UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA

International Doctorate School in

Clinical and Experimental Medicine-XXVIII Cycle

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CARNITINES AS NEW POTENTIAL BIOMARKERS FOR PROSTATE CANCER. METABOLOMIC STUDIES IN VIVO AND EFFECT OF L-CARNITINE ON PROLIFERATION OF HUMAN PROSTATE CANCER CELL LINE PC3

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TABLE OF CONTENTS

ABBREVATIONS

PSA = prostate specific antigen

PCa = prostate cancer

DRE = digital rectal examination

TRUS= transrectal ultrasound

miRNA = microRNA

CoA = coenzime A

HDAC =histone deacetylase

PAP = phosphatase

BHP = benign prostatic hyperplasia

PIN = prostatic intraepithelial neoplasia

HGPIN = high grade prostatic intraepithelial neoplasia

CT = computerised tomography

MRI = magnetic risonance imaging

EMA = European Medicine Agency

fPSA = free PSA

cPSA = complexed PSA

tPSA = total PSA

%fPSA = percentage of free PSA

PSAD = PSA density

ATP = adenosine triphosphate

tCho = total choline

MS = mass spectometry

NMR = nuclear magnetic resonance

ESI = electrospray ionisation

LC = liquid chromatography

UPLC = ultra performance liquid chromatography

SACI = surface activated chemical ionisation

NetCDF = network common data form

mzXNL = mz extensible markup language

LC= L-Carnitine

CoA = coenzyme A

OCTN = carnitine/organic cation transporter

AIDS = autoimmune deficiency disease

FA= fatty acid

PPAR = peroxisome proliferator-activated receptor

AMPK = AMP activated protein kinase

ACC = acetyl-CoA carboxylase

qPCR = real time PCR

LUTS = lower urinary tract symptoms

IRCCS = istituto di ricovero e cura a carattere scientifico, institute for treatment and research

CID = collision-induced dissociation

NIST = National Institute of Standards and Technology

IPV4 = internet protocol-version 4

ASCII = american standard code for information interchange

FBS = fetal bovine serum

FAK = focal adhesion kinase

p-FAK = phospho focal adhesion kinase

HRP = horseradish peroxidase

PBS = phosphate buffered saline

DMSO = dimethyl sulfoxide

MAP-LC3 = microtubule-associated protein light chain

1 INTRODUCTION

Prostate cancer is one of the most common cancers in Europe, accounting for 23% of all new cases of cancer in males. It is also estimated to be the third leading cause of cancer mortality (Ferlay et al., 2013). Prostate cancer therefore constitutes a significant public health burden, and like many other cancers, the burden is likely to increase due to the continued growth in the ageing population(Monnet et al., 2015). The principal cause of death from prostate cancer is due to recurrences or metastases, particularly to the skeleton, causing debilitating bone pain and pathological fractures.

Despite technological and scientific advancements, prostate cancer management remains a challenging field. Once the diagnosis of prostate cancer is made, both patient and physician are faced with complicated risk-benefit choices that range from conservative approaches such as active surveillance to invasive surgical and radiation therapy based treatments (Klotz, 2006)(Pomerantz & Kantoff, 2007). Definitive therapy with radical prostatectomy or radiotherapy for localized disease is, in many cases, not curative, with only 80% of patients remaining free of biochemical recurrence at 10 years(Pound et al., 1999)(Jhaveri, Zippe, Klein, & Kupelian, 1999)(Han, Partin, Pound, Epstein, & Walsh, 2001). Most patients who present with advanced and metastatic disease end up dying of prostate cancer (Wirth, Hakenberg, & Froehner, 2007). Furthermore, treatment modalities often impact significantly on quality of life. Complications of therapy such as incontinence and sexual dysfunction occur in up to 70% of patients post radical prostatectomy and occur even following less invasive therapies such as brachytherapy (Quek & Penson, 2005).
Unlike most solid tumours, prostate cancer management has long employed a biomarker that is prostate specific antigen (PSA). However, PSA is not cancer-specific and various non-malignant circumstances can cause a rise in PSA blood levels, e.g. benign prostatic hyperplasia and prostatitis. Despite its low specificity for the diagnosis of prostate cancer (PCa), PSA remains currently, in combination with a digital rectal examination (DRE) and transrectal ultrasound (TRUS), the most commonly used diagnostic method for PCa.

The treatment of prostate cancer has been further affected by the widespread use of PSA screening by physicians, despite the absence of clear evidence that such screening positively affects survival. Prostate cancer is unique in its natural history, because many cancers diagnosed on prostate biopsy are not destined to develop into clinically aggressive tumours, therefore the concern with the

widespread use of PSA is the problem of over-diagnosis.
Even when using the best prognostic factors, there is a significant heterogeneity in clinical outcome, while some patients have a dramatic and aggressive disease with short life expectancy, others may have slow tumour growth and treatment can be postponed for years.

Despite all the modifications and variations of PSA that have been investigated, there is still no consensus on what is the best use of PSA to obtain optimum diagnostic and prognostic information. This warrants new biomarkers to diagnose prostate cancer more accurately, decreasing the number of negative biopsies. The search for effective biomarkers has included gene expression profiling, miRNA expression profiling, serum proteomics and metabolomics. The latter represents a promising new approach that may allow for the development of low invasive tests for cancer metabolites to detect prostate cancer.

Metabolomics is the latest of the omics technologies that employs state of the art analytical instrumentation in conjunction with pattern recognition techniques to monitor and discover metabolic changes in subjects related to disease status or in response to a medical or external intervention. Metabolomics, like other omics technologies, is currently being used for the identification of biomarkers and metabolic pathways altered in cancer; it is also being used to evaluate the efficacy of medical interventions to cancer. Cancer is a disease that is known to alter cellular metabolism; therefore, metabolomics can play a major role in early detection and diagnosis of cancer and moreover in the evaluation of medical interventions and therapies to cancer.

