept for the total nutritional needs of men and animals was John Harvey Kellogg[60]. To improve the patients' diet, Kellogg developed, patented, and introduced a variety of new foods, including Granola and Corn Flakes; he also invented peanut butter, artificial milk made from soybeans, and a variety of imitation meats. His younger brother, Will Keith Kellogg, later built a successful company to market his brother's cereal creations; in so doing, he transformed the average American's breakfast.

We can evaluate and study different amino acids to achieve a similar Kellogg's scope: Which is the compound/ amino acids that help or overload the liver during TPN or PPN?

2.Chapter: Hypothesis and aim

HYPOTHESIS

1. Detrimental effects of different amount of amino acids solution on dogs' liver affected by different pathologies during parenteral nutrition.
2. BCAAs protecting role on model of liver failure.

AIMS

Considering the lack of data in the literature in veterinary medicine and the confusion and fragmentation of medical studies concerning the use of amino acids in PN the in vivo and in vitro studies purpose are[1]:

1. to evaluate a possible negative effect of a high dosage of solutions amino acidic ready prepared on a heterogeneous population of canine patients affected by different pathologies that needed parenteral nutritional therapy.
2. Long-term oral supplementation with branched chain amino acids (BCAAs) as adjuvant during liver injuries, both in humans and animals, is still not totally clear. Aim of the present study was to determine how BCAAs preserve liver functions in vitro.

Summary of the experimental design

The research is divided into two experimental projects:

1. An in vivo dog clinical model to assess the effects of high-dose amino acids solutions.
2. An ex vivo model to assess the effect of varying BCAA concentration on liver organoids.

The future aim of my PhD project will be to study the effect of the BCAA on the Normothermic extracorporeal liver perfusion. The intellectual process is starting from the clinical cases passing through an in vitro evaluation by three following steps: monolayer 2D culture, then 3D scaffolds culture and at the end the whole organ perfusion as the in vitro model.

3.Chapter : Partial Parenteral Nutrition in Malnourished Dogs:impact on liver damage

INTRODUCTION

Artificial nutrition (AN) is a therapeutic procedure which fully satisfy the nutritional needs of patients otherwise unable to feed naturally. Choosing the way to provide nutrition support is a key step in managing critical patients and select the combination of the artificial nutrition for each patient will be dictated by clinical conditions and nutritional status, duration of nutritional support as well as needs, dietary limitations, and the advantages and disadvantages of each type of nutrition[1].

Restoring an optimal body condition or physiological body weight is not necessarily the goal of nutritional support, the main purpose is to preserve lean tissues and organ function rather than completely resolving the state of malnutrition, a goal that occurs during convalescence.[2] In hospitalized patient, malnutrition is the result of an acute or chronic deficiency, both of calories as protein, causing the so-called "malnutrition protein-calorie " (MPC), characterized by lean mass loss and expansion of extracellular compartment (Tab.1).[3] Malnutrition refers to the progressive loss of body mass and adipose tissue due to an inadequate intake or increased protein and calories needs; possible consequences are: atrophy of organs and muscles, alteration of immune-responsiveness, delayed wound healing, anemia, hypoproteinemia, lower resistance to infections and death. The patient with protein deficiencies and calories should receive an integration until it is possible to eliminate the deficiency causes.[4] Malnutrition can be classified as primary or secondary to other causes. Primary malnutrition is characterized by the absence of one or more components of a diet, such as carbohydrates, fats (fatty acids), protein (amino acids), vitamins and minerals. In secondary malnutrition, dietary nutrient intake is adequate, but malnutrition is caused by malabsorption of specific nutrients, metabolic deficiencies and accumulation, excessive leakage or increased demand. The causes of secondary malnutrition are grouped into three categories, partly overlapping: gastrointestinal diseases, chronic cachexia and acute diseases.[5] Malnutrition indicators include weight loss, hair loss, muscle mass loss, hypoalbuminemia, lipopenia and coagulopathy. These alterations are however not specific to malnutrition and often do not appear soon during the process.[6]

Diagnosis of protein-calorie malnutrition
- weight loss> 10% of normal body weight
- increase nutrient losses (diarrhea, vomiting, wounds, severe burns)
- increased nutritional needs (traumas, surgical interventions, infections, burns, fever)
- anorexia or hyporexia (insufficient intake of food)> 5 days, or situations where it is expected that, for more than five days, the animal takes insufficient portions of food
- increased nutrient requirements (trauma, surgery, infections, burns, fever)

Table 1. Protein-caloric Malnutrition.

Simple fasting includes metabolic changes which may occur even in the absence of pathologies. The time it takes to metabolic changes and alterations associated with nutrient deficiency, should guide the nutrition of hospitalized dog. In fed properly animals, post-prandial nutrients are immediately used in metabolic pathways and stored as glycogen, fat and muscle protein. Any excess energy is converted into triglycerides deposited as fat in adipose tissue, liver and muscle. In a state of normal nutrition, when the serum glucose is high, the liver becomes a glucose importer. Patients with food deprivation show a complete reversal of the metabolic processes. Lacking the exogenous source of energy, the endogenous resources become the reservoirs to meet the metabolic needs. The glycogen storage becomes the primary source of energy and, to preserve for as long as possible the vital functions, the animals use stored fat and protein to maintain a constant concentration of glucose in the blood. The metabolism of carbohydrates is profoundly altered in the first week of fasting. In the early days, the dogs maintain constant blood glucose through glycogenolysis and gluconeogenesis. The muscle catabolism releases amino acids, lactic acid and pyruvate to produce glucose in the liver[6]. In an attempt to convert the circulating glucose, the liver releases ketone bodies. These are the product of the oxidation of long-chain fatty acids that originate from triglycerides packed in adipose tissue. Fatty acids, water insoluble, must be transported in the blood by albumin, while ketones, water soluble, have a wide distribution thanks to their ability to cross cell membranes. The insolubility and the dependence from the albumin limits the serum concentration of fatty acids. The advantage of the conversion of fatty acids into ketones is threefold: ketones are soluble in water, they do not depend on the albumin and they can cater to the lipid reserves of the cells for their high blood concentrations. Increasing ketones in serum helps to increase the endogenous proteins and glucose. In case of starvation ketosis is a correct response of the organism. From the fifth day of fasting decreases the

level of insulin and triggers lipolysis in an effort to preserve the stored proteins and maintain the level of glucose in the blood for glucose dependent tissues[7]. From the oxidation of fatty acids, the amino acids are partially spared to maintain muscle protein reserves during all the fasting period. Although, after two/three days the body proteins are partially spared because the muscles will catalyze the chains of amino acids. The reefing management in a patient depends by the number of days of the fasting period [8,9]. The collapse of protein breakdown is needed to address the imbalance between consumption and intake of energy. This catabolic phase continues until the neuroendocrine stimulus and the cytokines mediators are removed. Clinically we see the improvement of patients only when phase of anabolism starts. In this phase, the body to maintain its homeostasis requires energy and proteins. During catabolic fats and proteins are necessary for the patient so the diet will be administered with fewer carbohydrates and more protein and fat. Increase fat would lead to electrolyte imbalances and hyperglycemia. The phase of hospitalization therefore underlines the constant need to re-evaluate the patient to optimize the nutritional plan. However, for long-term fasting is meant the state of anorexia in which the patient is in a chronic illness. The ability to respond to this situation can be altered in the course of a disease process[6,7]. While plasma albumin and prealbumin are anti-inflammatory indicators in humans and their concentration is associated with the development of complications and mortality, in the veterinary medicine it is difficult to identify specific laboratory data to indicate a condition of malnutrition. Diagnosis of malnutrition can formulate through physical examination and some more generic laboratory tests. The number of red blood cells, the content of hemoglobin, urea and potassium, the concentration of albumin and total protein, and lipocytes number of white blood cells are useful to have a nutritional assessment of the patient and assess his state of hydration. Red blood cells, hemoglobin, albumin and total protein have a moderately long half-life, from one to eight weeks and they are difficult to evaluate in the early stages of malnutrition, while potassium and urea blood levels can be low in the anorexic patients due to the lack of food assumption, but urea tends to increase in the final stages of anorexia due to muscles catabolism[6-8].

In human medicine, many tools have been developed to assess the severity of complications, such as the Nutritional Risk Index (NRI) that combines albumin and weight loss, Geriatric Nutritional Risk Index (GNRI) that considers the current weight with the ideal weight and the Subjective Global Assessment (SGA)(Fig.1) [10].

GNRI = [1489 x albumin (g/L)] + [41,7 x (weight/ideal weight)]

NRI = [1,489 x albumin (g/L)] + [41,7 x (weight /usual weight)]

The best prediction index is the SGA and the value of albumin. The SGA is an excellent prognostic factor because diagnose malnutrition, giving emphasis to the causes and the consequences of malnutrition. The low albumin concentration (around 3/3.5g/dL) is associated with an increased long-term mortality. Both albumin and prealbumin pre PN predict the long-term risk of mortality. But to have a more precise and accurate prediction, levels of albumin and prealbumin must be integrated with GNRI or NRI. First thing to do is to investigate the patient nutritional status with a proper dietary history to reconstruct the informations on the diet habits. Than the energy requirement must be assessed.

RER = Resting Energy Requirement = 70x (BW kg) = kcal/die

PER = Partial Energy Requirement = 70% RER = kcal/die

RERs are the number of calories required to keep homeostasis resting in a thermally neutral environment while the animal is in an absorbing post state. In addition to specific dietary questions, the history should also include information regarding appetite, alterations in weight or appearance and gastrointestinal symptoms such as vomiting or diarrhea[8]. Clinical examination including nutritional evaluation is a crucial step in identifying the most appropriate nutritional support. Body Condition Score (BCS) and Muscle Condition Score (MCS) are the hubs of that assessment. BCS and MCS are evaluation criteria based on visible and tactile measurement indices to quantify the level of adiposity and muscle development; are parameters that affect the subjectivity of the operator. The BCS allows to give a score to the nutritive condition. It is a morphometric system based on inspection and palpation of zygomatic crests, thoracic and transverse apophyses of the lumbar and thoracic vertebrae, on the ribs and intercostal spaces, on iliac and ischemic tuberosity and on the side ditch to quantify the amount of adipose deposits and hence the nutrition status of the animal. A score from 1 to 5 or from 1 to 9 is assigned, classifying the animal from very thin to obese. The MCS evaluates muscle mass and tone through inspection and palpation of the muscular masses of the rear train, column, front train, neck and head. Muscle tone is what allows the animal to lift from the decubitus and support the body while maintaining the normal relationships between body and limbs; it is the degree of resistance that a particular muscle group opposes to the passive movement. Muscle mass loss affects resistance, immune response, wound healing and is associated with a higher mortality rate[11](Fig.3). Clinically, BCS and MCS are not directly related. An animal may be overweight but still lose muscle mass. Body weight assessment is used to check if it has remained unchanged or if there has been a decrease. Malnutrition can be supposed when a significant involuntary weight loss in the past 6 months is greater than 10% to the usual weight, or greater than 5% in one month. In absence of habitual weight, malnutrition is indicated by a 20% lowering from the ideal weight[12].

Abnormal or pathological weight loss should not depend on dehydration, but it should affect muscle mass or lean mass. No hematologic parameters such as albumin, prealbumin, potassium and lipocytes may be considered in the patient's assessment as their alteration may be indicative of an altered nutritional state[8]. Hypoalbuminemia occurs due to hepatic production deficiency because in physiological conditions 50% of the total protein daily synthesis is dedicated to the production of albumin, so inadequate protein intake can cause less production of these. However, albumin has a 8-day half-life in the dog, so before a parameter falls, few days passed since there is a steady decrease in protein intake[8]. Parenteral nutrition (PN) involves administering the intravenous nutritional support by attaching a venous catheter to the central or peripheral position, thus bypassing the gastrointestinal tract. The PN purpose is to correct the state of malnutrition, prevent progressive caloric-protein malnutrition, optimize the patient's metabolic state, minimize the risk of disease, death and hospitalization times[13]. Parenteral nutrition is used in those patients unable to take food spontaneously or in which the gastrointestinal tract has to be bypassed due to pathologies or general surgery procedure. The decision to undertake a period of parenteral nutrition is based on several parameters, often subjective[8]. PN in the dog is usually used as a short-administration to prevent further nutritional deterioration until enteral nutrition restoration. The short duration of the PN, usually intended less than 5 days, can reach the goal, in most animals, although, in humans, administration for extended periods is reported to lengthen the patient survival[9,10]. PN can be total or partial. Total Parenteral Nutrition (TPN) provides all the daily energy requirement, through a central venous catheter inserted into the jugular vein. The power supply for central venous route must be taken into consideration in severely catabolic patients, with severe depletion, or simply when TPN for prolonged periods is indicated. In order to obtain a positive nitrogen balance, in those patients a calorie-nitrogen ratio of at least 100/150 of non-protein calories per gram of nitrogen is advised[19]. With partial parenteral nutrition (PPN) only part of the daily energy, from 40% to 70%, is delivered through a peripheral venous route associated with a solid diet per os.. The PPN is indicated in patients with a moderate catabolic depletion. The peripheral route of administration is easier to establish using of low osmolarity solutions 600/750 mOsmol / L to prevent thrombophlebitis. The osmolarity is the osmotic pressure generated by the solutes present in 1 L of solution. It is a physical quantity that measures the concentration of the solutions used. In particular is the total number of molecules and ions present in one liter of solvent; therefore, expresses the concentration of a solution, emphasizing the number of particles dissolved[11]. The central venous access instead, although more complicated to establish and maintain, imposes fewer limitations in the formulation being able to get to 1400 mOsmol / L with a lower risk of thrombophlebitis. The parenteral solution is comprised of lipids, dextrose and amino acids that compose the "three-in-one solution" or "total nutrient admixture" (TNA) since the three

components make up the "one-fluid bag" then a single bag that it represents the Complete solution for patient nutrition[11-12]. Components are always the same but their amount change in relation to the subject's RER and the patient's clinical needs. In hand-made PN formula it is important to remember that lipid are an excellent substrate for bacterial and fungal growth and it is of fundamental importance to follow an aseptic procedure during the preparation, working under hood, wearing sterile gloves, needles etc. The PN components can be mixed either manually within a sterile bowl, one at a time according to a precise order: glucose, amino acids and lipdis or with an automated system. The manual system is certainly the most used and diffused, because it is easily replicable even if slower and more susceptible to bacterial contamination than the automated ones. It always starts with the dextrose solution, than amino acids, introducing lipids only at the end[11]. Once prepared, the solution can be stored in a 4 ° C refrigerator for up to 3 days[14]. In qualitative and quantitative determination of nutritional needs, the nutritional and metabolic state of the patient, as well as the basic pathology and related therapies, must be taken into account. For each macronutrient, the intake must remain within well-defined ranges, to avoid errors by defect or excess. First, it is necessary to calculate the daily caloric needs specific to the patient, determined by the baseline energy expenditure and the degree of physical activity, and varies with the intake of food and pathological conditions. Accordingly proteins, lipids and glucose requirements are then calculated[14]. The daily energy requirement (ER) of an animal is given by the intake of proteins, lipids, carbohydrates and fiber. It can be calculated as RER. The protein requirement can be calculated as Protein to Calorie Ratio (PCR) ratio, which corresponds to the protein g / 1000 kcal EM.

gr Protein = PCR x RER (Mcal)

The protein requirement is derived from estimating the amount of high quality protein needed to maintain nitrogen balance in the presence of adequate energy input. The needs are different depending on sex, age and physiological conditions; while the quality of a protein is determined by comparing the quantity of each essential amino acid contained in a certain food with that contained in a reference protein.

Dog	PCR	Cat	PCR
< 10kg	PCR 55	adult	PCR 70-80
11/25 kg	PCR 60	senior	PCR 80
26/45 kg	PCR 65	diabetes	PCR > 70

> 45 kg	PCR 70	liver failure	PCR < 60
senior	PCR > 70	kidney failure	PCR < 60
gestation	PCR > 65	anemia	PCR > 80
lactation	PCR > 75	obesity	PCR > 90
active	PCR > 75		

Table 2. RPC in dog and cat in physiological and pathological conditions.

	gr/BW kg	PCR Protein to calorie ratio gr/1000 kcal
LOW	1,9 - 3	50 - 60
MEDIUM	3-5	50 - 60
HIGH	5-7	50 - 60

Table 3. PCR Classes

Although there is no literature regarding the PCR use in parenteral nutrition, we can consider three protein levels: low, medium and high. Proteins are supplied by an amino acid solution with a concentration ranging from 3.5% to 15%, with a pH range of 5.3-6.5 and an osmolarity of between 300 and 1400 mOsmol / L, to which electrolytes can be added if necessary. The most commonly used solutions have a concentration of 8.5% with an energy density of 0.34 kcal / ml and osmolarity of 706/880 mOsmol / L. 44A amino acid solution contains essential amino acids and non-essential amino acids such as: L-Isoleucine, L -Leucine, L-Lisin acetate, L-Methionine, L-Phenynalanine, L-Threonine, L-Tryptophan, L-Valine are essential AAs, while non-essential AAs are L-Alanine, L-Arginine, L-Proline, L-Serine, L-Cysteine HCl and Acetic Acid[45]. The range of the proteins to be delivered are very wide and vary greatly depending on the patient condition and its eventual pathology; it goes from a minimum of 2-3 gr / 100 Kcal to a maximum of 4-5 gr / 100 Kcal for the dog, and a range from 3-4 gr / 100 Kcal to 6 gr / 100 Kcal for the cat. It is therefore up to the veterinarian to determine the right amount of protein needed for his or her patient, for example increasing it in all that situations where a high protein requirement is needed or diminishing it in case of kidney or liver failure. Intravenous amino acids are distributed to all organs through the plasma

and captured in different quantities as needed. Muscles and liver are the major amino acid regulator filters in the blood. Aromatic amino acids (phenylalanine, tryptophan), methionine, lysine and threonine are largely captured by the liver, while branched amino acids (leucine, isoleucine, valine) are largely captured by the muscles. Part of the amino acids are deaminated, with urea formation, while the carbonaceous skeleton is catabolized for energy purposes. The body then needs energy to eliminate the excess nitrogen, which could be a problem especially for patients with liver or kidney disease and could lead to hyperammonia with clinical signs of encephalopathy, while providing a low quantity of amino acids could counteract to lead to hypoalbuminemia, lower immune response, and alter the healing times[9,15]. Here we are to the main point of the use of amino acid during PN. The general thought of the hyperproteic diet could contrast the amino acidic lost during cachexia. There is no much information about the correct dosage and utilization range both in veterinary medicine and medicine in literature but the evidence that the high amino acids amount gave to starved patients is a growth in the nutrition science. The researches in experimental animals with liver disease or diet protein excess are mainly focused on what a diet can give an improvement in their liver function[16-19]. The general thought is that an hyper protein diet could contrast the amino acidic lost during cachexia, but there are not many informations about the correct dosage and range of utilization in either in veterinary and human medicine literature, but there is an evidence in nutrition science that an high amino acids diet administered to starved patients carries to a better clinical condition. Examples comes from some animal studies showing how proteins has a protecting role on the liver in common induced hepatic diseases such as alcoholic or nonalcoholic fatty liver[16-19]. It seems that a high dose of amino acids or proteins can counteract the degenerative processes that are on the base of liver steatosis. Especially hepatic content of BCAA-derived monomethyl branched-chain fatty acids (mmBCFAs) 14-methylpentadecanoic (14-MPDA; valine-derived) and 14-methylhexadecanoic acid (isoleucine-derived) where higher in the animal feed with a hyperproteic feed. Liver is the main organ handling ingested macronutrients and it is associated with the beginning of different pathologies. The science aimed to deepen our knowledge on molecular pathways affected by long-term intake of an HP diet. Transcriptome analysis performed on rats liver chronically fed with a casein-rich HP diet showed up-regulated processes related with amino acid uptake/metabolism and lipid synthesis, promoting a molecular environment indicative of hepatic triacylglycerol (TG) deposition. Moreover, changes in expression of genes involved in acid–base maintenance and oxidative stress indicate alterations in the pH balance due to the high acid load of the diet, which has been linked to liver/health damage. Up-regulation of immune-related genes was also observed. In accordance with changes at gene expression level, we observed increased liver TG content and increased serum markers of hepatic injury/inflammation (aspartate transaminase, C-reactive protein and TNF-alpha). Moreover,

the HP diet strongly increased hepatic mRNA and protein levels of HSP90, a marker of liver injury. Thus, long-term consumption of an HP diet, resulting in a high acid load, results in a hepatic transcriptome signature reflecting increased TG deposition and increased signs of health risk (increased inflammation, alterations in the acid–base equilibrium and oxidative stress). Persistence of this altered metabolic status could have unhealthy consequences[16-19]. The mechanism by which HP diet carry to an eventual improvement of liver function or a limitation of liver damage is still not well understood. Beside the positive effect of PN and their inside nutrients, direct damage of parenteral nutrition to the liver is mostly focused on lipid overload and the lack of transit of the duodenal ingests. The wide spectrum of clinical, biochemical and pathological manifestations may include benign increases in liver enzymes to liver disease histologically characterised by centrilobular, intrahepatic cholestasis, portal fibrosis, reactive bile duct proliferation, non-specific portal and periportal inflammation, and hepatic steatosis. The incidence of hepatic steatosis has decreased thanks to the improvements of TPN solutions and technology, including modifications of the TPN regimen, such as a greater content of lipids and lesser content of glucose. However, hepatic steatosis still remains the most common complication of TPN[20]. Steatosis consists of a hepatic accumulation of micro- or macrovessicular fat associated with metabolic diseases, diabetes, obesity[21], drugs and toxins, surgical procedures, and parenteral nutrition (PN)[22], as well as non-alcoholic steatohepatitis, in which case oxidant stress is thought to play a pathogenic role[23-24]. The precise pathogenesis of TPN-associated hepatic steatosis is still unclear. TPN-related hepatic dysfunctions [19] have been linked to oxidant stress as well as to genetic[25-27], nutritional, environmental and inflammatory factors. More specifically, the following nutritional factors have been associated with steatosis and/or steatohepatitis: an excessive infusion of glucose or lipids, a carbohydrate to nitrogen imbalance[27-33] , and the source of infused lipids, an amino acid imbalance, enteral starvation, photo-oxidised products of amino acids (AAs)[33-40]. Increased serum hepatic aminotransferase concentrations have been commonly observed within the first 2 to 3 weeks of TPN infusion in upwards of two-thirds of patients. Typically, this is a transient increase without significant elevation in serum bilirubin, at least in the adult patient. The serum bilirubin concentration often becomes elevated in children, particularly in preterm infant. It is important to recognize, however, that serum hepatic aminotransferase concentration elevations are both insensitive and nonspecific indicators of hepatic dysfunction. In fact, it is not uncommon for a patient to have cirrhosis in the face of normal serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations. When patients follow a longer period of time receiving TPN, total serum bilirubin concentration tends to increase slightly, but more importantly, the AST often increases significantly beginning after approximately 10 weeks. The alkaline phosphatase often increases as well, although some of this increase may relate to the