On the other side, given the constant increase in global cancer incidence with its associated morbidity and mortality, together with the rising healthcare costs of treatment, there is an increased interest in developing new strategies for disease prevention.
A possible approach with huge potential is chemoprevention, which is defined as the use of natural, synthetic or biological agents to reverse, suppress or prevent either the initial phases of carcinogenesis or the progression of premalignant cells to invasive disease (Sporn, 1976).
Various types of prevention approaches may be applied in prostate cancer including both primary and secondary prevention methods. Primary prevention (i.e. preventing the disease before it occurs) is a less suitable approach in prostate cancer where the aetiology is multi-factorial and risk factors are not well defined. Conversely, secondary prevention refers to interventions that prevent or minimize the progression of a disease at an early stage, thereby limiting disability once the disease is diagnosed.

Therefore, while the underlying prevalence of the disease may not be altered by secondary prevention measures, progression to clinically apparent disease may be reduced. The biological features of prostate cancer define it as a suitable target for secondary prevention.

Metabolic processes essential for tumour development are potential targets of cancer chemoprevention. L-carnitine is a trimethylated amino acid that plays an important role in cell energy metabolism by mediating the transport of long-chain fatty acids across the inner mitochondrial membrane. Carnitines exist in biological materials either as free carnitines (non-esterified molecule in relatively high concentrations) or as acylcarnitines (esterified form). In addition to its role in facilitating mitochondrial fatty acid metabolism, it has been established that L-carnitine has critical functions in regulating the mitochondrial ratio of free CoA to acyl-CoA in the transport of short- and medium chain acyl groups from the peroxisome to the mitochondria, and in the removal of excess acyl groups from the body via the preferential renal excretion of acylcarnitines.

Following the onset of a cancer, several metabolic changes are present in the whole body. Energy depletion, metabolism and cell cycle abnormalities, immune dysfunctions, proliferation, invasiveness, migration, cachexia, and death are results of neuro-bioenergetic imbalances that occur during cancer (Cuezva et al., 2002). L-carnitine may help overcome a neuro-bioenergetic crisis, enhance mitochondrial function, and effectively produce energy via oxidation of fatty acids which help the cells return to their normal metabolism, cycle, and function (non-malignant cells).
In vitro studies have shown that L-Carnitine can suppress cells cancer growth. Molecular mechanisms include increased apoptosis of cancer cells under certain conditions (Roscilli et al., 2013)(Wenzel, Nickel, & Daniel, 2005).
Additional mechanisms may also be implicated in the antitumorigenic effects of L-carnitine. It can exert inhibitory effects on cancer by virtue of its anti-inflammatory properties(Fortin et al., 2009)(Savica et al., 2005), or mediates histone acetylation inhibiting HDAC, which at least partially contributes to its cytotoxicity (Huang et al., 2012). Therefore, L-Carnitine may well be a promising tool in cancer therapeutics.

1.1 THE PROSTATE

1.1.1 PROSTATIC FUNCTION AND STRUCTURE

The prostate is the largest accessory gland in the male reproductive system. It is a compound tubuloalveolar exocrine gland which is located below the bladder and in front of the rectum and is pierced by the urethra and the ejaculatory ducts(Tortora & Derrickson, 2000).

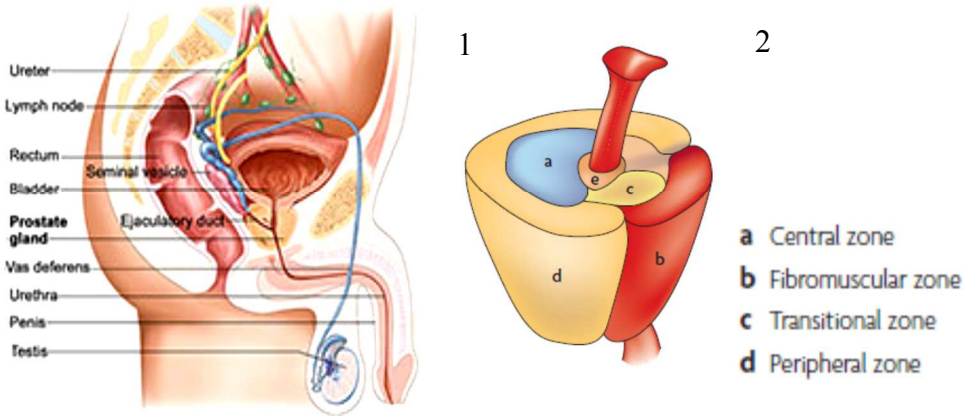


Fig.1.1 . (1) Anatomy of male reproductive and urinary system(Http://www.cancer.gov & http://www.cancer.gov/types/prostate n.d.), (**2**) Prostate and zones (De Marzo et al. 2007)].

The gland is a composite organ consisting of several glandular and non-glandular components. The non glandular tissue includes the indistinct fibromuscular capsule and the fibromuscular stroma, in which the numerous glands are embedded(McNeal, Redwine, Freiha, & Stamey, 1988). The prostate is responsible for producing a slightly white fluid that makes up part (25-30%) of semen(Tortora & Derrickson, 2000)(Ayala, Ro, Babaian, Troncoso, & Grignon, 1989). This fluid, which protects, carries and nourishes sperm cells, is rich in lipids, proteolytic enzymes, acid phosphatase, fibrolysin and citric acid. The majority of seminal fluid is produced by seminal vesicles, which are located directly behind the prostate gland. The prostate also contains some smooth muscle that helps expel semen during ejaculation.

The gland is divided into three zones: the transitional, central and peripheral zones(McNeal et al., 1988). The anterior fibro-muscular zone is the fourth zone of the prostate, but is usually devoid of glandular components and is composed only of muscle and fibrous tissue. Each glandular zone has specific architectural and stromal features.

The transitional zone forms 5% of gland's volume and contains moderately compact fascicles of

smooth muscle. The central zone comprises 25% of the prostatic volume and surrounds the transitional zone. Unlike the peripheral and transitional zone, the ducts are large and irregular. The stroma is densest in the central zone, followed by transitional zone and is least dense in the peripheral zone(Lalani, el-N, Laniado, & Abel, 1997). The peripheral zone is the largest and constitutes 70% of gland's volume. The stromal cells, smooth muscle and fibroblasts, which comprise the connective tissue framework, maintain the structure of glandular portion of the prostate. The glandular component of this organ is composed of large peripheral ducts. The acini and ducts contain secretory, basal, and neuroendocrine cells. The secretory/glandular epithelial cells secrete PSA, prostatic acid, phosphatase (PAP), acid mucin, and other secretory products in addition to expressing the androgen receptor(Lalani, el-N et al., 1997)(Bostwick, Pacelli, & Lopez-Beltran, 1997). The basal cells of the prostate form a flattened layer of inconspicuous cells at the periphery of glands, separating epithelial cells from basement membrane and stroma. These cells are thought to act as stem cells that repopulate the secretory cell layer(Bonkhoff, Stein, & Remberger, 1994).

The basal cells also display epidermal growth factor receptors, suggesting a role in growth regulation. However they contain little or no PSA, PAP or acid mucin(Maygarden, Strom, & Ware, 1992).

The neuroendocrine cells are the least common cells type of prostatic epithelium. Their function is unknown, however it has been postulated that they can serve an endocrine-paracrine regulatory in growth and development, similar to neuroendocrine cells in other organs(Aprikian, Cordon-Cardo, Fair, & Reuter, 1993)(Bonkhoff, Wernert, Dhom, & Remberger, 1991).

1.2 PATOLOGY OF THE PROSTATE

Prostate disorders are usually associated with aging, and they are among the most common of men's complaints after the age of 50. Young subjects are rarely affected by prostate problems. The main prostate disorders include: inflammatory disorders, hyperplasia, prostatic intraepithelial neoplasia and carcinoma.

1.2.1 PROSTATITIS

Inflammation of the prostate is known as prostatitis. There are four categories of prostatitis(Sommers Sawyer, 2010):

- acute prostatitis, caused by the infection of Gram-negative organisms;
- chronic bacterial prostatitis, caused by bacteria localized in prostate secretions and it is the

most common recurrent urinary tract infections in men;

- chronic prostatitis/chronic pelvic syndrome, for which there is not identifiable organism.
- asymptomatic inflammatory prostatitis, that is a symptomless microscopic condition of the prostate where patients have not genitourinary pain complaints but leucocytosis is present in the gland.

The treatment is different for the four types of prostatitis, therefore, the correct diagnosis is very important.

1.2.2 BENIGN PROSTATIC HYPERPLASIA(BPH)

Benign prostatic hyperplasia is a very common condition in older men(Edwards, 2008). BPH is the most common non-malignant condition to affect men in developed counties and is the most frequent benign condition found in prostate, occurring in more than 70% of men aged 70 years or greater.

Due to the anatomical position of the prostate, its enlargement can cause bother some urinary symptoms, as urinary hesitancy, frequent urination, impaired voiding, caused by extrinsic compression of the prostatic urethra. BPH, as with other abnormalities of the urinary tract can predispose to infection and also stone formation(Carson & Rittmaster, 2003). Age, race, ethnicity, family history, smoking and chronic diseases such as hypertension, coronary artery disease and diabetes mellitus are some of potential risk factor for BPH(De Nunzio, Aronson, Freedland, Giovannucci, & Parsons, 2012).

There are several effective treatments for prostate gland enlargement, including medications, minimally invasive therapies and surgery.

Although the pathogenesis of BPH is not well understood, there is a general agreement that it begins with stromal alterations, which then stimulate growth and variably alter the differentiation of associated epithelial cells(McNeal et al., 1988). Interestingly, most prostate cancers arise in prostate that already has BPH. However BPH originates in the transitional zone, while the peripheral zone is the most prevalent for prostate cancer. BPH is easily distinguished from prostate cancer histologically, as hyperplasia is characterized by an increased number of epithelial and stromal cells in the periurethral area of the prostate, causing a rise in formation of atypical epithelial glands, with distinct stromal configurations(Bonkhoff & Remberger, 1996). Although the benign prostate hyperplasia is not a premalignant precursor of prostate cancer, this condition has other significant similarities with prostate cancer. Both show increased prevalence with age, require androgenic stimulation and may respond to androgenic deprivation. Another fundamental aspect of benign prostatic hyperplasia is that

the current clinical marker for prostate cancer, PSA, is known to increase with age and is associated with BPH. While age-specific reference ranges have been recommended, subsequent studies have demonstrated that cancer detection (sensitivity) is significantly higher when a high percentage of free PSA is recorded. Nevertheless, considerably uncertainty remains when distinguishing between BPH and prostate cancer using the PSA test. This will be discussed later in this thesis.

1.3 PROSTATE CANCER

1.3.1.1 Epidemiology of prostate cancer

The incidence of prostate cancer varies largely between racial/ethnic groups, being highest among Africans, intermediate among Caucasians and lowest among Asians(Center et al., 2012).
In Europe the incidence and mortality of prostate cancer are approximately 416,700 and 92,200 respectively in 2012, and this kind of cancer represents the first most frequently diagnosed cancer and the third most common cause of death from cancer in men(Organization for Economic Co-operation and Develop- ment, 2010)(Bray, Lortet-Tieulent, Ferlay, Forman, & Auvinen, 2010) .