development of concomitant metabolic bone disease. In a retrospective study of over 40 long-term TPN patients, some of which had received TPN for up to 16 years at the time of the study in 1991, a significant correlation between TPN duration and the serum alkaline phosphatase was observed, although TPN duration and both AST and ALT were not correlated.[66] While it is recognized that liver test abnormalities occur during TPN, the question is whether TPN is associated with chronic liver disease. Given that the first case of TPN-associated liver disease was described in a young child, it comes as no surprise the first case of TPN-associated hepatic failure was described in an infant[41]. The growing need for safe and effective PN therapies to stop the patient have led to a test the correct dosages and uses of the amino acid part of the parenteral. Enteral nutrition is preferred to PN for invasive nutrition support because of its simplicity, trophic effects on the gastrointestinal tract and a possibly reduced risk of infectious complications. PN is more resource-intensive, potentially riskier, and requires somewhat more expertise than EN. It is indicated when invasive nutrition support is required and EN is refused, inappropriate, or demonstrated (or strongly predicted) to be incapable of meeting the patient's nutritional needs. The major disadvantage of EN in critical illness is its slowness. EN commonly fails to achieve the patient's nutritional goal—especially for protein—within the first 7–14 days of therapy[42]. PN's chief advantage is its ability to deliver a substantial dose of amino acids very promptly[43]. PN's safety concerns are inadvertent calorie overfeeding, potential amino acid over-provision, and a possibly greater risk of infectious complications. However, the differences reported in complication rates between EN and PN reflect observations from older studies when calorie overfeeding was routine.The justification of the tests for the detection of hepatic damage occurs with the selection of appropriate markers. In human and veterinary medicine, there is a fairly large overview of liver panels for assessment through blood tests. Hepatocyte cell death during liver injury was classically viewed to occur by either programmed (apoptosis), or accidental, uncontrolled cell death (necrosis). Growing evidence from the increasing understanding of the biochemical and molecular mechanisms involved in cell demise has provided an expanding view of the different kind of cell death that can be triggered during both acute and chronic liver damage such as necroptosis, pyroptosis, and autophagic cell death. The complexity of non-invasive assessing the predominant mode of cell death during a specific liver insult in either experimental in vivo models or in humans is highlighted by the fact that in many instances there is significant crosstalk and overlap between the different cell death pathways. Nevertheless, the realization that during cell demise triggered by a specific mode of cell death certain intracellular molecules such as proteins, newly generated protein fragments, or microRNAs are released from hepatocytes into the extracellular space and may appear in circulation have spurred a significant interest in the development of non-invasive markers to monitor liver cell death. Measurement of serum levels of hepatobiliary enzymes, such as alanine

aminotransferase (ALT), the excised aminotransferase (AST), alkaline phosphatase (ALP) and the ɣ-glutamyl transpeptidase (GGT), total protein (TP) and albumin is commonly used as a screening test to detect the presence of hepatobiliary diseases. These enzymes are highly sensitive for the detection of hepatobiliary disease, the interpretation is hampered by their lack of specificity towards diseases specific disorders[40]. The central role in the metabolism of the liver and its high blood perfusion mean that it is extremely sensitive to secondary damage. So, there are a number of clinical conditions with of liver enzymes increase without that there is a damage clinically significant. A marked increase in serum levels of the enzymes indicate more substantial hepatobiliary injury but does not necessarily imply an unfavorable prognosis. We must consider that in severe chronic liver disease the serum levels of the enzymes may be normal or only slightly increased; this occurs due to the replacement of the hepatocytes by the fibrosis and / or to the leakage of enzymes organ with consequent depletion the total content of the enzymes themselves. Alanine aminotransferase (ALT) is an enzyme specific liver cytosol, because the greater amount is present in the liver tissue. Its serum rising therefore occurs only in liver disease. The maximum increase of its levels, as serum enzyme activity, it is observed in cases of hepatocellular necrosis and acute inflammation. The extent of the raising levels of ALT is about proportional to the number of damaged hepatocytes. In dogs, serum ALT may increase in the presence of severe necrosis muscle. In dogs under corticosteroid and/or phenobarbital treatment increase levels of spherical ALT may be present but it is unclear whether the enzyme elevations represent a direct enzymatic induction or reflect a possible hepatotoxicity drug induced. The serum ALT it may also increase in case of primary or metastatic liver cancer. The serum aspartate aminotransferase (AST) is an enzyme mainly linked to the mitochondria, ubiquitous (myocardium, kidney, liver, muscles), so it can increase due to different pathologies not only in case of liver disease. It is more sensitive for ALT the identification of hepatobiliary diseases, although it is considerably less specific, since significant amounts of this enzyme they are also found in muscle. In general, the serum AST increments have a trend parallel to the elevations and, like these, they are associated with a spill enzyme resulting in an alteration of membrane permeability. In cases where the serum AST is much higher elevations, must be sought muscle origin. Alternatively, since the AST it is present within the cytosol and mitochondria, a high ratio ALT: AST may be indicative of severe acute damage irreversible. The reference values for the AST are 20/50 IU /l[8,40,41]. AST and ALT increase, mainly hepatocyte necrosis, but also for damage to the cellular function, although a lesser extent. AST and ALT are sensitive indicators, capable of identifying the presence of lesions liver even in symptomatic patients. Alkaline Phosphatase (ALP) has a low liver specificity, as it is associated with the presence of several isoenzymes and a particular sensitivity Pharmacological induction. It locates at the cell membrane in the hepatocytes. The ALP isoenzymes are present in the liver, kidney, intestine, bone

and placenta. So, although the rise of concentrations serum ALP is a sensitive indicator of hepatobiliary disease, its low specificity for the liver disease is confusing. The Alkaline Phosphatase can then increase in case of liver disease, drugs hepatotoxic and in any form of obstruction of the biliary tract. In dogs, the total value of serum ALP contributes two isoenzymes liver, one produced by the liver (L-ALP) and one induced by corticosteroids (C48 ALP)[40,41]. Total plasma proteins are a collection of different molecular species; proteins, glycoproteins and lipoproteins. They originate mainly from the liver and they have nutritional function, buffer, coagulation and fibrinolysis, transport and colloid-osmotic pressure. The plasma concentration may be altered for both alterations that affect and alter protein metabolism and for alterations blood volume. The absolute value does not reflect the quantitative changes in the individual unit. The increase in total protein can be by dehydration, hemoconcentration, venous stasis during withdrawal; to increase gamma-globulins in certain situations of liver cirrhosis, autoimmune diseases or in the presence of abnormal proteins (monoclonal gammopathies or polygonal). The decrease in total protein can be by overhydration (reduction proportional of all fractions), to decreased synthesis for insufficient intake food (malabsorption for chronic liver diseases), for protein loss kidney, intestine, hemorrhage, malignancy or excessive catabolism endogenous protein.[42-44]

AIM OF THE RESEARCH

The aim of our work was to evaluate any liver damage in a heterogeneous population treated with two types of PN formulations for PPN. The named by us: “Low/Medium” and "High" amino acids formulas have the purpose to cover the caloric patients’ needs for the most part with the amino acids. The objective of our study was therefore to provide preliminary data that could indicate or suggest a greater use of the amino acid portion on the PN formulas, focusing our interest on one of the complications that may occur during the administration of this type of AN: the short and long-term liver damage[44,47]. Since any report from the literature concerning the administration of different amount of protein during nutritional therapy, nor amino acids effect on liver damage has been published, our study aims to evaluate the percentage of amino acid in PN solutions related to liver damage markers. The liver damage was monitored during the PPN administration through the quantification of three liver parameters, total protein (TP), Alanine Aminotransferase (ALT) and Alkaline Phosphatase (ALT). For the subjects with hepatic values in the physiological range the purpose was preserve them, while for those with liver values altered the aim was get as close as possible to the physiological ones. The results obtained could evaluate the safety could document an eventually not negative effect of the high amino acid formula on the liver damage. Moreover, the

purpose of the work is faithful to the clinical principle by decreasing the lipid amount in favor of amino acids, a fatty liver disease from lipid overload is disfavored.

MATERIALS AND METHODS

PATIENTS

The study is based on a heterogeneous sample of 20 dogs, race, age, gender and disease, all of whom received PPN. All patients included in the study showed an inability to feed themselves or a gastrointestinal tract non-functionality. These characteristics have brought the dogs to present a state of undernourishment more or less marked, which is clinically manifested with a BCS less than 5 out of 9. Our subjects were all hospitalized for a time, by a minimum of 3 days, to a maximum of 32. During hospitalization, all subjects were treated with PPN via peripheral venous access for or a gastrointestinal tract functional disorders. All the patient showed a marked undernourishment, which was clinically manifested with a BCS less than 5 out of 9. During hospitalization, all subjects were treated with PPN via peripheral venous access for a variable time, from a minimum of 1 day to a maximum of 20. They were, however, subjected to different protocols regarding the percentage of amino acids administered with the PPN solution. The quote of amino acid ranged between 16-50%. For each dog enrolled in the study data related to signalament, clinical anamnesis, food intake, and the eventual therapeutic regimen were collected. General physical examination with particular attention to body weight, BCS and MCS presenting at the patient admission has been carried out. The nutritional status was considered good if the animal had less than three days of inadequate food intake and no signs of muscle loss. The nutritional status was considered discreet if the animal had more than three days of inadequate nutrient intake without muscle loss. The nutritional status was labeled poor if the animal had more than three days of inadequate nutrient intake with obvious generalized muscle mass loss[121]. In the clinical reports previous illnesses, biochemical exams before PN, estimated nutritional requirements, nutrient solutions used, changes in body weight during the administration of PN, complications encountered during the treatment, the results of cultures from the tip of the catheter, duration of PN and final conclusions were noted. The values of total protein (TP), alkaline phosphatase (ALP) and alanine aminotransferase (ALT) were obtained from blood biochemistry (Tab.4).

The experimental design is a cross-sectional, prospective screening and case-control study, based on the following classification of the patient: Medium / Low Protein Level group were the subject who

were classify as good or discreet nutritional status, while the High Protein Level group were the dogs with a poor nutritional status were the animal had more than three days of inadequate nutrient intake with obvious loss generalized muscle mass. The dogs were randomized into two groups: GROUP 1 (Medium / Low Protein Level group) composed by 11 subjects, receiving a normoproteic parenteral solution with a percentage of amino acids ≤ 25%, corresponding to an average of 1.9 to 2.2 g / kg / day of protein, GROUP 2 (High Protein Level group) consisting of 9 subjects, receiving a Hyperproteic parenteral solution with an amino acids amount > 25%, corresponding to an average of 3.6 to 6 g / kg / day of protein. The protein portion varies not only from group 1 to group 2, but also within the same group according to the subject, while respecting the maximum limit of the interval to which belonged the subject. The total energy of parenteral solution, in both groups is calculated for cover the RER of the subject, so the number of calories provided by each component must always be equal to the daily energy requirements.

DOG	***SEX***	***BW KG***	***AGE MONTH***	***BCS 3/9***	***MCS 3/5***	***PATHOLOGY***	***AA PN SOL. %***	***Hospitalization lenght of stay days***	***Lenght of PN days***
1	M	6	192	4	3	Gastroenteritis, Paraprostatic cysts, Adrenal, Testicular and Liver nodule	16	6	3
2	F	15	24	4	3	Iatrogenic chlythorax	18	5	5
3	FN	30	60	3	2	Volvulus of the small intestine	18	4	3
4	F	16	96	4	2	Nefrectomy and renal pelvic stones	18	5	1
5	M	17	156	3	3	Thorax foreign body	18	5	2
6	M	6,7	156	3	3	Pancreatitis and adrenalectomy	18	8	6
7	FN	5,6	144	3	3	Surgical treatment of gunshot woud	18	4	1

8	FN	40	42	4	3	Canine parvovirus	25	20	7
9	M	6,3	10	3	2	Spontaneus hepatic rupture	18	8	5
10	M	18	9	4	3	Splenectomy	33	8	3
11	M	17	132	4	3	Idiopathic Megaesophagus	33	4	2
12	M	32	60	2	3	Vascular ring anomaly, (retroesophageal left subclavian artery	33	4	3
13	M	7,2	4	4	3	Idiopathic Megaesophagus	23	7	6
14	M	14	6	3	4	Canine parvovirus	25	7	6
15	M	25	42	4	4	Enterectomy	50	9	5
16	M	6,3	96	2	2	Idiopathic Megaesophagus	50	32	20
17	F	4,6	2	3	3	Gastroenteritis	50	3	2
18	F	27	12	3	2	Gastroenteritis	50	4	3
19	F	3	4	4	3	Gastroenteritis	50	7	3
20	F	1	4	3	2	Canine parvovirus	50	4	4

Table 4. Patients considered in our study

PERIPHERAL VENOUS ACCESS PREPARATION

The site from venous access was prepared in accordance with the surgical rules of asepsis and then fixed with a bandage. The PN venous catheter was used exclusively for the administration of parenteral solution. For intravenous fluid therapy and medications, a second venous access was used, while the blood samples were carried out in a third vein.

PARENTERAL NUTRITION FORMULATION

Each solution consists of an amino acid 8.5% solution (III Fremine), a 5% glucose solution (Glucose 5%) and a 20% lipid solution (Intralipid), present in different concentration in a Three-in-one final solution. Freamine III amino acid contain 8.5% of L-essential free amino acids and non-essential.

It contains essential amino acids such as, L-Isoleucine, L-Leucine, L-Lysine Acetate, L-Methionine, L-Feninalanina, L-Treonina, L-tryptophan and L-Valine, the amino acids such as non-essential, L-Alanine, L-Arginine, L-Histidine, L-Proline, L-Serine, L-Cysteine HCL. It has an osmolarity of 860 mOsm /L and provides 0.34 kcal / ml.

Parenteral solutions within the laboratories of Department of Veterinary Sciences for Health, Animal Production and Food Safety through the use of a laminar flow hood (TopSafe 1.2), from the single components to the three-in-one solution finished product. At 5% glucose solution were added first the solution amino acids followed by lipids. The solution, once ready for use was then stored at 4 C for a up to 24 hours. Each solution was calculated for one-time infusion of 12h.

SCHEME FOR PER CALCULATION[123]

1) the patient's daily calorie needs calculation

RER = 70 x (BW kg) = kcal / day

PER= 70% RER

2) Calculation of the energy density of the solution

Calories Sol.AA =% RER = kcal AA

Sol.Glu Calories = kcal% RER = Glu

Calories Sol.Lip =% RER = kcal Lip

3) Calculation of the daily volume of each nutrient

AA kcal x 0.34 kcal / ml = ml AA

Glu kcal x 0.17 kcal / ml = ml Glu

Lip kcal x 2 kcal / ml = ml Lip

Tot Volume (ml): 24h = ml / h

4) Ensure the osmolarity

ml AA x mOsml / l AA

Glu ml x mOsml / l Glu

Lip ml x mOsml / l Lip

Osmolarity Tot mOsml / l

The osmolarity should be <650mOsml / PN in the central and

> 1400 mOsml / l in that device.

PATIENT MONITORING

Patients were monitored daily during the period of hospitalization. The daily evaluation included assessment of vital signs such status sensorium, hydration status, body temperature, heart and respiratory rate, mucous membrane color and capillary refill time (CRT), body weight, BCS and MCS in order to monitor their eventual change as an evaluation index of assisted nutrition. Increases in body weight in animals receiving an assisted nutrition intravenously for short periods, may actually reflect an increase of extracellular water within the tissues. Monitoring of the body weight is therefore a rudimentary method of measuring the effectiveness of same assisted nutrition. The laboratory tests were carried out according to the specific patient's needs. The blood was analyzed a minimum of three times during the study, according to the hospitalization length: 1) before starting the nutritional therapy, 2) during the therapy, and 3) at the end of the treatment. The first withdrawal allowed us to know the set (T1), then the influence of parenteral solution was detected with the second (T2) and the third (T3) blood tests. In our study, to perform liver response monitoring to PPN total protein (PT), alkaline phosphatase (ALP) and alanine Aminotransferase (ALT) were considered. Every 6/12 hours the urine glucose was performed using strip test dipped in a urine sample, collected for spontaneous urination in a disposable container for the urine.

MONITORING OF LIVER RESPONSE TO PPN

Low specificity of the serum albumin as evidence of state of nutrition and liver damage, wasn't examined in our study. The values we considered were the alanine aminotransferase (ALT), the alkaline phosphatase (ALP) and total protein (PT). Evaluation of serum concentrations of these hepatobiliary enzymes, is commonly used as a screening test to detect the presence of hepatobiliary disease. Although these enzymes are highly sensitive for the identification of hepatobiliary diseases, interpretation is hindered by their lack of specificity towards specific hepatobiliary disease.

MONITORING FOR COMPLICATIONS

Eventual complications evaluated were: mechanical, septic or metabolic. The mechanical complications included all the technical problems that interfere with administration assisted nutrition intravenously. Sepsis was defined by the presence of fever, depression, and leukocytosis with or without positive microbiological culture.

The assessment of the presence of clinical complications was made following the American and the European Society of Parenteral and Enteral Nutrion, respectively ASPEN and
ESPEN guidelines, where human patients are evaluated based on the presence or absence of:
phlebitis, thrombus embolism syndrome post-PN lung, sepsis, systemic inflammatory response syndrome (SIRS), post-PN diarrhea, shock.

STATISTICAL ANALYSIS

The collected data were analyzed by using SAS software. The distribution of the data allowed the use of different types of analysis:

1) univariate analysis of variance (ANOVA) for each of the variables (“Gaussian" analysis) on the differences of values "post" and "pre" [SAS STAT, PROC GLM] {Pre, post}; the analysis comprises a single variable. The GLM studies the variance of a single variable and consider the measures of all subjects at various times without the repeated measurements, giving a variable response with normal distribution (Gaussian).

2) Analysis of variance univariate repeated measures (Repeated measures ANOVA) for each of the variables (analysis: "Gaussian") [SAS STAT, PROC MIXED] {pre, post}; It includes a single variable in the study. The MIXED considers a single variable in the study and includes the measures repeated, giving a response variable with normal distribution (Gaussian).

3) Analysis of variance univariate repeated measures (Repeated measures ANOVA) for each of the transformed variables in score, placing score 0 = no physiological value, score 1 = physiological value) (analysis "Binary") [SAS STAT, PROC GLIMMIX] {pre, post}; Consider the study a single variable. The GLIMMIX Procedures studying the variance of a single variable, including repeated measures of the subjects. The answer is a variable that has not normal distribution, so do not Gaussian but Binary.

4) Analysis of variance multivariate repeated measures (Repeated measures MANOVA) on all variables simultaneously "as is" (Analysis "Gaussian") [SAS STAT, PROC MIXED] {pre, during, post}; It includes then more variables.

The MIXED in Repeat Measures MANOVA considers more variables and includes in the study repeated measures, giving a variable response with normal distribution (Gaussian). Independent variables considered were: confounding variables: age and weight (continuous), BCS and MCS (Categorical); Variables of interest: % protein (categorical = "Cut", levels = [1,2]), time (Categorical = "Time", levels = [1 (2) 3]) Variable identifying the person: Dog (Categorical = "Dog"). The data were statistically significant with p-value< 0,05.

RESULTS

In this study 20 heterogeneous dogs for mixed race, gender, age and diseases, all in a state of malnutrition were treated with PPN. During the nutritional therapy, there weren’t any septic or metabolic complication PN-related. The only mechanical complication PN-related were little clot in the venous-catheter treat with NaCl 0,9% flushing within the venous line. The bacterial cultures

carried out in this study are not quantitative but qualitative. Only one animal, without clinical signs of sepsis, was found to be positive for bacterial growth (staphylococcus epidermis) suggesting bacterial colonization. In all other cases, the test was negative, thus excluding bacterial contamination of the catheter or the venous line. The results reported in the following tables were obtained by blood serum analysis.

	Pre	During	Post	% AA PN
Dog 1	5	5,3	4,8	< 25%
Dog 2	4,7	5	5,3	< 25%
Dog 3	2,7	3,5	5,2	< 25%
Dog 4	4,4	4,8	5	< 25%
Dog 5	5,1	5,4	5,5	< 25%
Dog 6	5	5,6	6	< 25%
Dog 7	5,2	4,8	5,4	< 25%
Dog 8	5	4,7	5,9	< 25%
Dog 9	5,4	4,6	6,4	< 25%
Dog 10	5,1	5	5,2	< 25%
Dog 11	6	4,3	6,26	> 25%
Dog 12	6	6,1	6,3	> 25%
Dog 13	3,6	4	5,5	< 25%
Dog 14	4,4	5,3	5,6	> 25%
Dog 15	5,3	5,4	5,6	> 25%
Dog 16	5,9	4,2	6	> 25%
Dog 17	4,8	4,3	4,9	> 25%
Dog 18	4	4,3	5,8	> 25%
Dog 19	5	5,2	5,5	> 25%
Dog 20	5,3	5,4	6	> 25%

Table 5. Values of individual subjects of Total Protein in phases Pre, During and After (g/dl)

The total protein values (Tab.5) were all lower than the maximum value of 7.5 g / dl. In 6 cases (Dog 1,2,3,4,10,17) the final values, however, were lower than the minimum value of 5.4 g / dl. In these dogs, 5 had an increasing in PT values From PRE-to POST without reaching the physiological value. Among these, only one (Dog 17) had amino acids amount of > 25% in PN solution.

	Pre	During	Post	% AA PN
Dog 1	5	5,3	4,8	< 25%
Dog 2	4,7	5	5,3	< 25%
Dog 3	2,7	3,5	5,2	< 25%
Dog 4	4,4	4,8	5	< 25%
Dog 10	5,1	5	5,2	< 25%
Dog 17	4,8	4,3	4,9	> 25%

Table 6. Cases in which the final value of the Total Protein was less than the minimum value of 5.4 g / dl

	Pre	During	Post	% AA PN
Dog 1	210	100	64	< 25%
Dog 2	54	50	51	< 25%
Dog 3	70	52	55	< 25%
Dog 4	906	310	237	< 25%
Dog 5	84	63	49	< 25%
Dog 6	114	93	58	< 25%
Dog 7	66	51	51	< 25%
Dog 8	56	29	23	< 25%
Dog 9	62	64	30	< 25%
Dog 10	201	275	861	< 25%
Dog 11	233	70	28	> 25%
Dog 12	103	70	43	> 25%
Dog 13	111	84	33	< 25%
Dog 14	28	30	28	> 25%
Dog 15	27	39	27	> 25%
Dog 16	120	60	37	> 25%
Dog 17	21	20	24	> 25%
Dog 18	44	43	58	> 25%
Dog 19	23	30	20	> 25%
Dog 20	100	70	56	> 25%

Table 7. ALT of individuals over time Pre, During and After, cut off for normal values is ALT<60 U/L.

ALT values fall at the end of the therapy within the maximum range, all subjects had lower value than 60 U / L except for the Dogs 1, 4 and 10. Dog 1 and 4 started from very high initial values during the therapy declined while failing to re-enter the physiological maximum value. Dog 1 received a 16% amino acids solution, Dog 4 an 18% one. Dog 10 ALT value increased, receiving a n 18% AA

solution. ALT levels greater than the maximum limits were obtained in only three subjects belonging to the group with parenteral nutrition < 25% (Tab.7).

In all subjects in the group with amino acid solution > 25% ALT levels at the end of the treatment was <60 U / L.

	Pre	During	Post	% AA PN
Dog 1	210	100	64	< 25%
Dog 4	906	310	237	< 25%
Dog 10	201	275	861	< 25%

Table 8. Dogs with ALT values greater than the maximum parameters

	Pre	During	Post	% AA PN
Dog 1	5845	5659	5359	< 25%
Dog 2	180	185	170	< 25%
Dog 3	90	100	95	< 25%
Dog 4	145	150	187	< 25%
Dog 5	112	140	160	< 25%
Dog 6	1755	1703	1643	< 25%
Dog 7	153	167	144	< 25%
Dog 8	95	2633	686	< 25%
Dog 9	357	312	250	< 25%
Dog 10	4699	10360	14840	< 25%
Dog 11	408	425	364	> 25%
Dog 12	116	121	93	> 25%
Dog 13	53	60	70	< 25%
Dog 14	221	212	185	> 25%
Dog 15	106	116	130	> 25%
Dog 16	115	114	120	> 25%
Dog 17	342	290	221	> 25%
Dog 18	95	97	93	> 25%
Dog 19	85	93	86	> 25%
Dog 20	88	77	80	> 25%

Table 9. ALP values for all subjects at the time Pre, During and Post, cut off for normal values is ALP <130 U/L.

ALP value falling within the physiological range in 8 cases (Dog 1,2,6,7,9,11,14,17), while in 11 patients (Dog 1,2,6,7,9,11,12,14,17,18,20) the value was diminished. In 6 animals (Dog 1,2,6,7,9,14) received a Medium / Low Protein Level Solution, while 5 (Dog 11,12,17,18,20) received a High

Protein Level Solution. Only 3 presented in the POST phase a value within the maximum limit accepted, all however already they showed a physiological value in the PRE-phase. In 2 they had High Protein Level group solution and only one the Medium / Low Protein Level.

	Pre	During	Post	% AA PN
Dog 1	5845	5659	5349	< 25%
Dog 2	180	185	170	< 25%
Dog 6	1755	1703	1643	< 25%
Dog 7	153	167	144	< 25%
Dog 9	357	312	250	< 25%
Dog 11	408	425	364	> 25%
Dog 12	116	121	93	> 25%
Dog 14	221	212	185	< 25%
Dog 17	342	290	221	> 25%
Dog 18	95	97	93	> 25%
Dog 20	88	77	80	> 25%

Table 10. Cases in which ALP is decreased

	Pre	During	Post	% AA PN
Dog 1	5845	5659	5359	< 25%
Dog 2	180	185	170	< 25%
Dog 6	1755	1703	1643	< 25%
Dog 7	153	167	144	< 25%
Dog 9	357	312	250	< 25%
Dog 11	408	425	364	> 25%
Dog 14	221	212	185	> 25%
Dog 17	342	290	221	> 25%

Table 11. Dogs in which the ALP is decreased while remaining beyond the threshold value from the PRE-to END.

In 9 cases (Dog 3,4,5,8,10,13,15,16,19) the final value exceeds the initials. 6 belong from the Medium / Low Protein Level Solution group (Dog 3,4,5,8,1013), while 3 (Dog 15,16,19) from the High Protein Level Solution group. 5 subjects (Dog 3,13,15,16,19) remain inside the physiological range.

	Pre	During	Post	% AA PN
Dog 3	90	100	95	< 25%
Dog 4	145	150	187	< 25%
Dog 5	112	140	160	< 25%
Dog 8	95	2633	686	< 25%
Dog 10	4699	10360	14840	< 25%
Dog 13	53	60	70	< 25%
Dog 15	106	116	130	> 25%
Dog 16	115	114	120	> 25%
Dog 19	85	93	86	> 25%

Table 12. Dogs in which the ALP end values increased

Only in 8 cases (Dog 3,12,13,15,16,18,19,20) the ALP values are always within the maximum level of 130 U / L for all three phases of monitoring. Among these in 5 (15,16,18,19,20) had a protein content> 25% and only 3 (Dog 3,12,13) <25%.

	Pre	During	Post	% AA PN
Dog 3	90	100	95	< 25%
Dog 12	116	121	93	> 25%
Dog 13	53	60	70	< 25%
Dog 15	106	116	130	< 25%
Dog 16	115	114	120	> 25%
Dog 18	95	97	93	> 25%
Dog 19	85	93	86	> 25%
Dog 20	88	77	80	> 25%

Table 13. Dogs in which the ALP has always remained <130

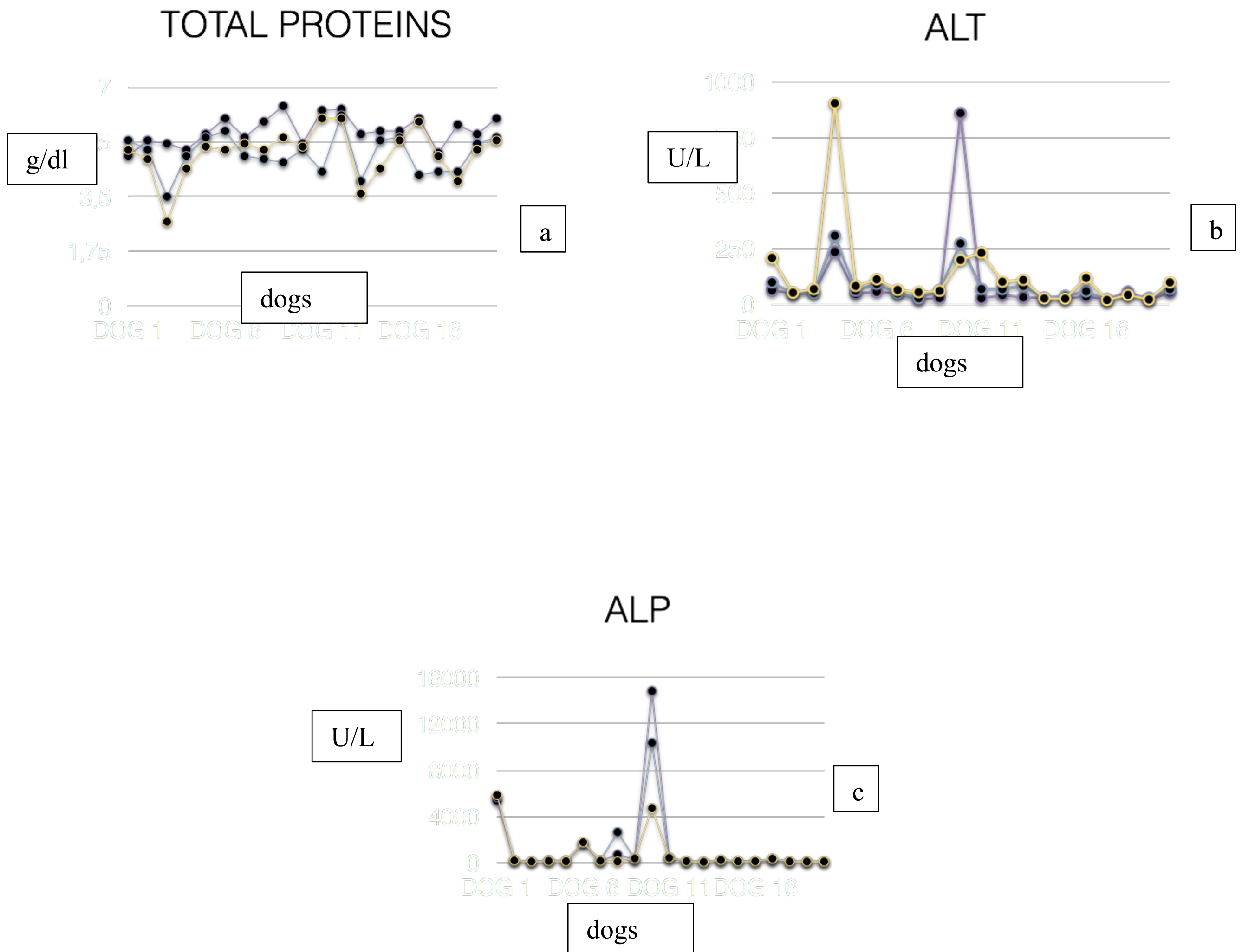

Chart 1. Graphical data of the Total proteins (a), ALT (b) and ALP (c) in each dog (full dot) from the patient number 1 to the patient number 20 in the different phases of the PPN: pre-PPN administration (yellow line), during PPN (blue line) and post-PPN (violet line).

STATISTICAL ANALYSIS

cut	time	N Obs	Variable	Label	N	Mean	Std Dev	Minimum	Maximum
1	1	11	alt	alt	11	180.0909091	246.4903465	54.0000000	906.0000000
			alp	alp	11	1227.73	2073.29	53.0000000	5845.00
			prot	prot	11	4.7454545	0.9059400	2.7000000	6.0000000
	2	11	alt	alt	11	110.1818182	92.0106713	50.0000000	310.0000000
			alp	alp	11	1723.36	3313.82	60.0000000	10360.00
			prot	prot	11	4.9181818	0.7236272	3.5000000	6.1000000
	3	11	alt	alt	11	139.2727273	246.1808648	30.0000000	861.0000000
			alp	alp	11	2091.91	4513.34	70.0000000	14840.00
			prot	prot	11	5.5090909	0.5166325	4.8000000	6.4000000
2	1	9	alt	alt	9	72.4444444	69.8303500	21.0000000	233.0000000
			alp	alp	9	172.7777778	123.0241620	85.0000000	408.0000000
			prot	prot	9	5.0777778	0.6457124	4.0000000	6.0000000
	2	9	alt	alt	9	43.4444444	18.7890334	20.0000000	70.0000000
			alp	alp	9	450.7777778	826.4375018	77.0000000	2633.00
			prot	prot	9	4.7888889	0.5301991	4.2000000	5.4000000
	3	9	alt	alt	9	33.4444444	14.1607831	20.0000000	58.0000000
			alp	alp	9	218.3333333	197.1287650	80.0000000	686.0000000
			prot	prot	9	5.7222222	0.3833333	4.9000000	6.2000000

Table 14. Blood exams results expressed in mean, standard deviation, minimum and maximum values. Medium / Low Protein Level group (cut 1), High Protein Level group (cut 2); pre-PPN administration (time 1), during PPN (time 2), post-PPN (time 3); number of subjects/observation (N Obs). Variable/label: aminotransferase (alt), the alkaline phosphatase (alp) and total protein (prot).

All the statistical test differences (observed probability (p value) at the alpha level = 0.05) in the analysis objective. Other none statistical variables significance, such as age, weight, BCS and MCS were found.

DISCUSSION

The appropriate protein-calorie determination in the canine patient during parenteral nutrition is fundamental for their clinical management. This allows for early recovery minimizing protein breakdown, without overloading liver and kidneys. The knowledge of the PN impact and the role of the amino acid on liver function (based on liver damage) allows the clinician to understand how these compounds may be employed as nutritional support without damaging the organs as liver or kidney. Studies relating to the parenteral nutrition refer to the use of parenteral solution which satisfy the minimum requirement needed in a dog. In spite of some clinical study showing the effects of PN on liver function in human patient with liver disease are present in the literature[45], there are no studies evaluating the use a high-protein parenteral solution (> 25%) and its effect on liver function in clinical veterinary patients. Effect of over amount of selected PN solutes on dogs were performed by Zentek et al that evaluated the effects of two parenteral solutions on liver function, one was hyperlipidic while, the other, had a hyperglucidic content. Complications occurred in dogs were hypertriglyceridemia with fever and nausea for the hyperlipidic one, and hyperglycemia necessitating insulin therapy for the hyperglucidic solution[46]. Our study was directed to evaluate how different amounts of amino acid could interfere with liver metabolism and, on the other hand, could substitute lipids and/or carbohydrates as energy source during PN in undernourished dogs. Starting from these premises and facing the scarcity of references in literature we clinical and hematological values. The study conducted did not bear statistically significant differences in liver function tests: ALT, ALP and PT between the two groups of animals that were administered using the two parenteral solutions denominated "Low-Medium" and "High" amino acids percentage. We were able to vary the amount of protein within a range so wide because no indication in literature was present about the PRC in a starving dog in the course of nutritional therapy, including PN. Lacking any kind of reference, we did change the RPC, starting from the values shown for a healthy dog, then we edited it basing on the protein daily requirement of the subjects, according to the metabolic and clinical response of the patient as a function of the results obtained up to that moment. We increased the percentage of amino acid to verify the liver response with the increase of the protein and look for an eventual correlation between high amino acid concentration and liver function, and to evaluate better results than in the first obtained. In group 1 (cut 1 the proportion of the three components the solution is very similar, so the energy comes from the three PN components equally. In group 2 with high-protein solution on the other hand most of the energy is provided by the amino acid level. To understand correctly the data, before analyzing other parameters first we evaluated the evolution of ALT, ALP and total protein in all subjects, in times T1 (Pre), T2 (During) and T3 (Post), always in reference to the group they belong to, Cut (1 and 2) (Tab.14). Subsequently in the study other variables, such as age, weight,

BCS and MCS were evaluated. Total protein increases in all subjects, while remaining within the limit accepted. The parameter hasn't exceeded the maximum value, regardless the percentage of amino acid administered. Only in one case the value is decreased for a subject belongs to the group 1, while in 5 cases the value has remained below the minimum accepted limit. This occurred mainly in group 1; while only a subject belonging to the group 2 has a value less than 5.3 g / dl, which would seem to indicate that the high amino acidic solution gives a better response. The ALT in the high-protein solution induced an improvement of the enzyme concentrations within physiological range. Only three subjects, in the POST, presented greater values compare to the maximum limit accepted, and all belong to the group 1, while two subjects have a decreased parameter. Only in one case belonging to the group 1, the value in the POST phase was greater than the phase PRE; the dog however presented hepatic rupture, so its increase could also be due to the disease. The same subject has a very high ALP that, also in this case it has gone to increase from stage PRE to POST. It would appear that the high protein solution had a better impact on ALT, although both solutions showed an improvement with a lowering of all the parameters evaluated in 19 out of 20 dogs.
The comparison between the PRE and POST liver values in relation to the amino acid percentage in the PN solution, did not suggest any statistically significant difference. The results are always greater than the p-value ($\alpha = 0.05$) in GLM, which consider the variables without the repeated measures obtaining a linear response. Both in MIXED studies and in GLIMMIX which instead it takes into consideration also the repeated values had a p-value> 0,05. This indicates that no group prevails over the other. In the GLM study, in addition to the statistical significance, the average between the POST and PRE-difference of the ALT, ALP and PROT TOT values in the two groups was calculated. It is therefore possible to claim that the two solutions have given the same results by affecting liver in a nearly identical way and that the two solutions gave the same results, influencing the liver function in an almost identical manner. No differences were found in any statistical procedure applied to our study. While it is recognized that liver test abnormalities occur during TPN, the question is whether TPN is associated with chronic liver disease. Total parenteral nutrition is considered to be an absolute risk factor for the development of biliary sludge and gallstones, often associated with hepatic steatosis. The incidence of total parenteral nutrition-related hepatobiliary complications has been reported to be very high, ranging from 20 to 75% in humans. All these hepatobiliary complications are more likely to occur after longterm total parenteral nutrition. End-stage liver disease has been described in approximately 15–20% of patients receiving prolonged total parenteral nutrition. The hepatic diseases referenced by the different studies in medicine attribute the liver damage and an its altered functionality during long-term TPN on the presence of an excessive lipid concentration or an excessive lipid peroxidation in the PN formula. The references in the literature therefore focus on the

full evaluation of the formulations with a higher content of glucose or lipids[41,44,47]. Chandler et al. determined the effect of PPN on nitrogen balance as an indicator of the effect on protein sparing, serum folate concentrations and serum insulin-like growth factor-I (IGF-I) concentrations in fasting dogs[48]. Hata et al. studied the effects of an amino acid mixture used for TPN in pediatrics founding that their amino acid solution for pediatrics has no significant effect on LFTs in neonates, even when high doses of amino acids (3.5g/ kg per day) were infused[49]. Olan et al. described the use of ProcalAmine as a source of parenteral nutrition in hospitalized dogs and its possible complications connecting the same idea of our purpose to evaluate a amino acids dosage for PN needs lower than us. Few clinical studies have examined the effects of specific amino acid mixtures on TPN-associated cholestasis. In one such study, Vileisis et al. reported the effect in humans of the amino acid intake in a high amino acid group (3.6g/kg per day) and a low amino acid group (2.3 g/kg per day)[50]. Although the incidence of cholestatic jaundice was almost identical in the two groups, the onset of cholestasis from the beginning of TPN occurred sooner and the peak value of direct bilirubin was higher in the high amino acid group. In experimental animal studies, Merritt et al. observed a direct cholestatic effect of tryptophan in suckling rat pups, and Belli et al. showed decreased bile flow in rats with amino acid imbalance[51,52].

In all the studies mentioned above the final discussion is directed to the future use of the amino acids solution as the only energy and structural nutritional compound during PN. Through the results of these studies in was found that all the hepatic marker was improving the liver state of function and damage.

As already discussed, a generous protein intake is usually recommended for dogs with cachexia or poor nutritional status. The goal is to support anabolic processes to the greatest degree possible, preventing protein-calorie malnutrition. However, providing dietary protein at levels above the animal’s needs is expensive and provides little benefit. Protein requirements are dictated by requirements for essential amino acids that cannot be synthesized endogenously. Many studies in numerous species indicate that these requirements are increased during critical illness regardless of etiology[8].

There are several limitations in this study to be considered. One is the small number of dogs in the study, which may have prevented detection of significant effects by other variables. To our knowledge, this is the first study to indicate the probable clinical advantages of amino acidic nutrition in dogs with poor nutritional performance. This preliminary data could be a first step for a clinical research focused on liver activity in a much larger trial for the veterinary practice and for translational medicine with the association of the dog as a model for infant patients.

The choice of only three blood makers for hepatic damage was done following the dictates of the most common and easily performed indices of liver suffering. Moreover, it is known as ALP, ALT and total proteins can be early markers of liver damage (from a few days to 3 weeks we can highlight any damage to the liver). In particular, the choice fell on the use of ALT as marker parenchymal marker and ALP as a biliary damage signal[47].

CONCLUSION

This evaluates the effects of two parenteral solutions enriched with different amino acid concentrations, on hepatic function in the critical canine patients. There are no studies in the veterinary literature that deepen the theme of the amino acid quota, which studies its effects on liver function. Up to now, texts and literature have always recommended a low-medium amino acids solution. The use of a high amino acids solution would reduce the lipid and glucidic fractions, thus reducing the risk. With this part of my doctoral project, the metabolic effects of a high amino acids solution on liver function compared to a low-medium amino acids solution have been evaluated. The aim was to verify a correlation between increased amino acids content and an increase in secondary complications with repercussions such as liver damage. Although data came from a limited sample of subjects, the results obtained has to be considered interesting. We started by giving the first group a standard, low-medium amino acids parenteral solution, then passing on to a high amino acids solution, departing from what recommended in the literature. We have investigated the increase of protein alone at the expense of the other two fractions. The results obtained show that there is no statistically and clinically significant difference between the two protocols. It would therefore seem that the administration of a low-medium amino acids parenteral solution affects liver function in the same way as a high amino acids solution. It would be interesting to continue to deepen the amino acid profile of parenteral nutrition not only from the quantitative point of view as a whole but also from a qualitative point of view, considering the individual amino acids and their role within canine protein metabolism. In fact, in this study, the amount of Freamine III in the solution was increased, thus increasing the amount of all the amino acids administered[113]. On the other hand, acting on the qualitative point of view, we may only vary the amino acid concentrations of our interest. In this way, the overall amino acid fraction would not increase much but would only interfere with the metabolic pathways of our interest. For example, integration of the solution with BCAA has already been partially tested in some preliminary studies, especially on patients affected by liver pathologies in which contradictory results have so far been, but this would be a field to be investigated.[53]

It would be desirable to broaden the sample of homogeneous subjects in order to obtain statistically more robust data and to evaluate the evolution over time of multiple parameters not only of liver function but also of renal function. In order to evaluate the effect of the solution on the whole organism. It would be also interesting to study the impact of parenteral nutrition in a homogeneous group of subjects on the basis of primary pathology due to malnutrition, in order to study whether cases with the same pathology respond similarly to the same protocol or whether there are specific variables subjects that influence the treatment response. The aim would be to identify a universal parenteral nutrition protocol based on the pathology or, if this is not possible, to identify which subjective variables cause a different response between the cases.

4. Chapter: Use of 2D and 3D rabbit hepatocyte cultures to investigate Branched-Chain Amino Acid effects in liver

INTRODUCTION

Hepatocyte culture and isolation started during the end of 1960s[1], where the isolation of the hepatocyte was carry out by simple incubation of the whole liver with enzyme digestion using collagenase and hyaluronidase followed by mechanical treatment. Hepatocytes serve as an in-vitro model for toxicological experiments and pharmacological testing[2,3,4] or as the basis for regenerative liver medicine[5].

Moreover, the present and future prospectives for hepatocyte in vitro culture are the application in clinical cells transplantation[6,7] using decellularized matrices[10,11] or 3D-printed scaffolds recellularized with primary hepatocytes as completely bioengineered organs[12,13]. These therapeutic options are still experimental and in vitro liver grow is one of the main field in regenerative medicine. Liver transplantation seems to be only successful treatment for patient with end stage liver pathology. Global liver cirrhosis deaths increase from around 676,000 in 1980 to over 1 million in 2010, about 2% of the global total[11].

Treatment for end-stage organ failure is restricted by the critical shortage of donor organs with the organ waiting list currently at 123 000 requests, a number that far exceeds the supply of available organs and that continues to grow by 5% each year. This situation has been the major driving force behind the rise of whole-organ engineering that aims to build transplantable organ substitutes to address the void in organ replacement therapies.

One limiting aspect in wholeliver engineering is the lack of a reliable cell source for primary adult hepatocytes. Pluripotent stem cells have the potential to generate an abundant supply of functional hepatocytes for use in cell-based therapies[12].

The main problem of the organs transplantation is their primary source. For these purpose, a novel technique as split liver transplants, living-related partial donor procedures and the increasing use of "marginal" organs such as older donors, steatotic livers, non-heart-beating donors, donors with viral hepatitis, and donors with non-metastatic malignancy.

To date, the availability of the liver grafts is no sufficient to meet their increasing demand.

Some studies have find that the decellularization of the liver parenchima can produce a liver organoids implantable in the future[13].

The liver is maily composed by two type of epithelial cells called hepatocytes and colangiocytes. Both cells synthesize essential serum proteins, control metabolism, and detoxify a wide variety of endogenous and exogenous molecules[14] LIVER[15] (Fig.1).

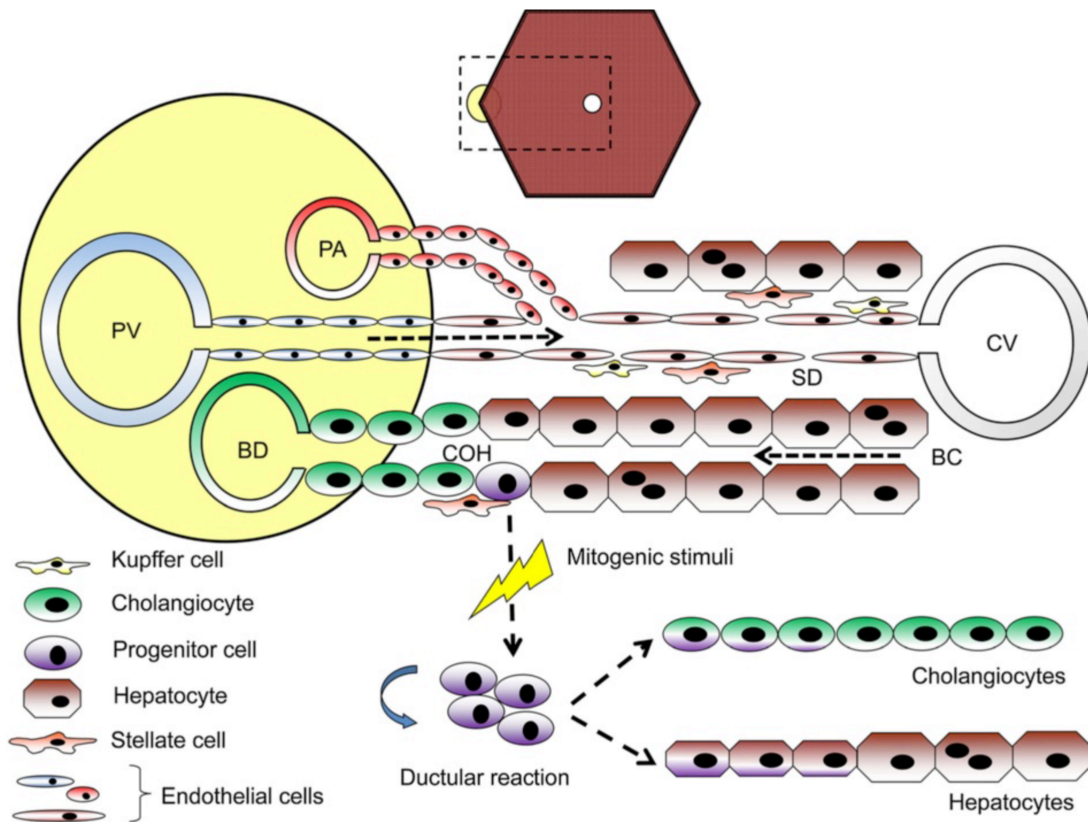

Figure.1 Liver lobular organization. Anatomical localization of the hepatic progenitors cells (HPC).