Similarly to the European trend, prostate cancer in Italy is currently the most common cancer among males (over 20% of all cancers diagnosed) older than 50 years (AIRTUM, 2013).
The prostate cancer has shown in recent decades a constant upward trend, particularly around the 2000s, coinciding with the increased use of the PSA test as a tool for early detection of prevalent

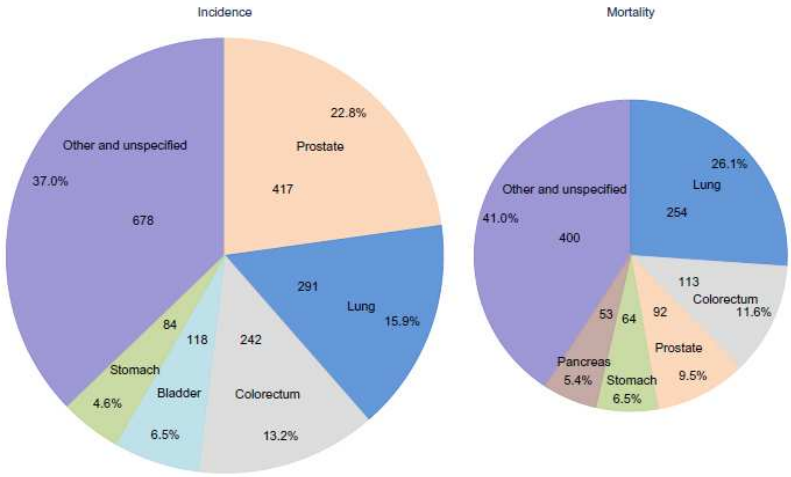


Fig.1.2 Distribution of expected cases and deaths for the 5 most common cancers in Europe in 2012 in males(AIRTUM, 2013).

cases. As for other cancers, there is a North-South gradient among the different Italian regions: 1,117 cases per 100,000 inhabitants in the North-West, 1,016 in North-East while in the Centre 803 and

South 393, due to the geographical diversification of multiple factors, primarily the spread of the PSA test(AIRTUM, 2013). Prostate cancer, despite being in the first place of incidence in Italy, it is in the third place for mortality, in almost all cases concerning males above 70 years. In 2013 in our country about 9,000 deaths from this cancer were expected, despite having to point out that the comorbidities generally found in older people can make it complex to separate the deaths from prostate cancer from those with prostate cancer.

Both genetic and environmental factors could account for the differences in worldwide prostate cancer mortality rates. The contribution of environmental factors is clearly substantiated by epidemiologic migration studies that show a change in the prostate cancer mortality rate for men migrating from low to high-risk regions. For example, men migrating from Japan to the United States have been shown to acquire the high risk of the local population as early as one generation after migration. This provides the best evidence to date of the role of environmental and lifestyle factors in the risk of prostate cancer.(Ferlay et al., 2013)

1.3.2 TYPES OF PROSTATE CANCER

Prostatic intraepithelial neoplasia (PIN)

PIN is defined as a cytological alterations in gland architecture that comprise an atypical proliferation of the secretory epithelial cells, ranging from minimal changes to those that areindistinguishable from carcinoma.

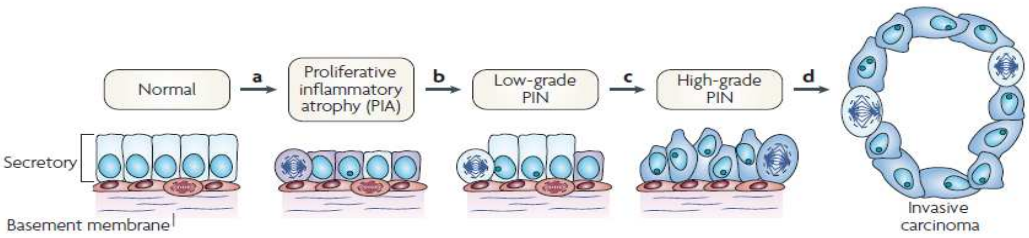


Fig.1.3 Cellular and molecular model of early prostate neoplasia progression(De Marzo et al., 2007).

PIN is subdivided into low and high grade lesions, with the distinction between on the degree of architectural and cytological changes. In low grade PIN there is proliferation of secretory cells with irregular spacing, pleomorphic nuclei and an intact basal cell layer. High grade PIN is typified by enlarged cells with increased nuclear/cytoplasmatic ratio, prominent nucleoli, coarse chromatin clumping along the nuclear membrane and variable degrees of disruption of the basal cell layer. PIN,

especially high grade, is considered to be a precursor to malignancy. It has been shown that prevalence of both HGPIN and carcinoma steadily with advancing age. HGPIN is more frequent in prostate gland that harbour carcinoma compared to benign prostate. As high grade PIN has a elevated predictive value as a marker for adenocarcinoma, repeat biopsies are warranted if detected on initial biopsy(Dabbs, 2013).

Prostate cancer

Prostate cancer is a clinically and genetically heterogeneous disease. Independent cancerous foci with distinct morphological features often coexist in a single prostate. (Shen & Abate-Shen, 2010). In effect, the presence of genomic lesions can vary between foci, including *TMPRSS2-ERG* fusion, *MYC* amplification, and *TP53* mutation(Mehra et al., 2007)(Jenkins, Qian, Lieber, & Bostwick, 1997)(Mirchandani et al., 1995). Moreover, whole-exome sequencing of over 100 primary prostate tumour–normal pairs revealed that the ubiquitin ligase complex subunit gene *SPOP* is among the most frequently mutated genes in primary tumours(Barbieri et al., 2012). This study also identified novel recurrent mutations in the fork-head transcription factor gene *FOXA1* and mediator complex gene *MED12*.

Since this tumour is composed of cancerous cells with alternative genotypes and phenotypes, it results in differentiated proliferation, aggressiveness and drug sensitivity, thereby affecting the patient's diagnosis and prognosis. In prostate cancer also some men progress rapidly to metastatic disease while others may be relatively asymptomatic for many years. In fact, portion not small (about 40%) of patients who are diagnosed with prostate cancer is destined to die "with" and not "for" their cancer(Stangelberger, Waldert, & Djavan, 2008).

Acinar adenocarcinoma is the most common form of prostate cancer which accounts for about 80%-90% of all prostate cancer(http://www.cancerresearchuk.org, n.d.). This cancer is an uncontrolled and abnormal malignant growth in the glandular tissue of the prostate. Adenocarcinoma mainly involves the peripheral zone of the gland but may grow to involve the entire gland and then metastasize to other parts of the body, especially lymph nodes and bones. There are other types of adenocarcinoma, which include atrophic, foamy, colloid and signet ring carcinoma. They are all treated in the same way as acinar adenocarcinoma.

A diagnosis of prostate cancer is adenocarcinoma to most urologists or pathologists. However, there are other forms of prostate cancer, including(http://www.cancerresearchuk.org, n.d.)