Despite their considerable replication capacity in vivo, hepatocytes present a limitation in some in vitro culture for their expansion resistence[16]. In recents study there are new culture strategy developed to maintain longer viability and vitality of this parenchimal cells. Culture system that allows the long-term expansion (>1 year) of single mouse adult liver[17], remaining committed to their tissue of origin. First of all, before introducing the in vitro studies to better understand the mechanism of liver rigeneration and approach the experimental design of my PhD thesis, I start from an anatomy and embriology background of the liver.

During the embryo phase, the first anlage of the bile ducts and the liver is the hepatic diverticulum or liver bud. The extrahepatic biliary tree develops through lengthening of the caudal part of the hepatic diverticulum. This structure is patent from the beginning and remains patent and in continuity with the developing liver at all stages. The hepatic duct develops from the cranial part of the hepatic diverticulum. The distal portions of the right and left hepatic ducts develop from the extrahepatic ducts. The proximal portions of the main hilar ducts derive from the first intrahepatic ductal plates. The extrahepatic bile ducts and the developing intrahepatic biliary tree maintain luminal continuity from the very start of organogenesis throughout further development, contradicting a previous study in the mouse suggesting that the extrahepatic bile duct system develops independently from the intrahepatic biliary tree and that the systems are

initially discontinuous but join up later. The normal development of intrahepatic bile ducts requires finely timed and precisely tuned epithelial–mesenchymal interactions, which proceed from the hilum of the liver toward its periphery along the branches of the developing portal vein[18]. The vascular architecture of the liver is established at the end of a complex embryological history, is in contact with two major venous systems of the fetal circulation: the vitelline veins and the umbilical veins. The portal vein is formed from several distinct segments of the vitelline veins; the portal sinus, deriving from the subhepatic intervitelline anastomosis, connects the umbilical vein, which is the predominant vessel of the fetal liver, to the portal system; the ductus venosus connects the portal sinus to the vena cava inferior. At birth, the umbilical vein and the ductus venosus collapse; the portal vein becomes the only afferent vein of the liver. Hepatic sinusoids progressively acquire their distinctive structural and functional characters, through a multistage process[19].

After the embriological developement, in the adult rabbit, we can find all the liver gland formed under different projection areas. The rabbit has 12 pairs of ribs, the last three are short and cartilage-free. The liver occupies the xifoid region, on a triangular area, whose summit is covered by the xifoids process of the sternum and whose base protrudes obliquely from the ventral end of the ninth (accasionally tenth) left to the seventh (rarely eighth) right coast. The right liver comes under the penultimate coast, rarely under the last, only with the process caudate. The right lobe has reduced projection area compare to the left side lobe that is projected to the dorsal end of the tenth coast. The medial lobe reaches the right lobe near the right costal arch. The gallbladder is located deep and not far from the medial plane, a level of the ventral end of the sixth space intercostal. Vascularization of the lumbar region:

The celiac trunk is born under the caudal end of the last thoracic vertebra. The mesenteric cranial artery begins a level of the intervertebral disc of the first two vertebrae lumbar.

The caudal mesenteric artery is much thinner than the mesenteric cranial artery and originates under the sixth vertebra lumbar.

The aorta ends at the level of the seventh lumbar vertebrae, bifurcating in the two iliac arteries common. The rabbit liver weighs in the 80 to 120g average. The liver margins give attack to the peritoneal coronary ligament and prolonged in the addominal cavity.

The liver is divided into a left lateral lobe, left medial lobe, right foot

The quadrate lobe is subdivided and it is narrow and not voluminous. The caudate lobe, also, is subdivided by the incision of the caudal vena cava in a caudate process (very developed) and in a papillary process[20].

During development or a pathological insult to the liver has a unique regenerative capacity of its kind. The repair-development process can be associated and equated although the literature does not differentiate clearly still embryonic cells from progenitor cells are still not well defined by modern.

LIVER REGENERATION

The wide array of functions performed by liver towards the rest of the body has been safeguarded by evolutionary events which imparted to liver a phenomenal capacity to regenerate. This process allows liver to recover lost mass without jeopardizing viability of the entire organism. The phenomenon of liver regeneration following loss of liver mass is seen in all vertebrate organisms, from humans to fish. It is also triggered when livers from small animals (e.g., dogs) are transplanted to large recipients of the same species. It has been recorded and mythologized in ancient times from the myth of Prometheus and libraries of clay tables picturing scarred livers of sacrificial animals, used to foretell the future in ancient Babylon and Rome[21].

Loss of liver mass can be induced by administering hepatotoxic chemicals (e.g., carbon tetrachloride) or by a surgical procedure which removes 2/3 of the liver mass in rodents (rats and mice), a technique known as 2/3 partial hepatectomy (PHx)[22].

These procedures are followed by an inflammatory response which removes tissue debris, followed by the regenerative response. A 2/3 PHx leaves a residual leading to 60% of hepatocytes and they undergo one mithosis in the average of 24h and 36h for rat and mouse respectively.

Only a small percentage of cells enter in G0, associated to a wave of apoptosis that seems to be the own liver control to avoid an over-shooting of the regenerative response[23]. The proliferation of hepatocytes advances from periportal to pericentral areas of the lobule, as a wave of mitoses[24] the last cells that undergo replication are the pericentral veins hepatocytes[25]. In term of proliferation time, fist cells are the hepatocyte on days 2-3 than the endothelial cells occur on 4-5 days after Phx. Stellate cells are cells of myofibroblastic origin, surrounding hepatocytes, located under the sinusoidal cells, producing extracellular matrix and several cytokines including HGF, and having a gene expression pattern substantially similar to the astrocytes of the brain[26]. The normal weight of he liver is reestablished within 5-15 days in human and animals. The entire liver mass is restored thanks to the manys mature adult hepatocytes instead of selective subpopulation of stem cells. To approach the entire signaling pathways triggered during liver regeneration strongly we have to think about those mechanism of wound healing, seen in other tissues. The main differencies are concernig the whole organ, thus the restore of a single lobe provoke changes in trophysm and metabolysm in the entire gland. Even though there is no damage to the residual tissue, there are big changes in hepatic blood flow patterns. The hemodynamic alterations after PHx induce the arterial component of the blood

supply per unit of liver tissue does not change after 2/3 PHx; the portal contribution per unit tissue, however, triples. Portal vein continues to carry the entire outflow from intestine, spleen and pancreas. The entire flow now needs to traverse through a capillary bed whose cross-section is mathematically down to 1/3 of the original. The hepatic capillaries have fenestrated endothelial cells which bring direct access of plasma through the endothelial cells to the hepatocytes. If these changes ar not present there is a deficiency on the activation of the HGF[27] and an alteration of the oxygen concentration. Portal vein carry much lower oxygen amount compare to hepatic artery. The relative increase in portal blood per unit liver tissue after PHx should result in decreased oxygen pressure in the circulating blood, perhaps triggering a hypoxic response. The tripling of the portal vein contribution should also cause a mathematical tripling in the availability per hepatocyte of growth factors and cytokines derived from intestine and pancreas. All aspects of liver regeneration, the importance of the hemodynamic events and the change of relative nutrients supplied as the correct level of the grow factors like epidermal growth factor (EGF) in in the portal blood.

One of the earliest observed biochemical changes is increase in activity of urokinase plasminogen activator (uPA) (Fig. 2).

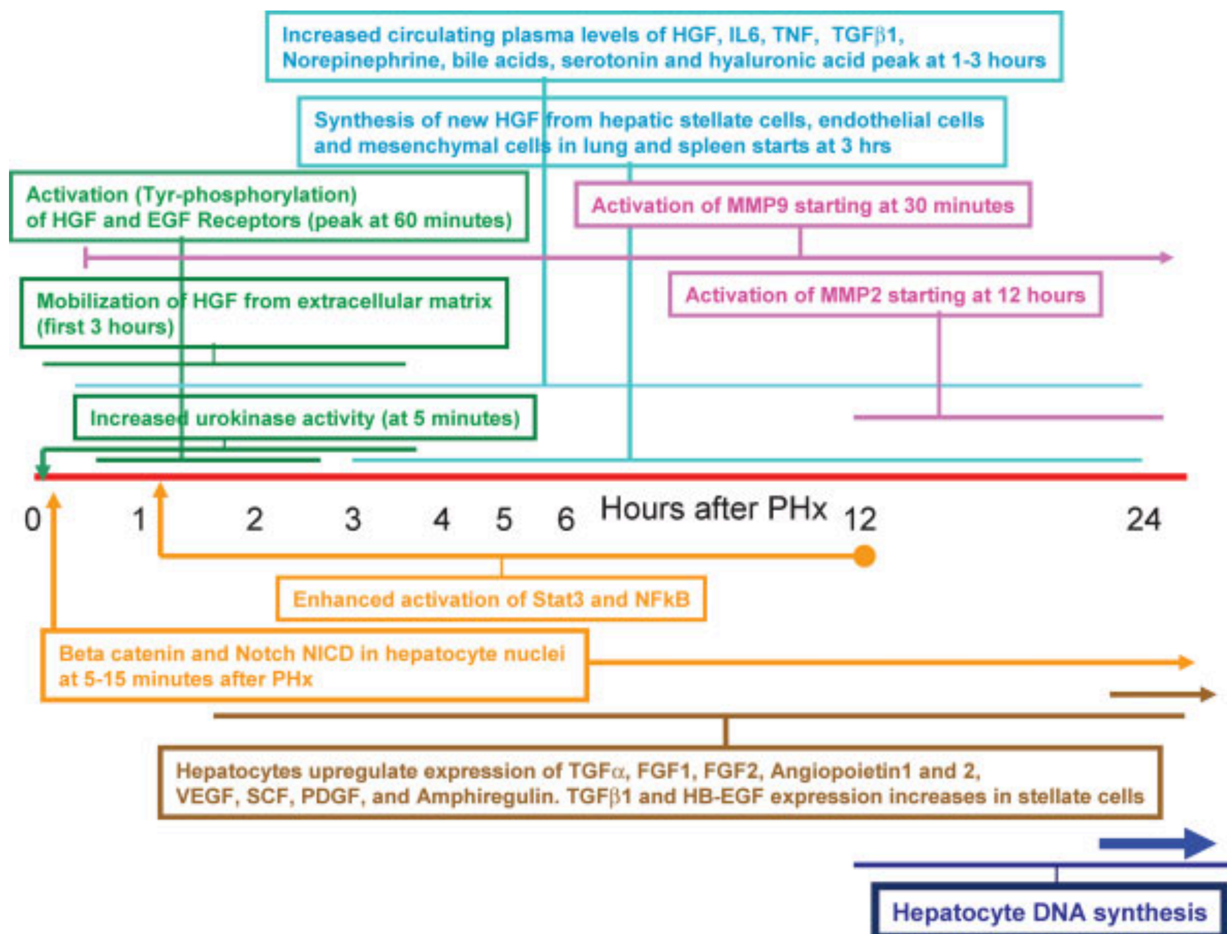

Figure. 2 Chronology during early stage of liver regeneration.

There is an increase in uPA activity throughout the entire liver starting as early as 5 min after PHx[29]. The relationship between increase in uPA and the hemodynamic changes seem to be an increase of uPA in several cell types including endothelial cells following mechanical stress associated with increased turbulent flow[30]. Thus, alterations of vascular flow patterns alone can trigger some of the early events. Increase in uPA activity is accompanied by activation of plasminogen to plasmin (within 10 min) and appearance of fibrinogen degradation products[31]. Urokinase is known to activate matrix remodeling, seen in most tissues during wound healing and also in liver regeneration. Many proteins of the extracellular matrix are subject to turnover. The activation of metalloproteinase 9 (MMP9) is seen at 30 minutes and further into the first 24-48 h afer PHx[32]. Wound healing and tumor biology have shown that matrix remodeling causes signaling though integrins and is associated with release of locally bound growth factors and peptides that have signaling capabilities. While there is not much proteinaceous matrix in the liver visible under the microscope, there is a great abundance of heavily glycosylated proteins in the pericellular space surrounding hepatocytes. Glycosaminoglycans are very abundant in liver and heparin, a shorter derivative, owes its name to liver (''hepar''). Several mechanisms are moving the repopulation and restituzion ad intergreum thanks to the extracellular matrix, where the metalloproteinase and their inibitors are comunicating[34] and binding many growth factors. The most rappresentative of the grow factos is the hepatic grow factors[35] (HGF).
Pre-existing stores of inactive and active HGF rapidly diminish with the first 3 h after PHX, as HGF rises in the plasma by 10- to 20-fold[36]. This HGF is bound to hepatic biomatrix, that relase activated uPA that is a highly homologous to HGF, HGF and plasminogen have the same consensus sequence (RVV) at their activation site, so HGF is available locally and in the circulation. Hepatocytes culture suggest that TNF may play a role in this process by inducing expression of MMP9 by hepatocytes[37]. Hyaluronic acid (a major component of hepatic biomatrix) and TGFß1 are release locally and in the sistemic circulation TGFß1is a known hepatocyte mito-inhibitor[38]. When the receptor TGFß1R is rendered inactive in normal, non-regenerating liver by injecting dominant negative DNA constructs, there is a noticeable increase in DNA synthesis of hepatocytes[39]. This suggests that TGFb1 exercises a competing tonic effect against opposing effects of matrix bound growth factors, keeping hepatocytes of the normal liver in a state of quiescence. The role of biomatrix on the cell expasion is achived by causing release (local and in the circulation) and activation of HGF while releasing TGFb1 massively in the circulation, where TGFb1 is bound and inactivated by alpha-2-macroglobulin[40]. Same procesed happen with the relase of EGFR, bounded by the EGF produced in the duodenum by exocrine glands, and cMet[41]. The similar time kinetics of activation of the HGF and EGF receptors

suggest that EGF, now targeting fewer hepatocytes, has an enhanced effect; or that the EGFR becomes more sensitive to EGF. Independent work suggests that the latter may be true. Norepinephrine, which also increases in the peripheral circulation, is known to enhance the effect of HGF and EGF receptors in hepatocyte cultures through the alpha-1 adrenergic receptor. Blockade of the alpha- 1 receptor suppresses liver regeneration[42]. Other studies have also shown that there is a cross-talk between Met and EGFR and it is possible that activation of Met enhances activation of EGFR[43]. The events occurring in the early period of 0–5 h after PHx have often been called ‘‘priming’’[44]. The term is a useful one, in that it denotes not only events associated for preparation for entry into the cell cycle, but also events and strategies of hepatocytes aimed at modifying patterns of gene expression so that they continue to deliver their homeostatic functions. The terminogy of ‘‘priming,’’ has also been used to denote a time in which events occurring are induced only by cytokines, with events induced by growth factors occurring after the cytokine-mediated events. The findings described above, however, clearly denote that there is no demarcation point that can be ascribed to separate events induced only by cytokines or only by growth factors. Quiescent hepatocytes in normal liver express a variety of growth factor receptors. These include receptors for PDGF, VEGF, fibroblast growth factor receptors, c-Kit. Primary culture however has shown that despite the expression of many mitogenic receptors, the only mitogens for hepatocytes in chemically defined serum-free media are HGF and ligands of the EGFR (EGF, TGFalpha, Amphiregulin, HB-EGF, etc). These ligands are direct mitogens, in that they induce a strong mitogenic response in hepatocytes in primary culture and clonal expansion of their population[47]. HGF, EGF, and TGFalpha also induce hepatocyte proliferation and liver enlargement when injected alone into intact normal mice and rats[48]. In addition to these proteins, however, there are other substances which, although not directly mitogenic to hepatocytes, enhance the effect of the direct mitogens. Despite to the entire signaling pathway originated throw the extracellular matrix and the different cells type, the consecutive mechanis to evaluate is base on the role of the adult hepatocyte in relation to the hepatic progenitor’s cells (HPCs). HPCs are present in adult liver, in small numbers in the Canal of Hering, the smallest ramifications of the intrahepatic biliary tree[49]. Extrahepatic peribiliary glands is described as the prime location for HPCs[50,51]. Some publications even speculate on a hematopoietic origin of HPCs, which is also highly debated. Different HPC-markers, such as keratin albumin, Alpha-Feto protein and HNF4α are collected in some studies[52]. Others marker as Keratin (K) 7 and K19, CD133 and EpCAM are shared with cholangiocytes, hematopoietic stem cells and embryonic stem cells. HPCs are epithelial cells that can display mesenchymal characteristics, depending on their activation status[52]. Recent studies have also focused on pro-regenerative effects of components of complement, bile acids, and serotonin, substances not known or tested to have direct or indirect

mitogenic effects. Some of the above signaling molecules were implicated based on decreased regeneration when their signaling is eliminated.

One of the molucules recently studied are the branched chain amino acids (BCAAs), leucine, isoleucine and valine (Fig.3). Recent studies have revealed the functions of these BCAAs, and they have been administered for the treatment of advanced liver diseases. In mice, BCAA-rich diets have shown to up-regulate the expression of peroxisome proliferator-activated receptor (PPAR) γ coactivator-1α (PGC-1α), a master regulator of mitochondrial biogenesis and the defense system against reactive oxygen species (ROS), and of sirtuin-1, a member of the sirtuin family linked to life span extension, enhanced mitochondrial biogenesis, and decreased ROS production, leading to the prolongation of the lifespan of male mice[54]. BCAAs have also been shown to induce the activation of genes involved in antioxidant defenses and inhibition of ROS production, as well as to induce the hepatic expression of mRNA encoding 8-oxyoguanine DNA glycosilase 1, an enzyme involved in repair of oxidative DNA damage, in a rat model of liver injury, indicating that BCAAs are involved in the induction of antioxidant DNA repair[55]. In various cell lines, BCAAs, especially leucine, have been shown to activate the mammalian target of rapamycin (mTOR) signals, stimulating the synthesis of proteins, including albumin[56]. BCAA activation of mTORC1 has also been associated with cell growth[57] and PGC-1α- mediated mitochondrial gene expression[57]. BCAAs have been shown to up-regulate PPAR-γ and uncouple (UCP) 2, reducing triglyceride concentrations in mouse livers[58]. These findings suggest that BCAAs may have a therapeutic effect on metabolic disorders and/or obesity. Apoptosis and regeneration of hepatocytes BCAA supplementation was shown to delay the progression of CCl4-induced chronic liver injury in a rat model by reducing hepatic apoptosis[59].

On the other hand, BCAAs promoted hepatocyte regeneration in a rat model of hepatectomy[60]. Moreover, BCAAs were reported to stimulate the production of hepatocyte growth factor[61]. Taken together, these findings indicate that supplementation with BCAAs, by reducing hepatocyte apoptosis and promoting liver regeneration, may result in rapid recovery from liver injury. BCAAs activate mTOR and subsequently increase the production of eukaryotic initiation factor 4E-binding protein-1 and ribosomal protein S6 kinase, which upregulate the synthesis of albumin[62,63]. Furthermore, leucine stimulates the nuclear importation of polypyrimidinetract polypyrimidinetract- binding protein, which binds to albumin mRNA and increases its translation[64].

BCAAs were shown to improve homeostasis model assessment scores for insulin resistance (HOMA-IR) and beta cell function (HOMA-%B) in patients with chronic liver disease, indicating that BCAAs can ameliorate insulin resistance. In mice lacking the gene encoding mitochondrial BCAA aminotransferase, an enzyme that catalyzes BCAAs, serum BCAA concentrations were elevated. In those mice, fasting blood glucose and insulin concentrations were decreased and HOMA-

IR was significantly lower than in wild-type mice[65]. Furthermore, administration of leucine or isoleucine improved insulin sensitivity in mice with high-fat diets[66]. BCAAs were also shown to temporarily increase plasma insulin concentrations in healthy young men, although plasma glucose concentrations were not altered[67].

The BCAAs play an important role also on the cancer biology, some culture system analyzed their effects on the hepatic cellular carcinoma (HCC) lines increasing their concentration in culture medium. BCAAs were found to accelerate insulin-induced vascular endothelial growth factor (VEGF) mRNA degradation at the post transcriptional level, downregulating VEGF expression during the development of HCCs[68]. BCAAs were also shown to induce apoptosis of liver cancer cell lines by inhibiting insulin-induced PI3K/Akt and NFκB pathways through mTORC1- and mTORC2-dependent mechanisms[69]. Moreover, BCAAs may inhibit obesity-related hepatocarcinogenesis by suppressing the stimulatory effect of visfatin, an adipokine with a critical role in HCC proliferation[70-72]. Insulin was found to induce cell proliferation through activation of the mitogen-activated protein kinase pathway[73], and BCAAs inhibit insulin signals by suppressing the expression of insulin-like growth factor[74].

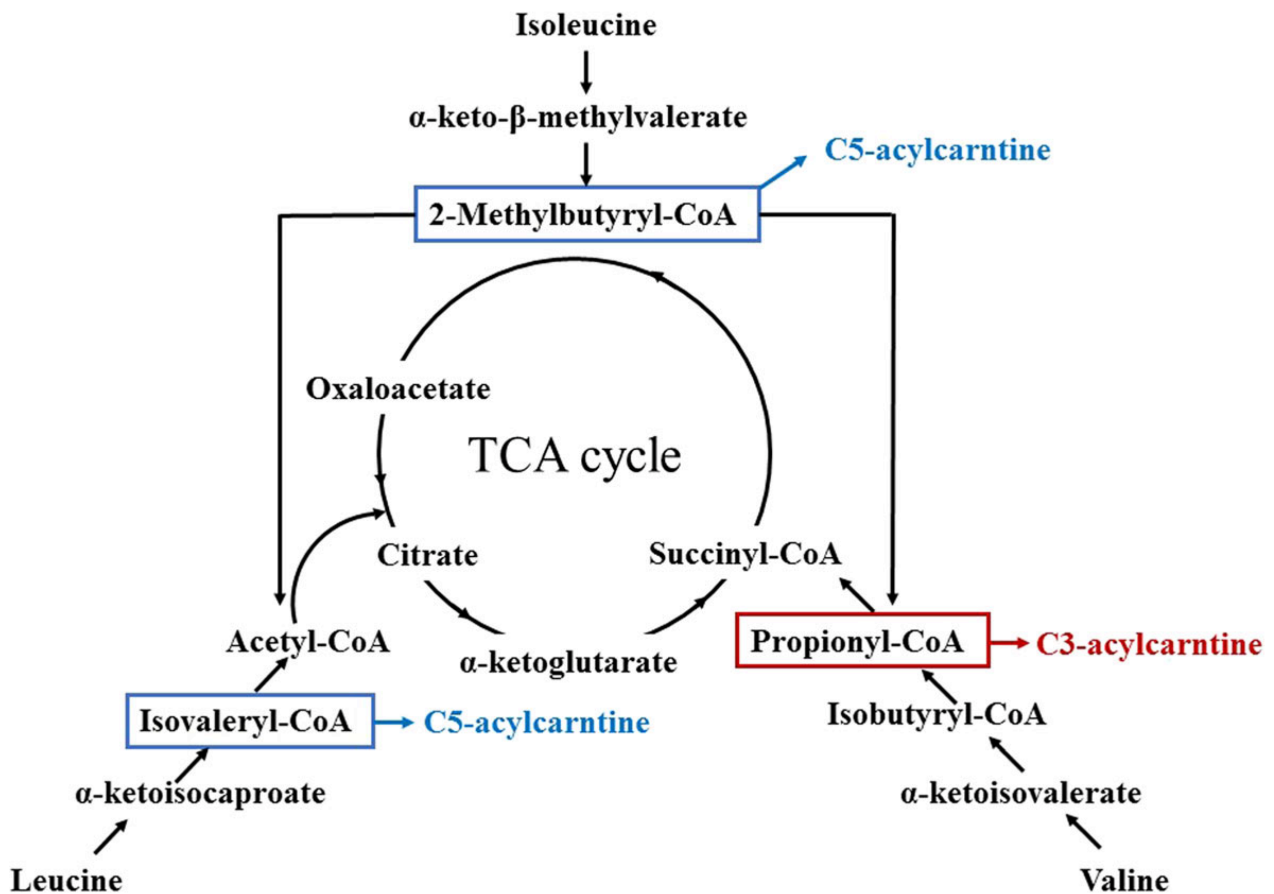

Fig.3

Pathway of branched chain amino acid catabolism. BCAA are catabolized to acetyl-CoA and/or succinate-CoA and subsequently enter the TCA cycle. The main steps of the catabolic reactions (transamination by BCAT and decarboxylation by BCKD) are shown. With the help of BCAT, BCAA are catabolized into branched-chain α-ketoacids which are subsequently decarboxylated by BCKD. Finally, all the BCAA metabolites are catabolized by a series of enzyme reactions to final products and enter the TCA cycle[74].

AIM OF THE RESEARCH

Aim of the present study was to determine how BCAAs preserve liver functions and cells proliferation in a novel 3D model, using a decellularized liver, as a most realible and faithful reproduction of the natural mechanisms in the liver compare with the standard 2D monolayer cell culture. Futhermore, in order to address wich cell type proliferation is present in relation of the BCAAs concentration we want to detect the mRNA expression in the liver organoid during three-times culture on the 7,14, 21 days. All the data and results collected wants to validate the hypothesis of positive BCAAs effects on normal or pathologic liver described in the recent in vitro studies. The main scope is to sustain the proliferative BCAAs effect on the HPCs like it happen in the liver bug during the embriogenesis[75], explaining and confirming the three amino acids active role on the hepatic regeneration also in the adult liver.

MATERIAL AND METHODS

ANIMALS AND LIVER HARVESTED

Two adult female New England White Rabbit 3 kg not eviscerated carcasses from a local slaughterhouse were collected, carrying it with a certified ice container. Before the incision, the abdominal wall was treated with topical chlorhexidine skindisinfectant with alternating chlorhexidine soaked gauze and dry gauze. Ventral mild line laparotomy extending from the pubis to the xyphoid combinewith a roof top incision using the #3 handle with #15 blade wasb perrformed.

We Dissected the falciform ligament with Metzenbaum scissor. A wet gauze bandage to hold the medial and left liver lobes cranially under the dome of the diaphragm was used. The upper omental liver lobe was mobilized than the stomach to the left was move. The stomach with a clamp was fixed and the minor omentum with Metzenbaum scissor and DeBakey forceps was dissect. The bile duct was detatched from the adipose and pancreatic tissue. The bile duct was cut 1.5 cm from the bifurcation. The portal vein tissue and the common hepatic artery were dissected from the surrounding tissue. The hepatic artery was tied up twice with silk 3-0. The inferior vena cava between the right kidney and the right lateral liver lobe were circumscribed. The vein was dissected from the retroperitoneal space and we injected 1,000 I.E. heparin in 1 ml 0,9 % saline solution into the inferior vena cava. We retracted the cannula and close the incision with a cotton pad than stoped for 1-2 min for the effect of heparin. Then we cannulated the portal vein with a Sheath introducer 8,5 Fr and a vein pick catheter introducer cutting the vein near the knots with a Potts scissor. After fixed the decellularization cannula with 3-0 silk sutures, we Injected a total of 2 mL heparin sodium (100 U/mL) through the vein to prevent coagulation. Then, infrahepatic inferior vena cava was transected to allow the outflow of the perfusate. Perfuse a total of 50 mL phosphate-buffered saline (PBS) slowly through the PV clear blood from the liver. The whole liver was isolate and transfer to the decellularization circuit (Fig.4). To collect the liver for the hepatocytes isolation protocol we cut the organ with Mayo scissor at the level of the grove liver for vena cava and its peritoneal ligaments. At the end, we transfer the whole liver to a 100 mm Petri dish.

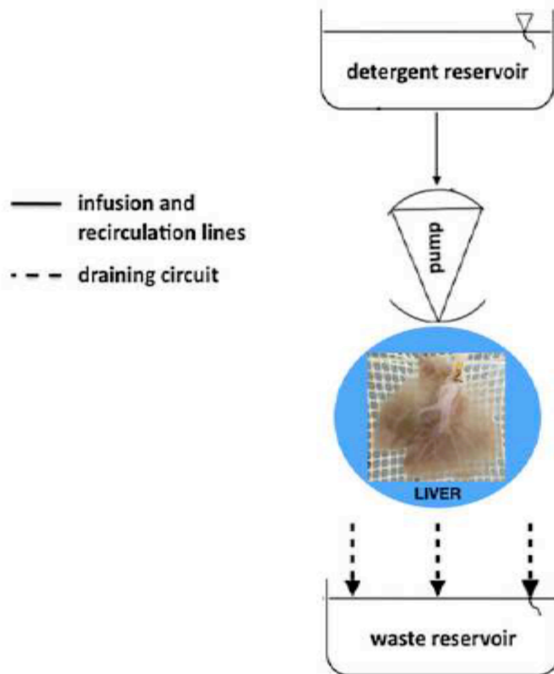

Fig.4

Schematics of the dynamic decellularization system. Lines represent the hydraulic circuit, which includes infusion and recirculation subsections (solid lines) driven by a peristaltic pump and draining subsection (dashed lines) driven by atmospheric pressure.

LIVER DECELLULARIZATION

For 1% Triton X-100 we added 10 ml to a 1.000 ml bottle, so 990 ml deionized water and we shake the bottle twice then we filtered the 1% Triton X-100 solution through a sterile filter. the portal vein was pre-inject through 400 ml NaCl 0,9% a 180 nd 800 UI heparin (see Fig. 1). The liver was placed in the high-density polyethylene basin and attached to the pump (ISMATEC 405 MCP-Z Standard). To performe the decellularization steps we made as followed: deionized (DI) water for 24 hours with a flow rate of 7 ml/min; Triton X 1% + Ammonium hydroxide (NH_4OH) 0,1% for 48 hours a flow rate of 10 ml/min; DI for 24 hours 10 ml/min; Triton X 1% + Ammonium hydroxide (NH_4OH) 0,1% for 24 hours; DI for 24 hours 10 ml/min. We disinfected the scaffold with peracetic acid for 30 minutes at 12 ml/min, then we washed the scaffold with sterile NaCl 0,9% for 2 h. Final procedure was a light source to trans illuminate the decellularized scaffold and check the macroscopic appearance of the parenchyma, its homogeneity and preservation of the vascular tree. (Fig.5).

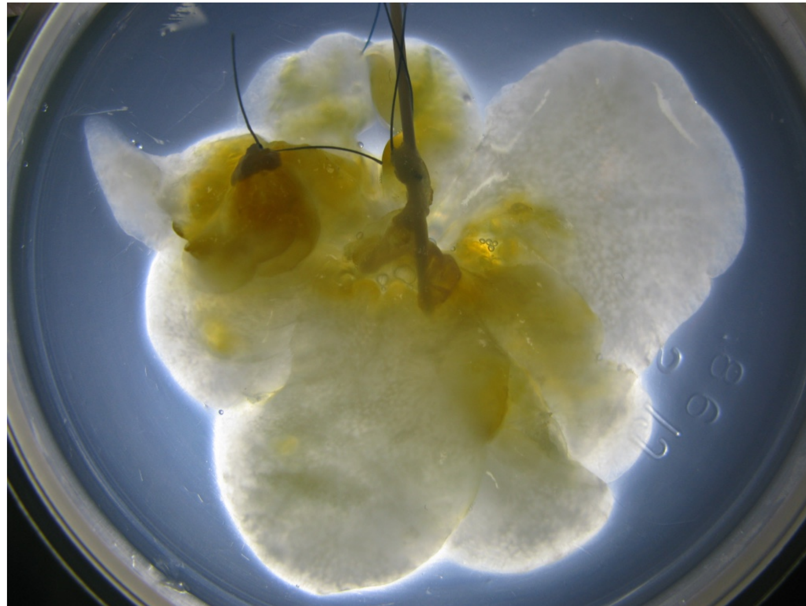

Fig.5

Trans illumination of the a decellularized left liver lobe. This technique shows the translucent color homogeneity after a complete cell removal from the liver parenchyma.

The Scaffold was trated with Peracetic acid for 30 minutes at 12 ml/min; than the scaffold was washed with sterile NaCl 0,9% for 2 h. The scaffold was cut in 30 pieces: 27 for BCAA experiment, 1 for histology and 1 for DNA contenet assay and 1 as control without cells store in the BCAA-free medium (Fig.6) (Tab.1). To detected the cells in the ECM the DAPI (4’,6’-diamidino-2-phenylindole) stain for fluorescence microscopy and Trypan blu were achieved. The decellularized liver scaffold was preserved in PBS at 4°C before the seeding.

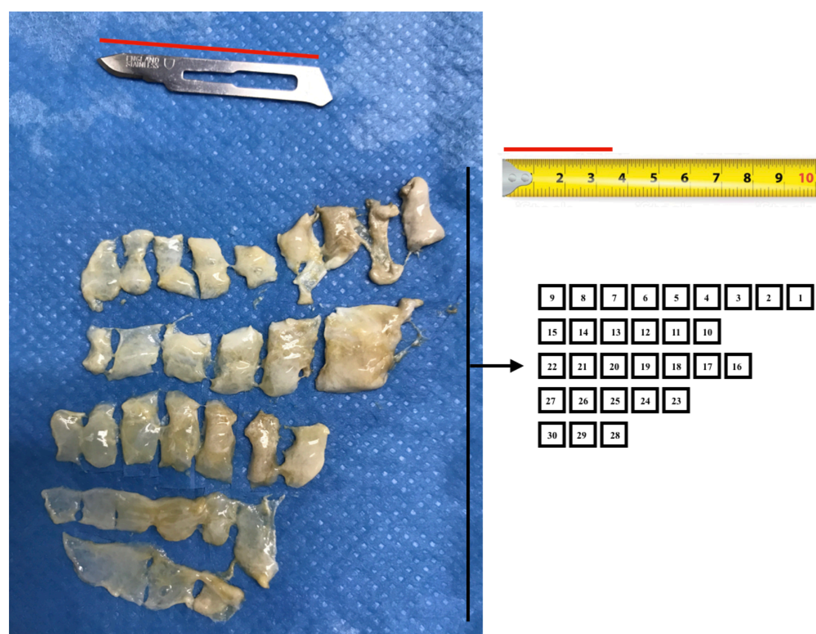

Fig.6 Left liver lobe sectioned il 30 ECM scaffolds. Each decellularized organoid has a ID number (see tab.1) for their traceability during and after the experiment.

ID NUMBER	Treatement	SCAFFOLD Area (mm2)
1	100 mM DAY 21	52
2	200 mM DAY 21	55
3	100 mM DAY 14	54
4	PCR_ 7 CTR	74
5	CONTROL DAY 7	44
6	PCR_7/ 100	83
7	200 mM DAY 7	51
8	100 mM DAY 7	66
9	100 mM DAY 7	53
10	CONTROL DAY 21	239
11	200 mM DAY 7	112
12	200 mM DAY 21	96
13	PCR_14/200	67
14	100 mM DAY 14	58
15	CONTROL DAY 7	43
16	CONTROL DAY 21	74
17	PCR_14/100	71
18	CONTROL DAY 14	73
19	CONTROL DAY 14	80
20	PCR_14 CTR	69
21	PCR_21/200	61
22	PCR_21/200	49
23	PCR_21 CTR	103
24	200 mM DAY 14	55
25	100 mM DAY 21	138
26	200 mM DAY 14	41
27	PCR_7/200	39

Table 1

The ID number for each scaffold correspond to a treatement with BCAAs or to a PCR sample. The Scaffolds area was espressed in mm^2

CELLS ISOLATION AND CELLS CULTURE

2D CULTURE

The rabbit liver cell lines were cultured in Dulbecco's modified Eagle's medium/ F12 (DMEM) (Life Technologies, Waltham, Massachusetts, USA) supplemented with 20% foetal bovine serum (FBS) (Life Technologies, Waltham, Massachusetts, USA), 2 ml Antibiotic Antimycotic Solution (100×) (Sigma-Aldrich, St. Louis, Missouri, USA), 1 ml L-Glutamine solution (Sigma-Aldrich, St. Louis, Missouri United States), 20 µl HGF (R&D systems Minneapolis, Minnesota, USA)[76].

The cells were plated in 4 well culture dishes, daily monitored and the medium was changed every two days. Upon reaching the confluence after 15 days, cells were trypsinized, centrifuged and resuspended in 5 ml of culture medium. From this solution, 6.6 µl were collected and loaded in a specific counting chamber (KOVA Glasstic). Based on the number of cells counted, a diluition with the culture medium was carried out in order to obtain a concentration of 120'000 cells per ml. Subsequently, cells present in this solution were plated in 4 wells plates (Nunc) on 0,1% porcine gelatin (Sigma), with a volume equal to 0,5 ml per well (60'000 cells per well). The various amino acids were solubilized to reach final concentrations of 2 mM, 10 mM, 50 mM, and 100 mM as compared with complete media alone.

3D CULTURE

T 75 flask was prepared adding 1.5 ml of sterile 0.1% porcine gelatin and waiting 2 hours to coat the surface at room temperature. We washed liver biopsies with new PBS, supplemented with 2% antibiotic-antimycotic solution and we placed it in a 100 mm Petri dish and cut into approximately 2 mm^3 fragments with sterile scalpels. Than we place fragments in tube containing 30 mL of Trypsin solution and gently shake from 10 to 30 min at 37°C. At the end of 30 min, the digested tissue was filtered with cell 205 strainer 100µm and collect cells in a new 50 mL tube. Filtered cells were centrifuged at 1250 g/min for 5 min at room temperature. Supernatant was remove and the cells were resuspended in 11 mL of culture medium. Total 15 ml (11ml medium+ 4 ml pellet). The cells were plated in gelatin pre-coated T 75 flasks and culture for 6 hours. After 6 hours centrifugation at 1250 g/min for 5 min at room temperature and the pellet was resuspended to obtain $1x10^6$ cells concentration in 500 µl. Place every single decellularized liver scaffolds in a 35 mm Petri Dishes. We seeded the cells directly onto scaffold using a 1000 µl pipette and we inserted the 35 mm Petri Dishes containing the ECM and cells in a 100 mm Petri Dish with another 35 mm Petri Dish containing deionized water to prevent exsiccation. The scaffold was transfered to the incubator under aseptic conditions with (5%) CO2 at 37°C, to encourage cell engrafting and ECM repopulation (Fig. 7). As in the 2D culture the the rabbit hepatocytes BCAAs-free medium was: Dulbecco's modified Eagle's

medium/ F12 (DMEM) (Life Technologies, Waltham, Massachusetts, USA) supplemented with 20% foetal bovine serum (FBS) (Life Technologies, Waltham, Massachusetts, USA), 2 ml Antibiotic Antimycotic Solution (100×) (Sigma-Aldrich, St. Louis, Missouri, USA), 1 ml L-Glutamine solution (Sigma-Aldrich, St. Louis, Missouri United States), 20 µl HGF (R&D systems Minneapolis, Minnesota, USA)[75].

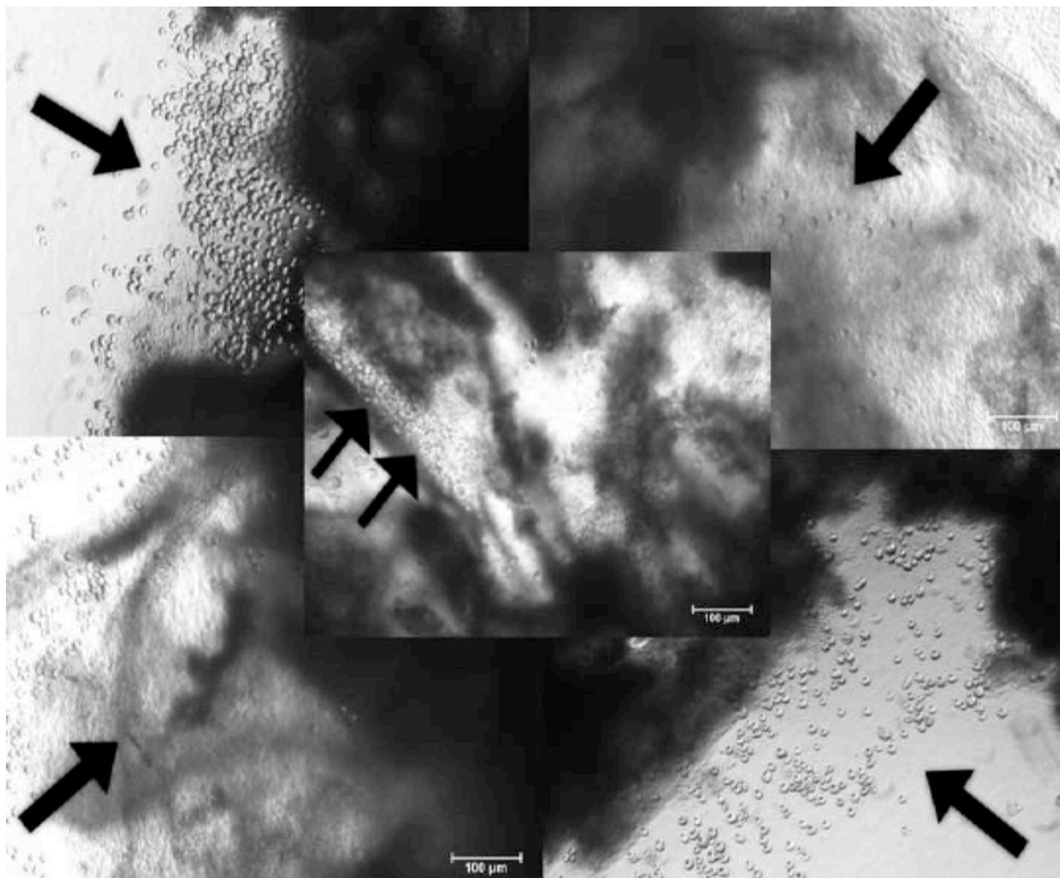

Figure 7.

Cells colonize the scaffold (arrows) and form clusters bordering and entering the recellularizing matrix.

BCAA EXPERIMELTAL DESIGN

The branched chain amino acids with 2:1:1 ratio was solubilized into the cell's monolayer medium culture to reach final concentrations of 2 mM, 10 mM, 50 mM, and 100 mM as compared with complete media alone.

In the 3D hepatocyte model with decellularized liver the BCAAs addiction with 2:1:1 ratio was: 200 mM, 100 mM, 0 mM as control (Tab.2).

The liver organoids incubated with the different cell type were evaluate after 7, 14, 21 days of in vitro static culture.

DAYS OF CULTURE	NUMBER OF SCAFFOLDS CULTURED	NUMBER OF SCAFFOLDS WITH CULTURE MEDIUM WITH FBS	NUMBER OF SCAFFOLDS WITH CULTURE MEDIUM WITHOUT FBS	NUMBER OF SCAFFOLDS BLOCKED
1	27	27	0	
2	27	0	0	
3	27	27	0	
4	27	0	0	
5	27	27	0	
6	27	0	0	
7	27	18	9	
8	18	0	0	9
9	18	18	0	
10	18	0	0	
11	18	18	0	
12	18	0	0	
13	18	18	0	
14	18	9	9	
15	9	0	0	3
16	9	9	0	
17	9	0	0	
18	9	9	0	
19	9	0	0	
20	9	9	0	
21	9	0	9	
22	9	0	0	3

Table 2

Time table for the recellularized liver scaffold.

ANALYSIS

FUNCTIONAL ASSAY

The albumin (Albumin Rabbit ELISA Kit, abcam) and Factor VII (Rabbit coagulation factor VII ELISA Kit, CUSABIO) content was determined by enzyme-linked immunosorbent assay. Absorbances were measured with a Thermo Scientific Multiskan FC microplate reader (Life Technologies, Waltham, Massachusetts, USA).

Morphological characterization, biochemical analysis medium during culture were evaluated. The supernatant of the cell culture was analyzed for transaminase enzyme activity, urea and albumin content after overnight culture. The supernatant of was therefore centrifuged at 3000g for 5 minutes (4°C).

Total protein ammount inthe culture medium were misured with chromatographic analysis (Cobas Mira, Roche, Basel, Swizerland).

The cells and organoids damage were misured detecting the ALT, GGT, LDH and ALP enzyme in the culture medium with chromatographic analysis (Cobas Mira, Roche, Basel, Swizerland).

PCR ASSAY

Total RNA was extracted using Trizol (Invitrogen) and DNAse I (Invitrogen) was added in Lysis solution at 1:100 concentration as indicated by manufacturer's instruction. DNA content assay of the decellularized liver scaffolds (ID number 28) were achieved with DNA Thermal Cycler.