- Ductal adenocarcinoma, which starts in the cells that line the ducts of the prostate gland.
- Transitional cell (or urothelial) cancer, starting in the cells that line the urethra. The urethra passes through the prostate to carry urine from the bladder to the outside of the body. More commonly, this type of cancer may start in the bladder and spread into the prostate.
- Squamous cell cancer that starts from the flat cells covering the prostate gland, called squamous cells.
- Carcinoid tumours, that start from cells of the neuroendocrine system, which is made up of specialized nerve and gland cells.
- Small cell cancer, a type of neuroendocrine tumour that is made up of small round cells.
- Sarcomas, starting from muscle cells, while sarcomatoid cancers have a mixture of sarcoma and adenocarcinoma cells.

The treatment of prostate depends on the anatomical and aggressiveness of the disease, but also the life expectancy of the patient and the presence of comorbidity that may constitute a risk of death greater than that represented from the same prostate cancer(Damber & Aus, 2008).
Treatments options are therefore based on factors such the patient's life expectancy and tumour characteristic, including tumour stage (see section 1.3.4) and aggressiveness (see section 1.3.5).
The types of standard treatments used for prostate cancer are watchful waiting, active surveillance, surgery, radiation therapy, hormone therapy and chemotherapy, hormone therapy and chemotherapy (Brawer et al., 2001):

- Watchful waiting involves monitoring a patient's condition without giving any treatment, because the disease is asymptomatic. The patients are less likely to die "for" their prostate cancer, for both the relative sluggishness of their disease and the relatively short life expectancy (under 10 years), because of advanced age or the presence of comorbidities with higher lethality of prostate cancer(Albertsen, Hanley, Gleason, & Barry, 1998).
- Active monitoring may be a solution for very elderly patients with symptomless, low-risk, non-aggressive prostate cancer. With this approach, the patient has very regular check-ups to track the disease's evolution and allow treatment to be arranged if and when necessary.
- Radical (or total) prostatectomy is the surgical removal of the prostate and seminal vesicles. This treatment is an option for patients in good health. The main side effects are impotence and incontinence(Chin, Dave, & Rajfer, 2007).
- In the external radiotherapy the patient is subjected to high-dose rays directed at the prostate.

These rays destroy the cells by causing lesions in their DNA. The treatment with radiation from inside the prostate gland is called brachytherapy, where seeds implantation and high dose rate therapy can be used. If the cancer has not spread beyond the prostate gland radiotherapy is used to try to cure it. For more advanced prostate cancer radiotherapy is used in two main ways: treating individual areas of cancer that has spread, or treating the whole body to control pain. The main side effects of radiotherapy, both external and internal, are impotence, colic and bleeding.

- Hormone therapy is used for patients with cancer that extends beyond the limits of the prostate. Hormone treatment decreases the quantity of male hormones, especially testosterone, which in turn slows the disease's progression. The effect of hormones on prostate cancer can be only temporary, and after around two years on average, patients could develop a resistance to the hormones that makes the treatment ineffective.
- Chemotherapy is used to treat patients who are resistant to hormones. Chemotherapy slows tumour growth and in some cases eases the pain associated with cancer.

1.3.3 DIAGNOSIS AND CLASSIFICATION OF PROSTATE CANCER

The main diagnostic tools to diagnose PCa include digital rectal examination (DRE), serum concentration of PSA, and transrectal ultrasound (TRUS)–guided biopsies. However, definite diagnosis of prostate cancer depends on the histopathological verification of adenocarcinoma in prostate biopsy cores or operative specimens.

1.3.3.1 Digital rectal examination (DRE)

A DRE is a physical examination doctors use to examine the prostate through the wall of the back passage (low rectum). DRE is a long-established test used by physicians to detect changes in prostate gland but it shows low sensitivity because can only detect cancer that are relatively large and the majority of cancer occur in regions that are not accessible by DRE.(Selley, Donovan, Faulkner, Coast, & Gillatt, 1997).

1.3.3.2 PSA

The measurement of serum levels of prostate-specific antigen (PSA) is the most widely used indicator to detect prostate cancer. PSA, a kallikrein serine protease encoded by the KLK3 gene, is secreted

almost exclusively by the epithelial cells of the prostate(Lilja, Ulmert, & Vickers, 2008). Its physiological role is believed to be liquefying the seminal fluid. The normal prostate architecture keeps PSA tightly confined, so that only a minute proportion leaks into the circulatory system. In healthy adult males aged ≤50 years, the concentrations are 10^6-fold higher in seminal fluid than in blood, in which the median PSA level is ~0,6 ng/ml.(Savlom et al., 2005). In blood, PSA manifests little or no catalytic activity(Niemelä, Lövgren, Karp, Lilja, & Pettersson, 2002). This is mainly due to a $\geq 10^5$-fold excess of protease inhibitors such as α1-antichymotrypsin (ACT) and α2-macroglobulin, which inactivate any catalytic PSA by forming stable covalent complexes(a Christensson, Laurell, & Lilja, 1990)

In effect PSA in blood exists in multiple forms: free or in complexes with the various serum protease inhibitors (complexed PSA); as pro-protein pro PSA (a precursor form of PSA) or mature protein (complete protein with no modification), intact or nicked(Mikolajczyk et al., 1997)(Mikolajczyk et al., 2000). The increased blood levels of PSA in men with cancer cannot be explained by increased PSA expression. The increased blood PSA levels must instead be caused by increased release of PSA into blood. Although there are no experimental data on the mechanisms of increased release, it is believed to result from the disruption of prostate architecture seen in prostate tumours, such as disordering of the basement membrane and loss of basal cell layer, ductal lumen architecture and epithelial cell polarity(Lilja et al., 2008).

1.3.4 NEEDLE BIOPSY

A prostate biopsy is a procedure to remove samples of suspicious tissue from the prostate. During a prostate biopsy, a fine needle with ultrasound guidance is used to collect a number of tissue samples from prostate gland, through the rectum (transrectal biopsy). Less frequently, a transperineal, ultrasound-guided approach can be used in patients who may be at increased risk of complications caused by using a transrectal approach(Loeb et al., 2013).

1.3.5 GLEASON SCORE AND TUMOUR GRADING

The Gleason Scoring System is the most common prostate cancer grading system used. Tumour grade is the description of a tumour based on how abnormal the tumour cells and the tumour tissue look under a microscope, analysing samples from prostate biopsies. Histological grading correlates with tumour aggressiveness, with patient survival and helps with determining the most appropriate treatment options. There are five types of prostate cell: type 1 cells are normal and type 5 are those in which cancer is most advanced.(Humphrey, 2004).

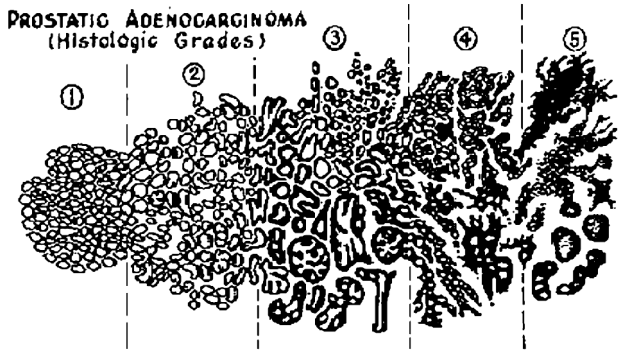


Fig.1.8 Simplified drawing of five Gleason grades of prostate cancer(Humphrey 2004).

Since prostate cancers often have areas with different grades, a grade is assigned to the 2 areas that make up most of the cancer. These 2 grades are added to yield the Gleason score (also called the Gleason sum, i.e. 3+4). The higher the Gleason score, the more likely it is that the cancer will grow and spread quickly(Humphrey, 2004):

- Cancers with a Gleason score sum of 6 or less are often called well-differentiated or low-grade. Cancers with a Gleason score sum of 7 may be called moderately-differentiated or intermediate-grade.
- Cancers with Gleason scores sum of 8 to 10 may be called poorly-differentiated or high-grade.

Gleason grading remains one of the most significant factor in the clinical decision-making on needle biopsy specimens and after radical prostatectomy is performed. However, due to its heavy reliance on human interpretation, Gleason grading is prone to subjectivity and limited-intra and inter-pathologist reproducibility. Some cases exist right at the interface between two different patterns according to the original description of the Gleason grading system, leading to inter observer and possibly intra observer variability(Lopez-Beltran, Mikuz, Luque, Mazzucchelli, & Montironi, 2006). Distinguishing between Gleason grade 3 and 4 and Gleason grade 4 and 5 can be particularly difficult when determining the percentage of each Gleason grade in the specimen(Epstein, Allsbrook, Amin,

& Egevad, 2005). Over and under grading of specimens in particular on biopsy is a major issue also. Because radiation therapy, radical prostatectomy, other therapies and overall prognosis of the disease are initially based on the Gleason score, these problems are a huge concern in prostate cancer diagnosis and better molecular markers that can make the distinction between Gleason grades would be hugely beneficial.

1.4 BIOMARKERS OF PROSTATE CANCER

1.4.1 BIOMARKERS OF DISEASE

According to the National Cancer Institute of U.S.A, a biomarker is "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease"(Http://www.cancer.gov, n.d.),such as cancer. Biomarkers typically differentiate an affected patient from a person without the disease. There is tremendous variety of biomarkers, which can include proteins (e.g., an enzyme or receptor), nucleic acids (e.g., a microRNA or other non-coding RNA), antibodies, and peptides, among other categories. A biomarker can also be a collection of alterations, such as gene expression, proteomic, and metabolomic signatures. Biomarkers can be detected in the circulation (whole blood, serum, or plasma) or excretions or secretions (stool, urine, sputum, or nipple discharge), and thus easily assessed non-invasively and serially, or can be tissue-derived, and require either biopsy or special imaging for evaluation. Cancer biomarkers can be also processes such as apoptosis, angiogenesis or proliferation.

The markers are produced either by the tumour itself or by other tissues, in response to the presence of cancer or other associated conditions, such as inflammation. Biomarkers can be used for patient assessment in multiple clinical settings, including estimating risk of disease, screening for occult primary cancers, distinguishing benign from malignant findings or one type of malignancy from another, determining prognosis and prediction for patients who have been diagnosed with cancer, and monitoring status of the disease, either to detect recurrence or determine response or progression to therapy. An ideal diagnostic biomarker should have a sensitivity and specificity close to 100% and a high predictive value in order to be clinically relevant. It should be accurate, economical, easy to perform and non-invasive collected specimen.

Many biomarkers have been approved by European Medicine Agency (EMA), however, the majority of discovered biomarkers are not sensitive and/or specific enough to be used for population screening(Issaq, Waybright, & Veenstra, 2011).

The development of non-invasive methods, to detect and monitor tumours continues to be a major

challenge in oncology. A liquid biopsy, or blood sample, for example can provide the landscape of all cancerous lesions. In 1948, the publication of a manuscript that described circulating free DNA (cfDNA) and RNA in the blood of humans was, without knowing it, the first step towards the 'liquid biopsy'(Mandel & Métais, 1948). Clearly other components of blood hold promise, including exosomes, micro-RNA, metabolomics, as well as immune system analysis.

1.4.2 PROSTATE CANCER AND PSA

The measurement of serum levels of prostate-specific antigen (PSA) is the most widely used indicator to detect prostate cancer(Brawer et al., 2001). Moreover, prostate-specific antigen (PSA) is one of the few molecular markers routinely used for detection, risk stratification and monitoring of a common cancer. The level of PSA as an independent variable is a better predictor of cancer than suspicious findings on DRE, however diagnostic confirmation always requires a prostatic biopsy(Monnet et al., 2015)]. In the context of detecting prostate cancer, an increased PSA level prompts a recommendation that the man undergo prostate biopsy, with PSA of 4 ng/ml being the traditional threshold level(Lilja et al., 2008)(AIOM, 2013). Although serum PSA test has emerged as the most useful marker in all of oncology, its poor specificity has led to prostate cancer not being detected early enough in some cases. Studies have shown that about 20% of men with PSA levels below 4.0 ng/mL have prostate cancer and that many men with higher levels do not have prostate cancer(Aus et al., 2005)(Lucia et al., 2004)(AIOM, 2013).