The effective removal of genomic DNA from each RNA batch was then confirmed performing a standard PCR amplification for β-actin, using genomic DNA as positive control. Only negative samples were then reverse transcribed with Superscript™ II Reverse Transcriptase (Invitrogen). Activation of transcription factor Oryctolagus cuniculus albumin (ALB), Oryctolagus cuniculus alpha fetoprotein (AFP), Oryctolagus cuniculus KIT proto-oncogene receptor tyrosine kinase (KIT), Oryctolagus cuniculus hepatocyte nuclear factor 4 alpha (HNF4A), Oryctolagus cuniculus coagulation factor VII (F7), Oryctolagus cuniculus IL-6, Oryctolagus cuniculus hepatocyte growth factor (HGF), Oryctolagus cuniculus vascular endothelial growth factor (VEGF) and Oryctolagus cuniculus transforming growth factor alpha (TGF-α) were examined using consensus oligonucleotides of:

Oryctolagus cuniculus albumin (ALB), mRNA

NCBI Reference Sequence: NM_001082344.1

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	1488	20	59.97	55.00	3.00	3.00	atctgtccgtggtcctgaac

RIGHT PRIMER 1693 20 59.99 55.00 4.00 2.00 ctccgtttctggaagagtgc
SEQUENCE SIZE: 1996
INCLUDED REGION SIZE: 1996

PRODUCT SIZE: 206, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00

Oryctolagus cuniculus alpha fetoprotein (AFP), mRNA

NCBI Reference Sequence: XM_002717026.3

OLIGO start len tm gc% any 3' seq
LEFT PRIMER 1633 20 60.06 50.00 6.00 0.00 ATCTTCCACGCGGATCTATG
RIGHT PRIMER 1839 20 60.07 50.00 4.00 1.00 AATCAGCTTTGGACCCTCCT
SEQUENCE SIZE: 1958
INCLUDED REGION SIZE: 1958

PRODUCT SIZE: 207, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 1.00

Oryctolagus cuniculus KIT proto-oncogene receptor tyrosine kinase (KIT), mRNA

NCBI Reference Sequence: NM_001329070.1
OLIGO start len tm gc% any 3' seq
LEFT PRIMER 1646 20 60.03 50.00 5.00 0.00 ccattaagatgggcagagga
RIGHT PRIMER 1932 20 60.07 55.00 4.00 2.00 gtgattccccaggtagctca
SEQUENCE SIZE: 2910
INCLUDED REGION SIZE: 2910

PRODUCT SIZE: 287, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

PREDICTED: Oryctolagus cuniculus hepatocyte nuclear factor 4 alpha (HNF4A), mRNA

NCBI Reference Sequence: XM_008274469.2

OLIGO start len tm gc% any 3' seq
LEFT PRIMER 1001 20 59.98 55.00 3.00 2.00 gagtatgcctgcctcaaagc
RIGHT PRIMER 1215 20 59.90 50.00 6.00 3.00 tggatctgctcgatcatctg
SEQUENCE SIZE: 4465
INCLUDED REGION SIZE: 4465

PRODUCT SIZE: 215, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00

Oryctolagus cuniculus coagulation factor VII (F7), mRNA

NCBI Reference Sequence: NM_001082679.1

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	314	20	59.66	55.00	5.00	0.00	gctcctgtgaggaccaaatc
RIGHT PRIMER	544	20	59.99	50.00	4.00	2.00	cgcatgggtaatcaactgtg

SEQUENCE SIZE: 1619

INCLUDED REGION SIZE: 1619

PRODUCT SIZE: 231, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

PRIMER DISEGNATI per analisi sangue su conigli

Oryctolagus cuniculus IL-6 mRNA, complete cds
GenBank: DQ680161.1

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	385	20	59.98	55.00	4.00	0.00	CTTCAGGCCAAGTTCAGGAG
RIGHT PRIMER	583	20	60.12	55.00	4.00	0.00	AGTGGATCGTGGTCGTCTTC

SEQUENCE SIZE: 726

INCLUDED REGION SIZE: 726

PRODUCT SIZE: 199, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 2.00

Oryctolagus cuniculus hepatocyte growth factor (HGF), mRNA
NCBI Reference Sequence: NM_001168707.1

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	1924	20	59.99	50.00	5.00	0.00	GAGAAATGCAGTCAGCACCA
RIGHT PRIMER	2128	20	60.07	55.00	3.00	0.00	GGACGAAGATACCAGGACGA

SEQUENCE SIZE: 2193

INCLUDED REGION SIZE: 2193

PRODUCT SIZE: 205, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00

Oryctolagus cuniculus vascular endothelial growth factor (VEGF) mRNA, partial cds
GenBank: AY196796.1

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	10	20	59.99	55.00	4.00	1.00	CTACCTCCACCATGCCAAGT
RIGHT PRIMER	245	20	59.83	50.00	3.00	2.00	CACACTCCAGGCTTTCATCA

SEQUENCE SIZE: 361

INCLUDED REGION SIZE: 361

PRODUCT SIZE: 236, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00

Oryctolagus cuniculus transforming growth factor alpha mRNA, partial cds

GenBank: AF333183.1

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	44	19	60.40	57.89	3.00	0.00	AAGCCCTGGAGAACAGCAC
RIGHT PRIMER	205	20	60.05	55.00	5.00	3.00	CAGAGTGGCAGACACATGCT

SEQUENCE SIZE: 206

INCLUDED REGION SIZE: 206

PRODUCT SIZE: 162, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 3.00

MORPHOLOGY

Briefly, the samples to be processed for standard histology were further fixed in 10% (v/v) phosphate-buffered formaldehyde for 24h at room temperature, then dehydrated in a graded 50% (v/v), 70% (v/v), 95% (v/v) and 100% (v/v) ethanol series, embedded in paraffin and cut into 4 µm-thick sections by a microtome. Finally, the sections were stained with Hematoxylin and Eosin, using a standard staining protocol, for the evaluation of the morphological structure and their relationships. The observations were carried out through the use of an optical microscope Nikon ECLIPSE E600 (Olympus, Milan, Italy), equipped with digital camera and software for image analysis.

QUANTIFICATION OF THE REPOPULATION OF THE ORGANOID ECM

After the measurement of the total Area (mm^2) of each scaffold, the organoids were sliced at 4 different levels: first superficial slice at 4 µm, second superficial-mild slice at 104 µm, third deep-mild slice at 204 µm and fourth deep slice at 304 µm (Fig.8). Each slice was 4 µm thickness as standard histological evaluation. Cells count with optical micrioscopy for each slice were achieve. To compair the repopulatin onto organoids the total ratio of cells number/Area (mm^2) per slice were evaluated.

SCAFFOLD SAMPLING

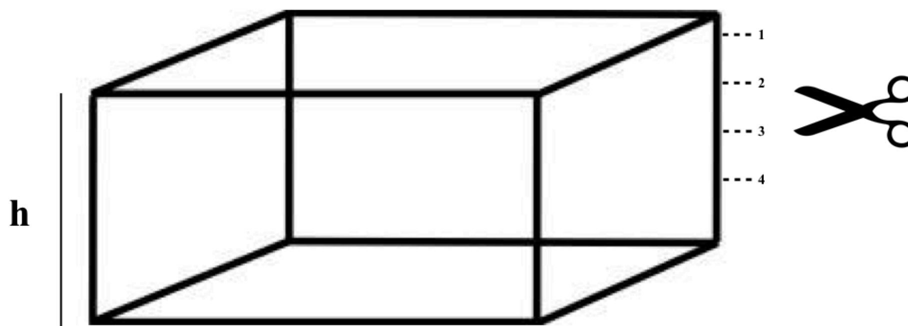

Figure.8

Graphical scheme of the scaffold sampling obtained to count and detect the ECM repopulation. The number represent the slice leves: 1= histological slice, 4 µm; 2= histological slice, 100 µm; 3= histological slice, 200 µm; 4= histological slice, 300 µm; h= scaffold thickness, 500 µm.

IMMUNOCYTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

Cells were fixed in 4% (wt/vol) paraformaldehyde in PBS (Sigma), rinsed three times in PBS and permeabilized with 0,1% and 0,4% (vol/vol) Triton X-100 (Sigma) in PBS, for 20 min. Samples were treated with blocking solution containing 5% (vo/vol) BSA and 5% (vol/vol) goat serum in PBS, for 30 min. Then, cells were incubated with anti-albumin (Abcam) overnight. The day after, cells were incubated with suitable secondary antibodies (Alexa Flor® 488) for 60 min. Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI, Sigma). Samples were observed under a Nikon Eclipse TE200. For immunohistochemistry the samples were treated with the following procedures: deparaffinized and hydrated through xylenes or other clearing agents and graded alcohol series; washed for 5 minutes in tap water. The unmasking was perform using a Vector® Antigen Unmasking Solution, Citrate-based, pH 6.0 (H-3300) or Tris-based, pH 9.0 (H-3301); washed in buffer for 5 minutes; incubated for 20 minutes with diluted normal blocking serum; bloted excess serum from sections; incubate for 30 minutes with primary antibody diluted in buffer; washed for 5 minutes in buffer; incubated for 30 minutes with diluted biotinylated secondary antibody; washed for 5 minutes in buffer; incubated for 30 minutes with VECTASTAIN® ABC Reagent; wash for 5 minutes in buffer; incubate in peroxidase substrate solution.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA). Statistical differences were evaluated by the Kruskal–Wallis test. A P value <0.05 was considered significant. The median values of, albumin synthesis, factor VII, total protein AST, ALT, LDH secretion were expressed with

charts.

RESULTS

During surgical explant procedures, the liver was considered healthy. It was reddish-brown in color, with a soft consistency, and is highly vascular and easily friable. The histology of the normal liver reveals a uniform red color sample with lobular architecture present and without any sigh of inflammatory infiltration. The central and sublobular veins were normal and presents. Hepatocytes were normal with no sign of atrophy or hypertrophy, uniformity and no pigments were noted.

MORPHOLOGICAL OBSERVATION ON THE MONOLAYER HEPATOCYTES CULTURE.

Figure 9 shows the morphology of hepatocytes on day 15. As seen in Figures 9A, hepatocytes formed an extended monolayer in collagen-coated plastic dishes. The cell attachment was slightly different between BCAA-free and BCAA-containing cultures; the cells stretched more on the surface of the dish in the latter case compared to the former (Figure 9B). On the BCAAs-fre medium dish, the cells were initially weakly attached, agglomerated, and formed densely fusiform packed monolayer. However, when BCAAs were added to the medium epithelial layer was not formed (Fig.9).

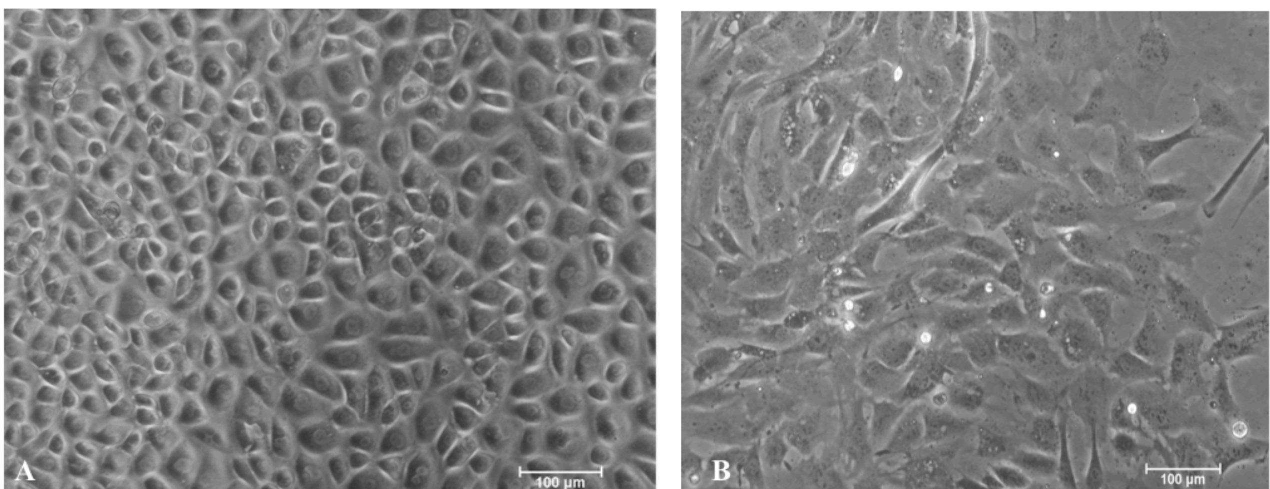

Figure 9

Primary rabbit hepatocyte 2D culture before BCAAs treatment.

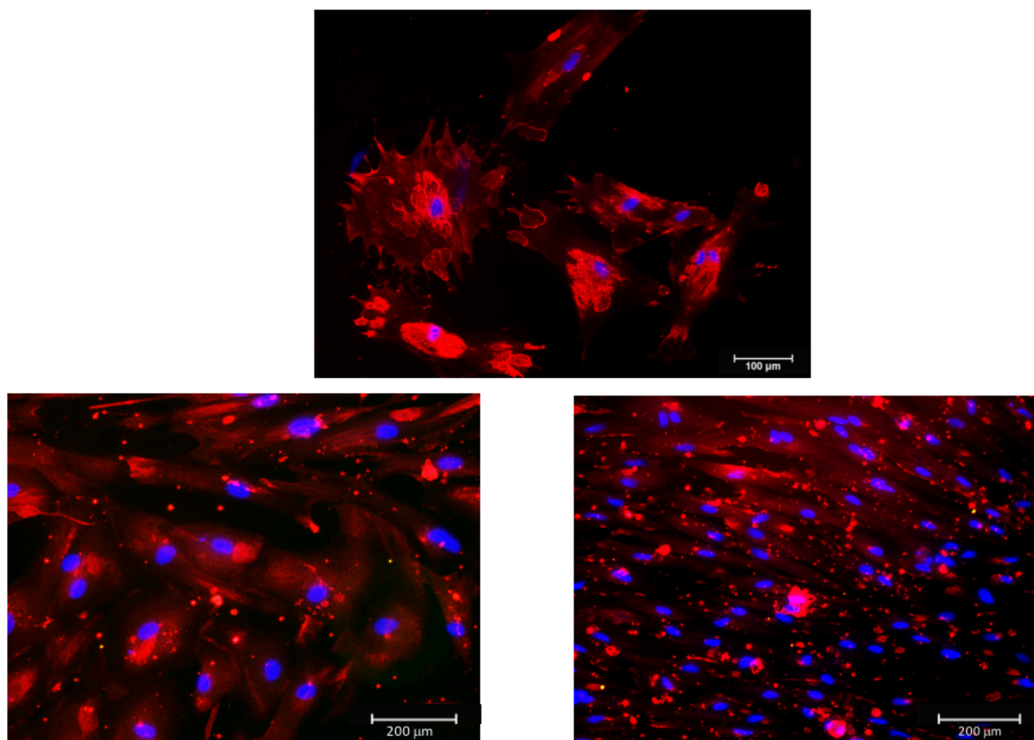

Figure 10

Immunofluorescence images of primary rabbit hepatocyte 2D culture for 7 days, the cells expressed albumin (red cytoplasm) confirming the cells characterization as hepatocytes.

EFFECTS OF THE BCAAS ADDICTION IN MONOLAYER HEPATOCYTES CULTURE

We studied the effects of BCAA on cell growth, albumin and FVII secretion. Figure 2 presents the changes in albumin and Factor VII in a collagen-coated dish culture, in which BCAAs were added in a FBS-free medium for 48h. No increase in cell prolifertion was observed when BCAAs was added to the medium for days of -free culture.

The albumin and Factor VII production ability was conserved from the all case-control studies, revealing a non-significant difference between five groups (p-value >0,05).

The cell damage marker represents by ALP and LDH were not detected. The total protein (TP) amount in the medium were not statistically different within the groups (p-value >0,05), except for ALT (p-value <0,05) (Charts1). Hence, our results revealed no difference between monolayer cultures in the BCAAs-free compare with BCAAs medium culture.

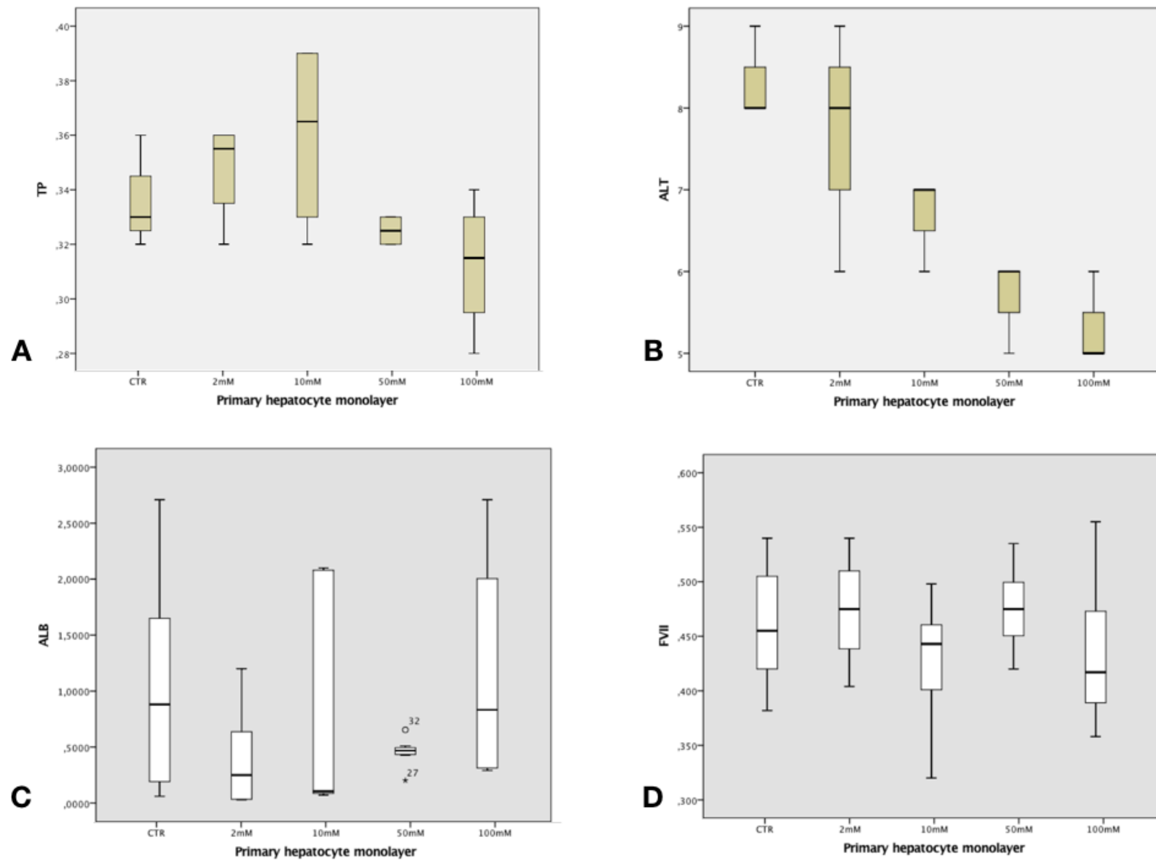

Chart 1

Box plot of TP (A) and ALT (B) cellular enzyme release (U/L) in the culture medium and the factor VII (A) and albumin (B) production expressed in ng/ml and µg/ml

PERFUSION DECELLULARIZATION OF THE RABBIT

Perfusion decellularization of whole rabbit livers liver was achieved by portal perfusion with Triton X-100 and other reagents. The PV was successfully cannulated with an 8,5-sheath introducer 8,5 Fr cannula and fixed with 3-0 silk sutures. The liver color turned soft red after the blood was washed out. After rinsing with distilled water, the liver turned yellowish brown and showed mild swelling. During the perfusion of 1% (w/v) Triton X-100/0.1% ammonium hydroxide, liver cells were washed out in great numbers, and the liver color quickly became semi-transparent. At the end of the process, the liver was transparent and the acellular scaffold retained the gross shape of the liver (Fig.11). The vascular system was visualized clearly through the capsule of the acellular scaffold.

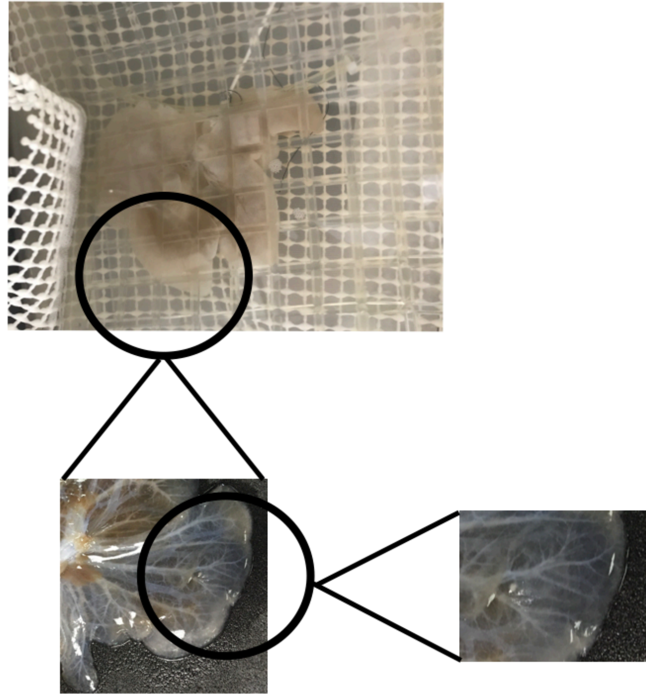

Figure 11

Decellularized liver lobe and an enlargement of the left lateral lobe where we can appreciate the intact vasculature structure

MACROSCOPICAL ORGANOIDS EVALUATION

During the growing time, some organoids took a reddish-brown liver color. This changing during the culture days were indipendent from the scaffold thikness or size but there was a correlation between the color and the number of cells.

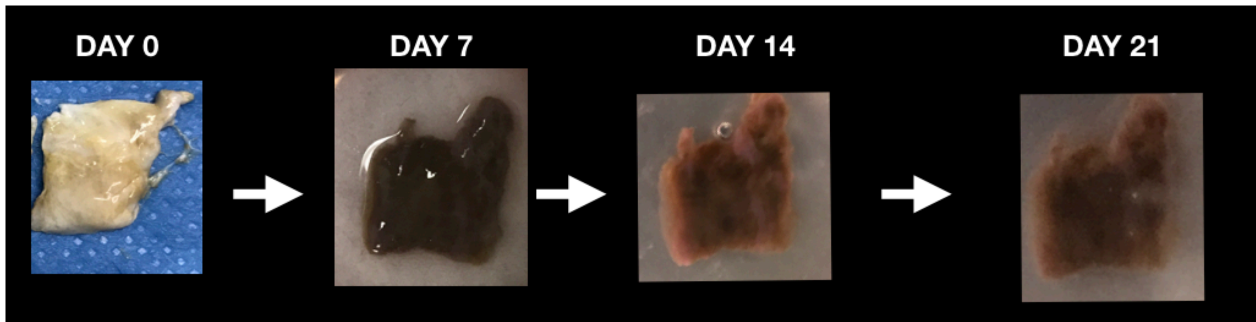

Figure 12

Organoid changing from the decellularized macroscopic aspect to the liver organoid aspect.

FUNCTIONAL ASSAY

The total protein presence in the all cuture conditions were produced/release in a non-significantly different amount (p-value< 0,05; Chart 1). The liver enzyme, normaly used as hepatic maker of liver damage where significantly higher in the 200-mM concentration on day 21 (Chart.1). The production of factor VII (FVII) and albumin was detected in all the cells cultured on the decellularized liver in the absence of BCAAs and in BCAAs-free medium. Both proteins were detected at low levels in the cells cultured in the BCAAs-free medium. The secretion of FVII increased steadily in both the 100 mM grups and reached a peak on day 21, and significant differences in the levels of albumin were observed between the cells cultured in the BCAAs and in BCAAs-free medium (Chart 3). In the presence of 200 mM, the production of ALB was significantly higher than in the cells cultured in the 100 mM and BCAA-free medium After day 7 a ALB production decrease was observed on the following days 14 and 21.