On the other hand, the number of cases of prostate cancer diagnosed each year is far higher than the number of deaths due to this disease. Autopsy studies indicate an even higher incidence of asymptomatic prostatic malignancy in elderly men(Martin, 2007)(Jha, Anand, Soubra, & Konety, 2014). This implies that rather than dying “of” prostate cancer, many patients are dying “with” this disease. In addition, various factors can cause a man’s PSA level to fluctuate. The presence of benign prostatic hypertrophy (BPH), numerous medications, inflammatory conditions, and physical manipulations of the male genitourinary tract can cause temporary and occasionally permanent alterations in serum PSA values. For example, some drugs, including finasteride and dutasteride, which are used to treat BPH, lower a man’s PSA level(Guess, Heyse, & Gormley, 1993)(Roehrborn et al., 2004)(Etzioni et al., 2005). PSA also varies with age, race and body mass index(Pater, Hart, Blonigen, Lindsell, & Barrett, 2012)(Henderson et al., 1997). Although PSA is relatively organ-specific to the prostate, it is not then specific for malignant neoplasms of the prostate. Moreover, this test can not help to distinguish aggressive versus non aggressive(Caplan & Kratz, 2002).

For these reasons, better molecular markers are urgently required. This will help to select patients

suitable for appropriate therapy and reduce unnecessary surgical interventions.

PSA derivatives

A number of strategies have been developed to address some of the pitfalls inherent in the use of PSA levels for the early detection of prostate cancer. Not every test has found wide acceptance in clinical practice, and most are still undergoing clinical trials.

Complexed and free PSA.

A majority of PSA in blood occurs in stable covalent complexes with protease inhibitors; these forms are collectively known as complexed PSA (cPSA). The non-complexed forms, known as free PSA (fPSA), are unreactive with plasma protease inhibitors. In proportion to tPSA, fPSA is lower in men with prostate cancer than in men with BPH(A. Christensson et al., 1993).

The ratio of fPSA to tPSA (generally expressed as the percentage of free PSA (%fPSA)) adds significant information when separating men with BPH from those with prostate cancer. The calculation of the percentage of free PSA to total PSA (the free PSA ratio) has been shown to be clinically useful by increasing specificity in patients with borderline or intermediate PSA values (4-10 ng/mL) and negative DREs. A number of studies have also shown the free PSA ratio to increase the sensitivity of cancer detection when PSA values are within the normal range(Partin et al., 1996)(Kuriyama et al., 2012).

PSA Density

PSA density (PSAD) was first introduced as a way to further distinguish prostate cancer from benign prostatic disease in patients with PSA values in the borderline range and negative DREs(Polascik, Oesterling, & Partin, 1999).

The definition of PSAD is the ratio of the serum concentration of PSA to prostatic volume as measured by transrectal ultrasound. The ratio relies on the fact that prostate cancer releases more PSA per volume of prostatic tissue into the circulation than does BPH(Polascik et al., 1999).

However, studies using PSAD for prostate cancer screening have led to conflicting results, and PSA density requires a transrectal ultrasound, which is expensive, time-consuming and causes significant patient discomfort(Lilja et al., 2008). This might help to explain why PSA density is not widely used in the clinical setting.

PSA dynamics: velocity and doubling time

PSA velocity, the change in PSA level over a specified time interval, has been much advocated as a means of identifying men with prostate cancer. PSA velocity is associated with diagnosis of prostate cancer and with risk of recurrence or cancer-specific death after treatment(D'Amico, Chen, Roehl, & Catalona, 2004). PSA doubling time, the time required for the PSA level to double, is mostly used to monitor disease progression for patients after initial surgery and radiotherapy and for patients who choose surveillance rather than definitive treatment(D'Amico et al., 2006).

Limitations of this method include the need for multiple PSA determinations and the necessity for cumbersome calculations.

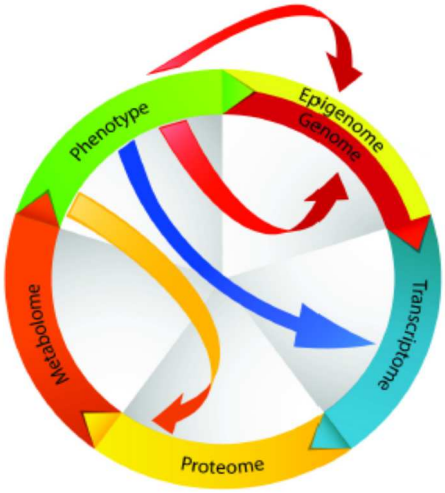


Fig.1.10 Model of sequential and synchronous events contributing to the phenotype. According with this model, the metabolome represents the closest molecular representation of phenotype(Bathe & Farshidfar, 2014)

Age and Race-Specific PSA Reference Ranges.

After age 50, the median PSA level increases, presumably as a result of the increasing frequency of benign prostate diseases with age: BPH, for example, is found in only a few percent of 40 year olds but in a quarter of 60 year old(Verhamme et al., 2002). Because of this rise in PSA, age-specific reference ranges have been proposed as a means of increasing the sensitivity of detection in younger men and the specificity in older men(Morgan et al., 1996)(Borer, Sherman, Solomon, Plawker, & Macchia, 1998) .

Age-specific ranges have, however, been criticized, mainly for missing clinically significant cancers in older men, and have not become uniformly accepted.

It has been reported that African men have increased PSA values compared with white men, regardless of age, clinical stage, and histologic grade(Morgan et al., 1996).

Some speculate that this difference is due in part to an increased volume of tumour at the time of diagnosis in African American men in comparison with their white counterparts. Despite their importance, understanding of racial differences in PSA and their clinical implications is far from complete(Lilja et al., 2008).