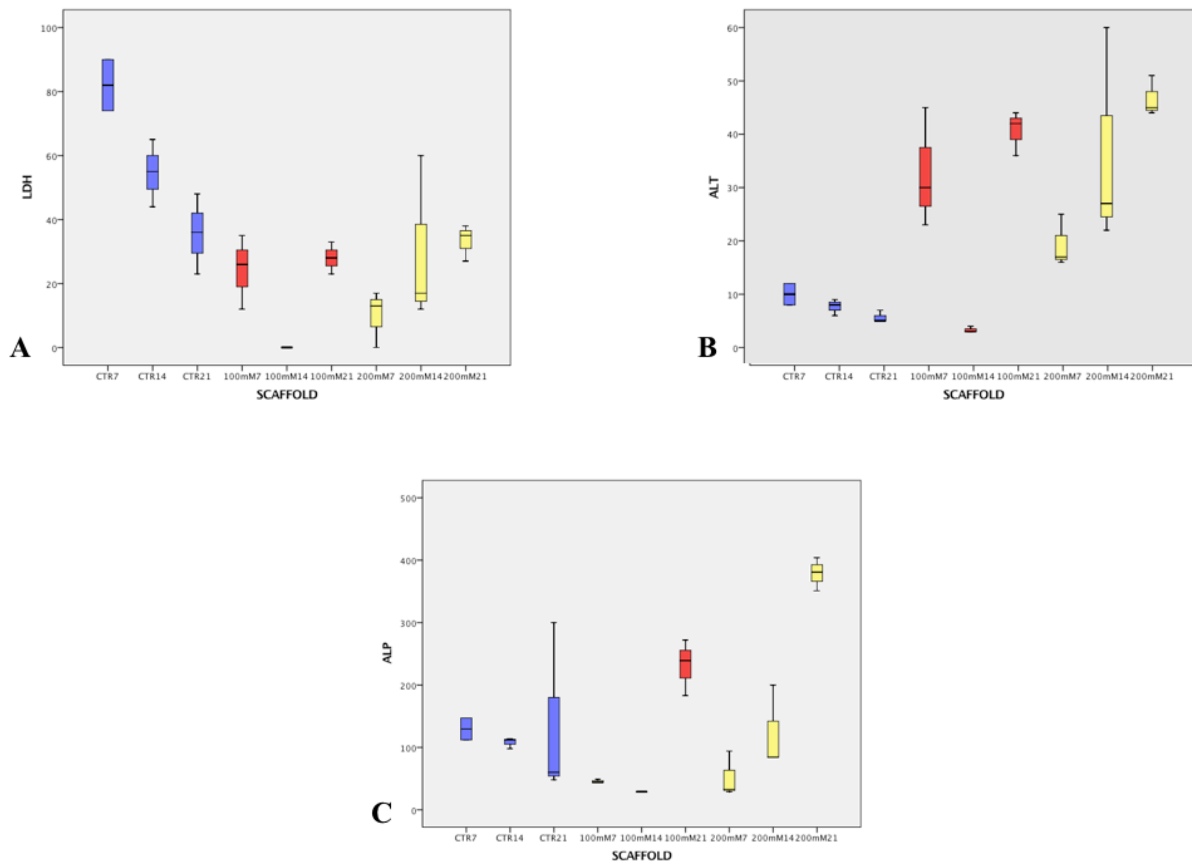

Chart 2

Box plot of LDH (A), ALT (B) nad ALP (C) cellular enzyme release (U/L) in the culture medium. Blue box plot: Controls (7-14-21 days); Red box plot: BCAAs concentration in the culture medium 100 mM concentration (7-14-21 days); Yellow box plot: BCAAs concentration in the culture medium 200 mM (7-14-21 days).

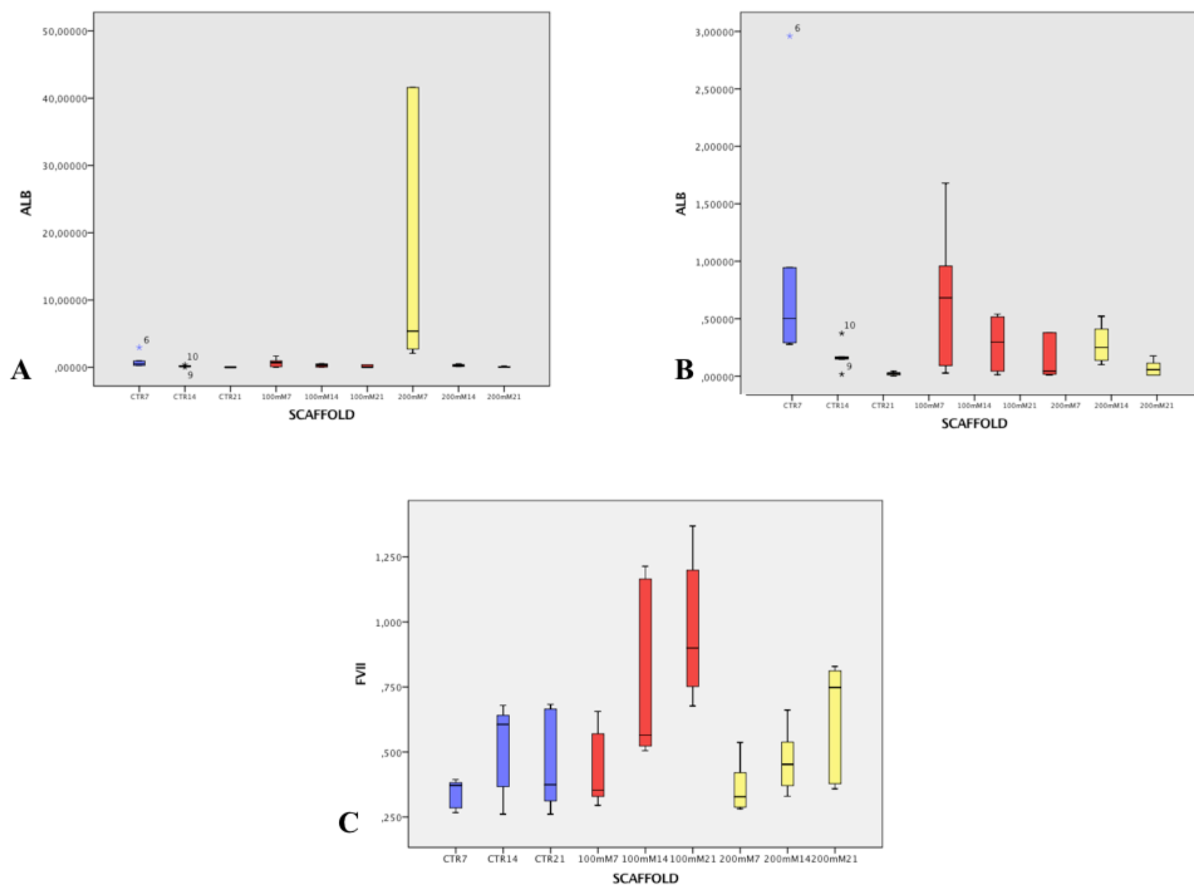

Chart 3

Box plot of the albumin (A, B) factor VII (C) production expressed in µg/ml and ng/ml. Interestingly for the albumin production there is a significant and impressive difference between the 200 mM BCAAs concentration at day 7 compared to others groups. Blue box plot: Controls (7-14-21 days); Red box plot: BCAAs concentration in the culture medium 100 mM concentration (7-14-21 days); Yellow box plot: BCAAs concentration in the culture medium 200 mM (7-14-21 days).

PCR

Reverse-transcription–polymerase chain reaction analysis showed the expression of Oryctolagus cuniculus albumin (ALB), Oryctolagus cuniculus alpha fetoprotein (AFP), Oryctolagus cuniculus KIT proto-oncogene receptor tyrosine kinase (KIT), Oryctolagus cuniculus hepatocyte nuclear factor 4 alpha (HNF4A), Oryctolagus cuniculus coagulation factor VII (F7), Oryctolagus cuniculus IL-6, Oryctolagus cuniculus hepatocyte growth factor (HGF), Oryctolagus cuniculus vascular endothelial growth factor (VEGF) and Oryctolagus cuniculus transforming growth factor alpha (TGFalpha) by day 7-14-21. Expression Oryctolagus cuniculus albumin (ALB), Oryctolagus cuniculus hepatocyte nuclear factor 4 alpha (HNF4A), Oryctolagus cuniculus coagulation factor VII (F7), Oryctolagus cuniculus hepatocyte growth factor (HGF), and Oryctolagus cuniculus transforming growth factor alpha (TGFalpha) was detected at all time points. Organoids were negative for Oryctolagus cuniculus

IL-6, Oryctolagus cuniculus alpha fetoprotein (AFP), Oryctolagus cuniculus KIT proto-oncogene receptor tyrosine kinase (KIT) and Oryctolagus cuniculus vascular endothelial growth factor (VEGF). Normal rabbit liver speciments were used as positive control and ECM as negative control. The DNA quantification is represented in the chart 4.

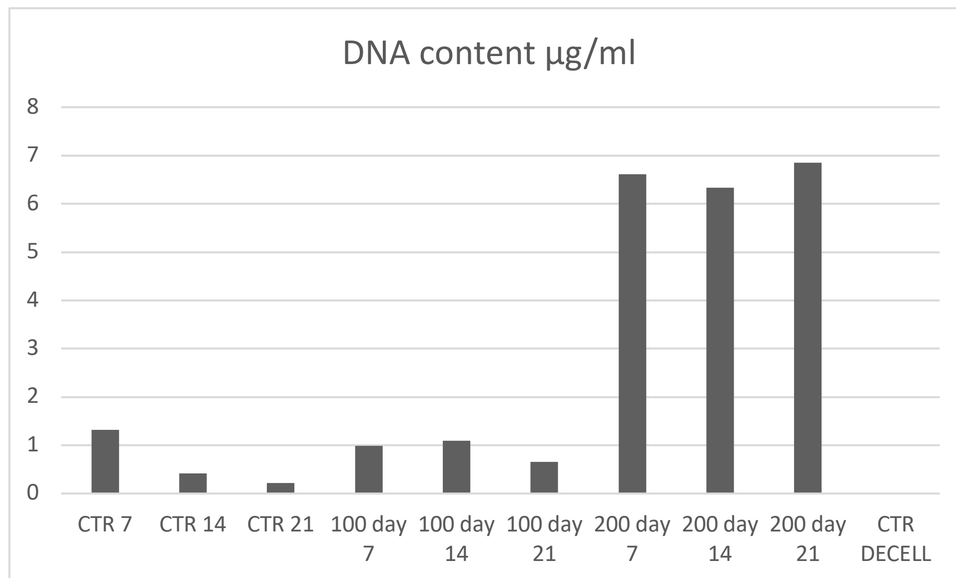

Chart. 4

DNA quantification demonstrated significant DNA reduction in the control groups, an high value in the the 200 mM groups.

MORPHOLOGY AND QUANTIFICATION OF THE REPOPULATION OF THE ORGANOID ECM

The number of cells decreased significantly (Chart 5) between 7, 14 and 21 days of culture ($p < 0.05$). Liver ECM were also successfully repopulated with hepatocyte cells, which rapidly engrafted and migrated through the scaffold. Repopulation with cells was also characterized by marked Albumin positivity indicating hepatocyte-like cells proliferation (Fig.14,15). Interestingly, there was a marked decrease in cell number between 7, 14 and 21 days for the control groups, a peak value at 14 days for the 100 mM and an increase number of cells between 7, 14 and 21 days for the 200mM concentration (Chart 5; $p < 0.05$).

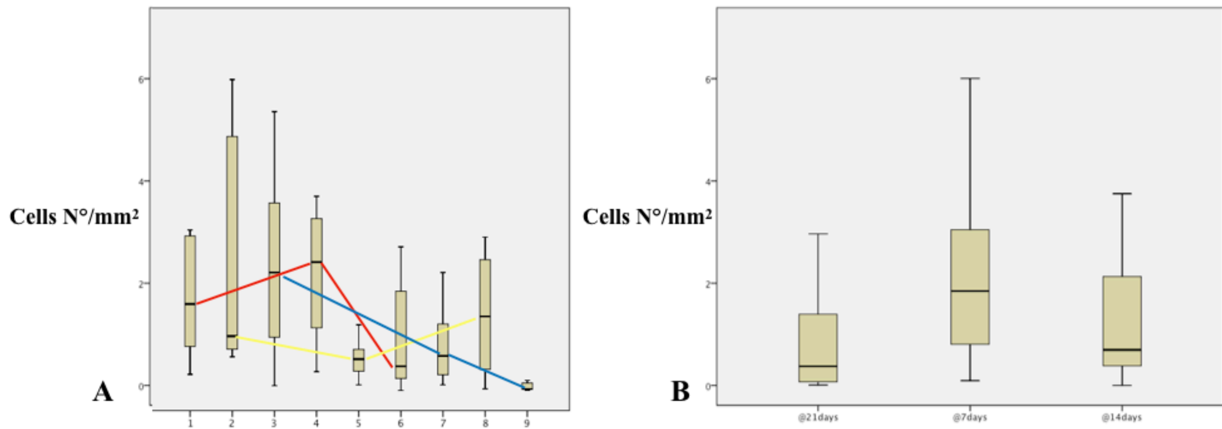

Chart 4

Box plot plot for the representation of cell proliferation. (A) All groups during 7-14-21 days; red lines connect the median values of 100 mM groups, yellow lines connect the median values of 200 mM groups, blue lines connect the median values of control groups; 1 = 100 mM 7 days, 2 = 200 mM 7 days, 3 = CTR 7 days, 4 = 100 mM 14 days, 5 = 200 mM 14 days, 6 = CTR 14 days, 7 = 100 mM 21 days, 8 = 200 mM 21 days, 9 = CTR 21 days. (B) cell proliferatio during 7-14-21 days considering all the groups.

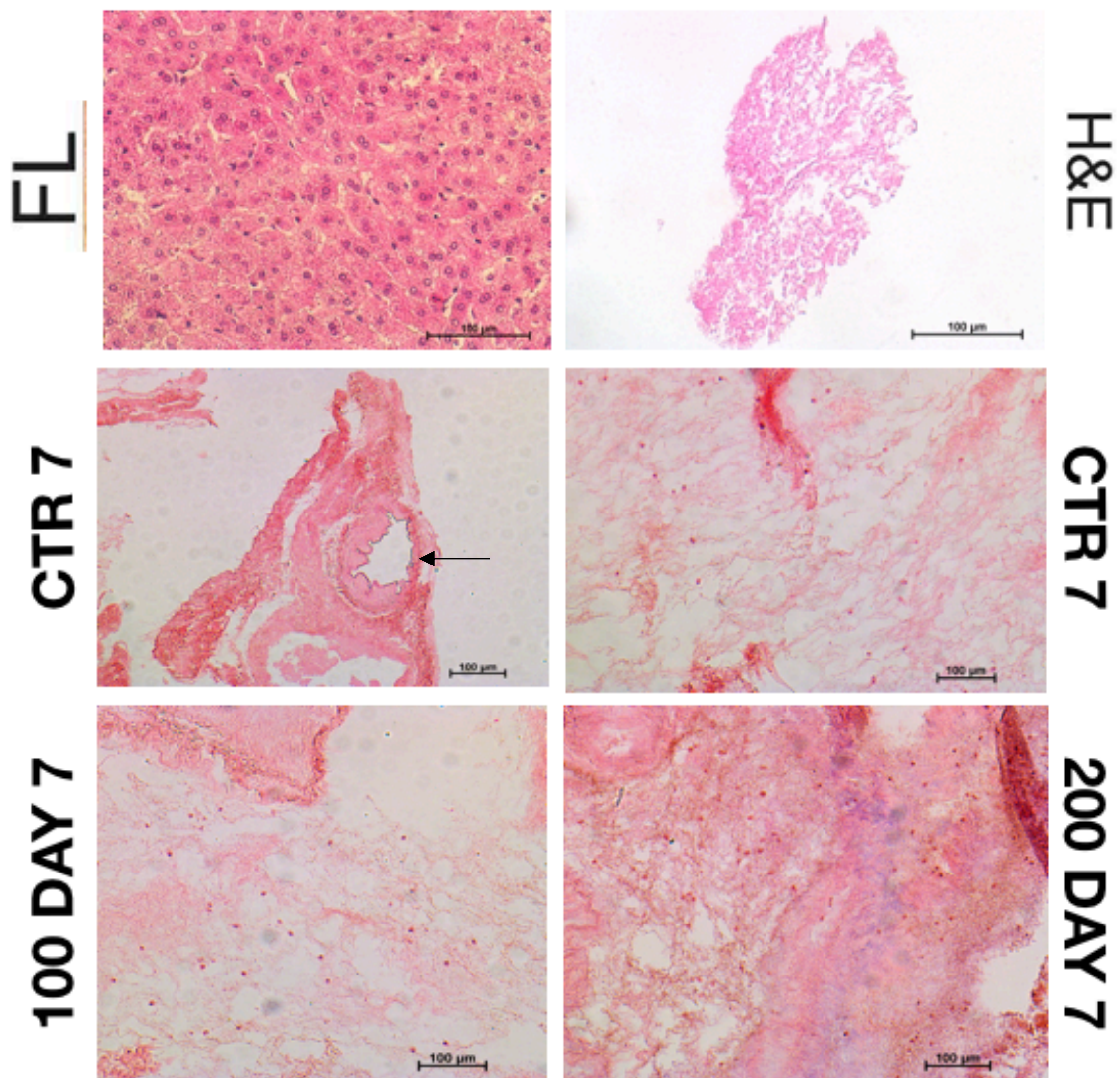

Figure 14

Histological comparison (10X and 20X magnification) of fresh liver (FL) and decellularized liver (H&E). Examples of recellularized organoids: control BCAA-free medium at day 7 (CTR7), 100 mM BCAA medium concentration at day 7 (100 DAY 7), 200 mM BCAA medium concentration at day 7 (200 DAY 7) by Hematoxylin and Eosin (H&E). In the CTR 7 on the left there is a vessels repopulated with endothelial cells (black arrow).

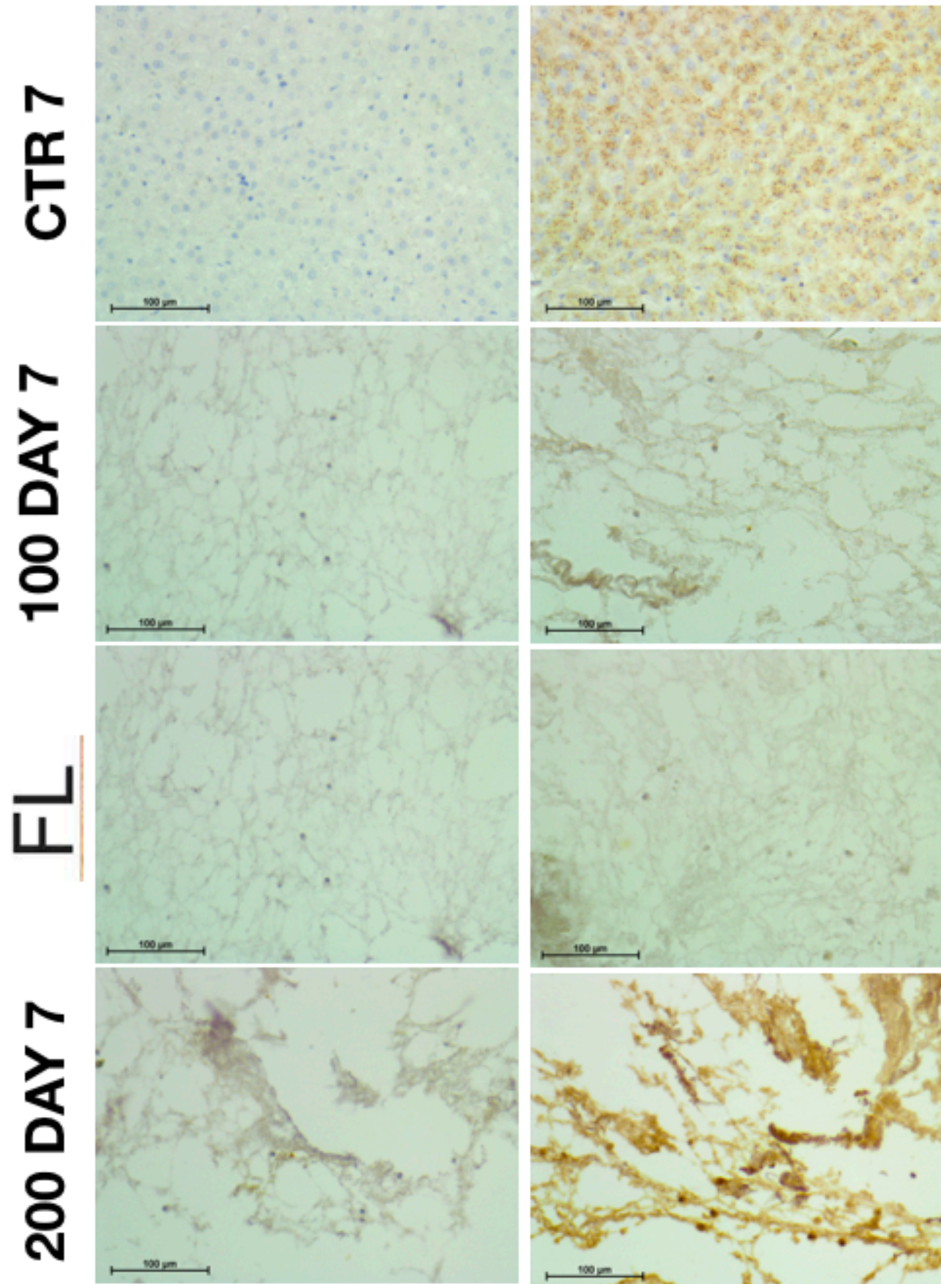

Figure 15

Expression and distribution of ALB staining in fresh liver (FL) control BCAA-free medium at day 7 (CTR7), 100 mM BCAA medium concentration at day 7 (100 DAY 7), 200 mM BCAA medium concentration at day 7 (200 DAY 7). Scale bar for 20X magnification 100 μm.

DISCUSSION

The BCAA efficacy to counteract different types of disease strongly debilitating have been studied in depth by using in vitro and in vivo clinical trials in medicine[77,78]. The complex mechanisms that underlie the catalytic and anabolic activity of these three amino acids are processed and discovered by recent studies that associate the regenerative and energetic ability to their dual atitude in the glucogenetic to produced energy and their structural role to produce proteins. Moreover, their broad constant presence in mammals in the form of essential amino acids is the key note in clinical nutrition, suffice it to think that in every animal and human constituent protein the BCAA constitutes 20% of the protein from primary to quaternary structures[78].

The connection between nutrition or intergration with BCAA and therapeutic support to counteract diseases is close and real. Indeed, the BCAA as a nutritional therapeutc option is well known to coadiuvant the treatement of acute and chronic hepatic failure[77,79]. Mouse and rat represent the most exploited and studied in vivo animal model, while for in vitro researches, murine cells culture or immortalized cell lines such as HepG2 or primary tumor cell lines[80-83]. Recently, more space and interest was developed for th 3D cultures, especially using artificial matrices or self-composing cells to mimic and facilitate an exchange of paracrine signals in mono cell cultures or in co-cultures. This latest type, especially hepatic liver culture, allows models to better investigate mechanisms and to promote cellular growth by different stem cell lines.

Technology for producing highly functional liver organoids is developing rapidly. The first successful decellularization of a liver tissue was reported in 2004 by Lin and Colleagues[84]. Scaffold repopulation with primary rat hepatocytes revealed a significantly higher albumin and urea synthesis when compared to monolayers of the primary hepatocytes cultured on collagen gels, thus suggesting a greater functional potential of cells cultured in a 3D scaffold. Since 2004, several other reports have described the successful decellularization of rat, rabbit or pig[85].

For the BCAAs evaluation on their role on the liver regeneration and hepatocyte metabolism we selected and set up two differents in vitro models: 2D monolayer hepatocyte culture and a 3D culture using the upper cited organoid generate by isolated hepatocyte from a native rabbit liver and ECM obtain from a eterologous rabbit liver. The decellularized matrix reveal a maintained net vasculature and a low DNA level as a good maker for decellularization process. This technique previously described[86-93] was moderately short, simple and cheap a let to recellularized the scaffold in an acceptable decell-recell time for a total 7 days to obtain a liver organoid cultured in a Petri dish. The liver cell isolation with a trypsinization method let to obtain a total of $\simeq 19 \times 10^6$ cells. The choice of this type of enzymatic method was done for its inexpensiveness and speed procedure, compare with the most used collagenase two-steps perfusion[94]. The enzymaric isolation with trypsin let us to obtain

a decellularized matrix faster than non enzymatic procedures, evaluated from a short isolotaion trial not reported in this thesis.

In the 2D cell culture, BCAAs were not significantly predominant on the hepatocyte viability, function and proliferation but morphology change drastically before and after tretments. Therefore, the difference in response to the BCAAs treatement may be due to the difference in signal transduction induced by the morphological change[95] induced by the amino acids. The fusiform/fibroblastic morphology know as short spindle shape of the hepatocytes is associate to a indifferentiated form of the livers parenchymal cells[96]. The main and most interestin resuls were obtained with the 3D culture. Moreover, the liver organoid model let us to compare precisely the BCAAs treatment in differents time culture. The monolayer model didn't allow us to evaluate the cells in culture for more than 15 days, where after that cell entered in G0 and sign of apotosys were visible. The scaffold recellularized let us to consider the efficacy of BCAAs and evaluate the cells capatity to re-populate differents volume of ECM. The scaffold size variable (Tab1)(Fig.6) gave us a results and conclusion that to repopulate hepatic scaffolds small variability in its dimensions are not a limitation for the cells colonization. The macroscopical evaluation reveal in some scaffold, after 7 days of culture, a modification in the colour aspect of the organoids turning brown, similar to liver parenchyma (Fig.12). In literature, there is not any information about specific colours of recellularized scaffold, we detected that this characteristic is correlated to cellular proliferation, where the darkest scaffolds were the most repopulated (Chart 4). Moreover, the chromatographic analysis of the enzyme activity in the culture medium revealed a highest ALT, ALP, GGT, LDH values in the darkest organoids. At the end, the colour changing show a peack at day 7 of culture, after that moment the colour decrese consistently till day 21. These data suggest that the highest enzyme level is correlated to the highet repopulation level and both are associated to the highest metabolism and organoid cell damage.

To compare the cell adhesion and proliferation in the differents scaffold we counted the number of cells in the different Areas (mm^2). Interestingly, cell proliferation at day 7 of BCAAs treatment does not create a substantial improvement. At day 14 cells/Area raio improved with 100 mM, contrariwise 200 mM concentration gave a higher cells number at day 21. In general, therefore, the best time for the cells groth turns out to be at day 7 with or without any BCAAs stimulation, while at day 14 there was a spread shown by medians with a considerable better value for the concentration of 100 mM. These results should be read as a kind of vitro reproduction of what happens in live patients.

The oxidative damage and cell growth continue over the times culture and increase more and more as in the sick animals or human patients. The action of BCAAs is against cellular and organ aging, especially against the depletion of those cells called HPC always through a mTOR activation

mechanism that allows the cell and organoids to dramatically improve their lives and vitality. Therefore, we obtained a decresing in the cells number at day 21 in controls and in 100 mM groups and an improvement in the numerosity in the 200 mM groups. A higher BCAAs amount correspond to a highest length of cell life without any biophysical cancer transformation. Several tumor suppressors, such as p53, p21, p16, Arf, and pRB, function as regulators of senescence, so they act as important tumor suppressors. An explanation for the molecular mechanisms of BCAAs to prevent cancer incidence was reported by Nakano et al., the BCAAs inhibited insulin-induced hepatic tumor cell proliferation by inducing apoptosis through their activities always on mTORC1 and mTORC2[96,97]. In the monolayer culture, we had not any differences between the results with or without BCAAs addiction. The data obtained from the monolayer primary cell culture revealed our initial hypothesis, where the cell metabolic exchange in 2D models is unefficacy to maintain a physiological cell metabolism. Indeed, the hepatocyte in monoculture substantially changes its morphology[95,98] but not expresses the most of its functional characteristics, hence the motivation and success redeemed from 3D culture methods such as Matrigel®[92]. Thanks to our 3D culture, we were able to get some interesting results regarding the different behavior of BCAAs. We can say that there is a direct correlation between cellular damage and functionality. While there is a consensual relationship between the number of cells that repopulated the scaffold and liver damage hepatocyte especially between controls and cells stimulated with 200 mM. While in the case of the 100mM concentration organoids they behaved exactly in the opposite manner. The cell number/Area ratio and the cell damage were inversely proportional. This can be considered valid and supported by the role of hepatocytes during the structured system of the liver regeneration[98,99]. It is further understood that the mechanism of replication is the basis of vicariate role of single hepatocytes and progenitor cell as explained by recent studies concerning cellular hepatobiliary therapy[101]. It is therefore important to study how the different liver organoids productions are modified without focusing the attention on the cell number evaluation for each scaffold. Regarding the difference between ALB production and FVII there is a considerable increase the ALB production in the 200 mM samples and a balance ratio between cell damagen and cell number in the 100 mM treatment at days 14.

The difference between albumin production and factor VII lies in the action and functionality that these two proteins have in their organization. In fact, the albumin is produced and released by the hepatocytes both progenytorse and mature and it is considered a Negative-phase protein. The doubt and the issue we raise is how this protein is released is released or produced in vitro, where the cells are subjected to extracellular stimuli that are completely different from its natural environment. Albumin is produced for many functions and reasons, among the most important we include intravascular oncotic pressure and its carrier role of substances. In the case of hepatic or systemic

pathologies and disorders, this protein is released in small quantities to allow for increased production of all inflammatory protein factors as IL1, IL6, IL8 and TNFα. We chose and selected another maker for hepatocytes function as the non-activated form of FVII. This protein is even more specific to evaluate the mature hepatocytes and HPC functionality of our organoids than ALB. Factor VII is normaly internalized in the hepatocyte or released into the bloodstream or vascular system, so we can certainly assert that the levels found in our cultures are unlikely to be overestimated[102,103]. To support our results and therefore the validity of the differences between the case-control groups was the statistical evaluation by the Kruskal Wallis test. The culture times are used to create different cellular environments. The evaluation beyond the ability to colonize the ECM created a kind of in vitro model of suffering cells. The role of BCAAs is evaluated and confirmed to counteract aging by recreating in vitro condition of cell damage. It is, therefore, on the basis of these considerations that at this stage of my doctoral project we can establish that for a recellularization of a decellulartized liver the best dosage of BCAAs was found to be the 200 mM concentration. While for a shorter and stabilized culture, the most effective dosage was 100 mM. In this last case, we can talk about stabilized culture as the best ratio between production, damage, and cell counts in term of median values. To complete and confirm the framework for the production, we have selected RNAm primers that physiologically are expresses during different moments of the hepatocyte replication and liver regeneration. The expression of albumin using standard PCR and FVII, instead, there are served for the further confirmation of the sought ELISA given. All organoids were positive for HNF4, HGF and TGF-α, while negative for IL-6, AFP, c-kit and VEGF. This RNAm transcriptional framework is an index of a replication activity of the second phase of liver regeneration, studied in literature mainly with in vivo murine post-epatectomy of 70% liver resection[100]. The possibility that selected cells during culture were HPC close to differentiation appears to be the most likely one. Indeed, the presence of HNF4, HGF, and TGF-α is an index of non-complete hepatocellular differentiation. The HNF4 marcher is a progenitor marker known. The replicative response and the production of HGF and TGF-α characterizes hepatocytes in phases not completely mature[96,97,100]. The HPC phase selection feature of the BCAAs may be that stage not yet investigated but explaned by Hiroyuki Koike et al. and Aezam Katoonizadeh et al [75,104]. Indeed, in literature, the difference between stem cells and HPC is not so clear. New published works are trying to evaluate the different role between the so-called stem cells and the HPC niche. Our contribut to the literature could be the PCR negative result on the AFP and c-kit expressions. AFP and c-kits are usually expressed in young HPCs[107] , and the one of the roles of BCAAs is given by the ability to select and amplify the cellular response to liver damage. Several studies report the ability to help the growth and development of stem cell niches such as MSC midollars[79]. Hiroyuki Koike et al. reported that the metabolic activity of BCAAs is direct and active

to the embryonic liver cells by catalytic pathways[75]. It is well known that all of the mainly catalytic pathways of the Bcaa are primarily limited in the adult liver. Instead, in the embryo until the first half of gestation Bcat1 and Bcat2, the transaminases catalyzing the first deamination reactions, are widely active, then after the second half of gestation drastically decrease[75]. It is thus clear that in the adult patients the BCAAs hepatotoxic protection is also carried out to their energetic role. The three amino acid amino acids are absorbed by the gastrointestinal tract, carried to the portal circle and partially captured by the liver but not depleted, retaining high post-prandial concentrations in the blood to be absorbed and used predominantly by nervous tissue and kidneys.
We can additionally add that the c-KIT, AFP and VEGF negativity may also be associated with the non-carcinogenicity of cultured cells on the scaffold, also reffering the protective factor that BCAAs may have against abnormal replication by mutation of the expression of VEGF and TGF beta 1. Thus, their anti-cancer role is act augmenting cell growth but inhibiting tumor growth through the expression of mTOR and diminishing the functionality in differentiated tumor cells[110].
The choice of the animal model for this study falls from the rabbit as large animal model. The rabbit is considered an excellent bridge between the fast metabolism of a murine model and swine model. The difficulty in finding material such as antibodies suitable for factor VII and AFP created a little gap to confirm more precisely our hypothesis. We believe that this model to date is the most accurate and precise to study the regenerative mechanisms of the liver. Moreover, recent studies[111] showed that the rabbit can be an excellent model to study disease mechanisms and treatments of acute liver.
This project evaluated the role of BCAAs in hepatocyte replication using 2D and 3D in vitro culture as a model allowing us to focus our attention on organ recellularization and discover the 200mM concentration with 2: 1: 1 ratio main BCAAs dosage. This study leaves and raises other questions that make up a small drop of an ocean of metabolic connections and paracrine signals. Above all, this study also wants to help and support the future of organ culture for transplantation purposes. The ultimate goal is to understand how the nutrition of the single cell can help to expand the organ's availability by creating real biobank for research and clinical purpose to ‘build’ an in vitro organ to bypass the great problem of organ rejection.

CONCLUSION

BCAAs as mentioned by Okobayashi and Hiroyuki Koike are active on cell proliferation and particularly on metabolism by activating mTOR-linked mechanisms to support production and metabolic activity of hepatocytes. Thanks to the results detected we can complete study of Hiroyuki et al., confirming the hypothiesis that BCAAs have a direct action on HPC cells. Moreover, these amino acids can be a valuable support for bioingeneering, hepatocytes cultures and organoid

construction helping to prolong the time culture counteracting the oxidative damage of the cells. The future of this research may be to evaluate whether further concentrations can give a further and exponential boost. With this part of my PhD project, I can assume that the usefulness and reliability of the 3D in vitro model allows greater accuracy and greater data to study the hepatocytes interactions.

5.Chapter: General Conclusion

The sign and hallmarks of liver damage both in clinical and in experimental in vitro studies are cellular enzyme predominantly presents in the hepatocytes cytoplasm (ALP, ALT, LDH). The hepatocytes in vitro function is mostly represented by albumin and factor VII production. We have decided to validate these damage markers and functionalities in clinical and experimental in vitro models to better investigate the best therapeutic choice in case of general illness and cachexia. Indeed, in the case of serious debilitating diseases, a principal therapeutic failure and a cause of death of patients is an incorrect nutritional intake. Choosing an effective diet plan is still a turning point in managing the continuous slimming, thus defining the term malnutrition. PN is defined in some cases as a true and proper nutritional therapy and not just as an energy support. PN is also a direct route to feeding the individual body compartments and organs bypassing the selective gastrointestinal tract. Of the three main components of parenteral solutions, amino acids are increasingly considered and used[1]. In this study we started from the evaluation of different amino acid solutions containing different amounts of BCAAs, revealing an interesting data not present in the literature. By increasing the proportion of amino acids at the expense of lipids and glucose, the liver doesn't get overloaded but has, also, some improvement in the damage markers. The following in vitro study on hepatocyte models has allowed us to fill and investigate a BCAA protection mechanism against the liver aligning with the recent study of Hiroyuki et al. Due to all the comforting data gathered from the literature we decided to select BCAAs as the most important amino acids components in the parenteral solutions. This project started from a close collaboration between nutritional and surgical ideas. PN interpolates between the two disciplines by combining nutritional knowledge with the metabolic needs of the liver during different pathologies. During the first part of my doctoral studies, however, it was always present the necessity to focus on the evaluation of the ready to use formulas available on the market. The needs to provide and select an in vitro model that can be valid repeatable and easy to use has required great efforts to set up and personally validate the different procedures that constituted the materials and the methods of the second in vitro project. The main challenge of the clinical study was to enroll patients due to manage the high costs for by the owners in relation to the normal clinical nutritional procedures. The in vivo project therefore does not want to be a point of arrival but a beginning of the evaluation of the PPN both as a scientific veterinary study and as a study in the field of translational medicine. As a mental path the doctoral project has seen the real deductive process starting from a clinical need to the inverse process of bench-to-bedside. Starting from the clinical situation of the animals the liver stress induced by the disease and, eventually by the PN, we built an in vitro 2D and 3D liver structures as a model of organ failure; attributing the homeostatic modification a "non-natural" environmental condition. In 2D and 3D cultures, cells reside in an altered environment that deprives them of those normal chemical exchanges such as hormonal

paracrine signals that belong to the living organism. This situation induces the cell to re-establish and self-regulate itself to avoid a death and an early cellular apoptosis.

In conclusion, this doctoral project hasn't only the peculiarity of creating finished information but has the characteristic of opening new doors and new ideas to the relatively new field in hepatology and nutrition in the PPN and the possible role of amino acids and in particular the increasingly investigated role of BCAAs and their positional effects on liver and hepatic cells. In this study we have investigated the importance of the interaction between BCAAs alone or integrated in PN solutions and the eventual roles that different amount of amino acids have on liver preservation. Results obtained in this study also suggest that the liver organoid culture using decellularized rabbit liver could be a valid model to study toxicological compound or, in particular, improve the emerging field of the regenerative medicine to grow organoids and transplantable organs.

6. Chapter: References

INTRODUCTION

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HYPOTHESIS and AIM

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PARTIAL PARENTERAL NUTRITION IN MALNOURISHED DOGS: IMPACT ON LIVER DAMAGE

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USE OF 2D AND 3D RABBIT HEPATOCYTE CULTURES TO INVESTIGATE BRANCHED-CHAIN AMINO ACID EFFECTS IN LIVER

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GENERAL CONCLUSION

1. Hoffer LJ. Parenteral Nutrition: Amino Acids. Nutrients 2017; 9, 3, 257.

7.Chapter:PhD Candidate activities

PhD Candidate activities

- COST Action BM 1308 “Sharing Advances on Large Animal Models – SALAM final conference. Advances on large animal models: bridging the gap between biomedical research and clinical translation September 28-29, 2017, Halle (Saale), DE
- First Annual Meeting,, COST Action CA 16119 CellFit , ‘IIn vitro 3 - D total cell guidance and fitness’12 - 13 September 2017 , Albena Resort,, Bulgaria
- Course C.O.S.T., Salaam Training school in Principles and Procedures of Tissue Sampling and Biobanking Including Legal and Ethical Aspects, Munich, Gemany, 27-31/03/2017.
- Trainig in Cardiothoraic surgery, Organ perfusion and transplantation at Veterinary Clinic JASMINE, Yokohama, Japan, from march 2017.
- Tutor for the course of Chirurgia Plastica (Veterinary plastic surgery), Prof. Fabio Acocella, Via Celoria 10, Medicina Veterinaria, V.E.S.P.A. 2016/2017.
- Trainig in Cardiothoraic surgery in small animal at Clinique Veterinary Bozon, Versailles, France from Ju
- Congress: LXX Convegno SISvet, SICV session, 13-16/06/2016, Palermo, Italy.
- Course C.O.S.T., Trait Ontologies and Systematic Phenotyping of Large Animals, Madrid, Spain, 10/04/2016-14/04/2016.
- Congress: BSAVA Congress, Birmingham, UK, 7-10/04/2016.
- Congress: Focus on organ perfusion for clinical transplantation, Hotel Michelangelo, 23/03/2016, Milan
- Course in Animal experimentation, Istituto Carlo Besta, 29 gennaio 2016
- Tutor for the course of Base Anatomiche e Tecniche di Chirurgia del Torace (Veterinary Anatomy and technique in thoracic surgery), Prof. Fabio Acocella, Via Celoria 10, Medicina Veterinaria, V.E.S.P.A. 2015/2016.
- Tutor for the course of Base Anatomiche e Tecniche di Chirurgia Addominale (Veterinary Anatomy and surgical technique), Prof. Fabio Acocella, Via Celoria 10, Medicina Veterinaria, V.E.S.P.A. 2015/2016.
- Tutor for the course of Alimentazione e dietetica animali da compagnia (small animal nutrition), Titolare Prof. Valentino Bontempo, Via Celoria 10, Medicina Veterinaria, V.E.S.P.A. da 16/10/2015 a 10/12/2015.
- Tutor for the course di Razionamento animali monogastrici (small animal nutrition), Titolare Prof. Valentino Bontempo, Via Celoria 10, Medicina Veterinaria, V.E.S.P.A. da 16/10/2015 a 10/12/2015.
- Veterinary service for: OPEN ABDOMEN course for surgeon, Brescia, July 2015.

- Veterinary service for: ATOM course for surgeon, Brescia, July 2015.
- Course in Animal experimentation, Istituto Mario Negri, 3-5 giugno 2015.
- Shift worker at the small animal hospital of the University of Milan Faculty of Veterinary Medicine.
- Collaborations with Veterinary Clinic Gambolò, Pavia, Italy, chef medical office dr. Giuliano Colli Lanz

Scientific dissemination:

- Scientific paper: Matteo Ghiringhell, Alessandro Zenobi, Stefano Brizzola, Fulvio Gandolfi, Valentino Bontempo, Sandro Rossi, Tiziana A.L. Brevini, Fabio Acocella. Simple and quick method to obtain a decellularized, functional liver bioscaffold. Methods in Molecular Biology Methods Mol Biol 2017 Nov 4.
- Thesis Advisor: 'VALUTAZIONE MORFOLOGICA QUANTITATIVA DI ORGANOIDE RICELLULARIZZATO DEL FEGATO', Luca Allevi, 2016/2017.
- Scientific Posters: Matteo Ghiringhelli, Stefano Brizzola, Georgia Pennarossa, Tiziana A.L. Brevini, Fabio Acocella. Use of 2D and 3D rabbit hepatocyte cultures to investigate Branched-Chain Amino Acid effects in liver. First Annual Meeting, COST Action CA16119 CellFit, "In vitro 3-D total cell guidance and fitness" 12-13 September 2017 Albena Resort, Bulgaria.
- Scientific Poster: Matteo Ghiringhelli, Stefano Brizzola, Filippo Consolo, Tiziana A.L. Brevini, Fulvio Gandolfi, Alessia Di Giancamillo, Marco Trovatelli, Camilla Mocchi, Angelica Stranieri, Tiziana Vitiello, Eleonora Fusi, Valentino Bontempo, Fabio Acocella. 3D culture and normothermic perfusion for liver animal model validation. COST Action BM 1308 "Sharing Advances on Large Animal Models – SALAM final conference. Advances on large animal models: bridging the gap between biomedical research and clinical translation September 28-29, 2017, Halle (Saale), DE.
- Scientific Oral presentation: Vitali F, Kariuki EK, Gakuya F, Limo C, Ghiringhelli M, Ravasio G. Comparison of dexmedetomidine-ketamine and medetomidine-ketamine immobilization in free-ranging and captive African lions (Panthera Leo). AVA Autumn Meeting Berlin Association of Veterinary Anaesthetists; November 9th-11th 2017.
- Coccolini F, Acocella F, Morosi L, Brizzola S, Ghiringhelli M, Ceresoli M, Davoli E, Ansaloni L, D'Incalci M, Zucchetti M. High Penetration of Paclitaxel in Abdominal Wall of Rabbits after Hyperthermic Intraperitoneal Administration of Nab-Paclitaxel Compared to Standard Paclitaxel Formulation. Pharm Res. 2017 Jun;34(6):1180-1186. doi: 10.1007/s11095-017-2132-4. Epub 2017 Feb 28.
- Scientific Posters: E Fusi, M Ghiringhelli, R Rebecca, A Crotti, C Bianchi, R Rice, R Rizzi, S

- Marelli, PA Martino” Effect of lactobacillus acidophilus strain supplementation on nutritional status and faecal quality in two canine breeds” ESVCN 20th Congress, 15-17 September 2016, Berlin, Germany.
- Scientific Posters: C Macchioni, M Ghiringhelli, ES D’Urso, E Lazzarini, F. Di Cesare, D Fonda, D Gioeni and G Ravasio “Resting metabolic rate (rmr) measurements in general anaesthesia: reduction provided by dexmedetomidine in dogs” ava Congress Lion, France, Prague, Czech Republic, 14-17 September 2016
- Oral Presentation: P Scarpa, C Palestrini, SP Marelli, M Giraldi, M Ghiringhelli, M Raja, E. Fusi” How does the nutritional assessment of dogs vary in a veterinary staff?”, 26TH ECVIM-CA CONGRESS, GOTEBORG, SWEDEN, 8-10 September 2016.
- Oral Presentation: Matteo Ghiringhelli, Stefano Brizzola, Fabio Acocella, Davide Caretti,” Clinical anatomy of the celiac trunk in the dog: application for elective surgery or surgical emergency”, 70° Congresso S.I.S.V.E.T., Palermo, giugno13-16, 2016.
- Oral Presentation: Matteo Ghiringhelli, Stefano Brizzola, Fabio Acocella, “Vacuum assisted closure system in the complex pleural empye- ma: a case report”, 70° Congresso S.I.S.V.E.T., Palermo, giugno13-16, 2016.
- Oral Presentation: Matteo Ghiringhelli, Stefano Brizzola, Fabio Acocella, “Evaluation of parenteral nutrition in malnourished dogs”, 70° Congresso S.I.S.V.E.T., Palermo, giugno13-16, 2016.
- Scientific Paper: Matteo Ghiringhelli, Stefano Brizzola, Gabriele Barella, Matteo Lodi, Stefano Faverzani, Fabio Acocella, “Octreotide as medical therapy of idiopatic chylothorax in 3 cats after surgery”, Journal of Small Animal Practice Vol 57 54, May 2016.
- Scientific Posters: Matteo Ghiringhelli, Stefano Brizzola, Gabriele Barella, Matteo Lodi, Stefano Faverzani, Fabio Acocella “Octrereotide as medical therapy of idiopatic chylothorax in 3 cats after surgery”- 2016 BSAVA Congress
- Birmingham, UK.
- Thesis Advisor: “APPORTO VASCOLARE REGIONALE DELL’ARTERIA EPIGASTRICA PROFONDA COME BASE DI SVILUPPO DI UN LEMBO MIOCUTANEO NEL CANE”, Sesana Alberto, 2014/2015.
- Thesis Advisor: “MISURAZIONE DEL METABOLISMO BASALE NEL CANE MEDIANTE UN CALORIMETRO INDIRETTO PORTATILE: VALUTAZIONE E COMPARAZIONE DI PROTOCOLLI ANESTETICO/SEDATIVI”, Eleonora Lazzarini, 2014/2015.

- Thesis Advisor: “VALUTAZIONE DELLA PENETRAZIONE NELLA PARETE ADDOMINALE DI ABRAXAME DOPO CHEMIOTERAPIA INTRAPERITONEALE IPERTERMICA (HIPEC) NEL CONIGLIO”, Marco Trovatelli, 2014/2015
- Scientific Abstract: Matteo Ghiringhelli, Georgia Pennarossa, Alessia Di Giancamillo, Stefano Brizzola, Tiziana A.L. Brevini, Cinzia Brevini, Fabio Acocella, Valentino Bontempo. Derivation of canine hepatocyte in vitro models to study Branched-Chain Amino Acid effects on liver functions. HAF journal https://doi.org/10.13130/2283-3927/7025
- Scientific Poster: ‘Computer tomography of airways and lungs during total liquid ventilation with perfloorocarbons” submitted - 41st Annual Congress of the European Society for Artificial Organs (ESAO), Gemelli Polyclinics, Rome (Italy), September 17-20, 2014
- Scientific Poster: “Chylothorax in cat: macroscopic and microscopic anatomical evaluation of the complex cisterna chyli - thoracic duct basing on a new surgical approach” - 2014 ACVS Surgery Summit in San Diego, California.