1.5 METABOLOMICS

Metabolomics is the latest of the omics technologies that employs state of the art analytical instrumentation in conjunction with pattern recognition techniques to detect small metabolites (<1500

Da) in biofluids or tissue, and follow their changes related to disease status or in response to a medical or external intervention. Metabolomics is a term that encompasses several types of analyses, including:

(a) metabolic fingerprinting, analysis of samples following minimal sample preparation and employing limited identification and quantification. Samples are analysed with the sole purpose of classifying them (eg, into diseased and healthy classes)
(b) metabolic profiling, the quantitative study of a group of metabolites, known or unknown, within or associated with a particular metabolic pathway
(c) target isotope-based analysis, which focuses on a particular segment of the metabolome by analysing only a few selected metabolites that comprise a specific biochemical pathway.

Metabolomics allows for a global assessment of a cellular state within the context of the immediate environment, taking into account genetic regulation, altered kinetic activity of enzymes, and changes in metabolic reactions. Thus, compared with genomics or proteomics, metabolomics reflects changes in phenotype and therefore function. The omics sciences are, however, complementary as "upstream" changes in genes and proteins are measured "downstream" as changes in cellular metabolism. The converse is that metabolomics is also a terminal view of the biological system, not allowing for representation of the genes and proteins that are increased or decreased.

The origin of metabolomics dates back decades, with initial key applications in the fields of inborn metabolic errors, toxicology, and functional nutrigenomics, but it has been recognized that cancer is a disease that is known to alter cellular metabolism(Griffin & Shockcor, 2004); therefore, metabolomics, like the other omics technologies, can play a major role in early detection and diagnosis of cancer and moreover, in the evaluation of medical interventions and therapies of cancer(Kim, Maruvada, & Milner, 2008)(Spratlin, Serkova, & Eckhardt, 2009)(Fan, Lane, & Higashi, 2004).

1.5.1 METABOLOMICS IN CANCER

The classic example of metabolic reprogramming is the Warburg Effect(Warburg, 1956). In normal cells, in the presence of sufficient oxygen, glucose is processed through oxidative phosphorylation, which is the most efficient means of generating ATP. Glycolysis only becomes a primary means to metabolize glucose in hypoxic conditions. However, in cancer cells, glycolysis is the dominant

pathway for glucose metabolism, and this is independent of oxygen supply. The advantage to the tumour cell is that this is a much more rapid means of ATP production, which is necessary to support rapid cellular proliferation.

Pattern recognition technologies in all omics have been used for the diagnosis of several tumour types using a variety of experimental platforms. Perhaps the best application of metabolomics thus far in cancer diagnostics is in breast cancer(Claudino et al., 2007).

Several studies have analysed breast biopsy samples and have identified over 30 endogenous metabolites in breast tissue. Breast cancers reliably showed elevated tCho (total choline-containing compound) levels (resulting from increased phosphocholine), low glycerophosphocholine, and low glucose compared with benign tumours or healthy tissue(Bathen et al., 2007)(Glunde, Jie, & Bhujwalla, 2004)(Sitter et al., 2006). In vivo, metabolites analysis of the breast was performed on 77 patients before biopsy, and precise differentiation of cancer and benign tissue is possible based on choline detection, with a sensitivity of 100%(Bathen et al., 2007). It is relevant to underline that a biopsy could have been prevented 68% of the time if only performed on the choline-positive tissue(Jacobs, Barker, Bottomley, Bhujwalla, & Bluemke, 2004)(Bartella et al., 2007).

Data in brain cancers is extensive with defined metabolomic biomarkers established from studies of brain tumour specimens(Florian, Preece, Bhakoo, Williams, & Noble, 1995)(Maxwell et al., 1998) . In vitro, cell lines from meningiomas, neuroblastomas, and glioblastomas showed metabolic patterns that reflect differences in alanine, glutamate, creatine, phosphorylcholine, and threonine that are distinct among the histologic subtypes. Clinically, metabolite levels have been compared with histology results from biopsy specimens in 29 primary glioma patients(Dowling et al., 2001) . Interestingly, in this study, the histologic presence of cancer correlated with abnormally elevated tCho and decreased N-acetyl aspartate levels.

Metabolomic differences between healthy women and those with epithelial ovarian cancer have been investigated(Odunsi et al., 2005). Serum metabolic profiles correctly separated 38 women with cancer from 53 normal premenopausal women and those with 12 benign ovarian disease in 100% of cases; there was also a 97.4% separation rate for cancer patients versus normal postmenopausal women. Interestingly, in another study, metabolic profiling of ovarian tumour tissue showed a statistically significant differentiation between invasive ovarian carcinomas and borderline tumours as reflected by differences in 51 metabolites(Denkert et al., 2006) .

1.5.2 METABOLOMICS ANALYTICAL TECHNIQUES

Currently, there is no single analytical technique which can be used to analyse the entire complex nature of the metabolome in any given biological sample(Aboud & Weiss, 2013). There are a number of techniques, which are available to researchers each of which may be employed for experimental purposes to analyse the same samples, in order to overcome the limitation in technologies. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) are the most frequently used to detect the mass of metabolites.

NMR exploits the behaviour of molecules susceptible to a magnetic field (nuclear spin $\neq 0\rangle$, allowing the identification of different nuclei based on their resonance frequency. This technique requires minimal separation and is non-destructive. It is, however, far less sensitive than other techniques and provides a much reduced coverage of the metabolome(Veenstra, 2012).

1.5.3 MASS SPECTOMETRY

Mass spectrometry, as the name implies, measures the mass of a molecule from which potentially the chemical structure can be discerned. To do this the mass spectrometer has to electrically charge, or ionize the chemical compounds, and thus generate charged molecules or molecular fragments, whose mass to charge ration (m/z) can be measured; the ionized samples, indeed, are accelerated through an electromagnetic field, which allows the detection of the differently charged ion species, or the sample molecules.

Mass spectrometry produce an output of data for each separated analyte, which has been eluted. The relative response of the detected analytes is measured as intensity of the signal against time. Individual spectra of each peak can be used to identify analytes, using internal standard or metabolites libraries stored in the devices in order to identify unknown peaks.

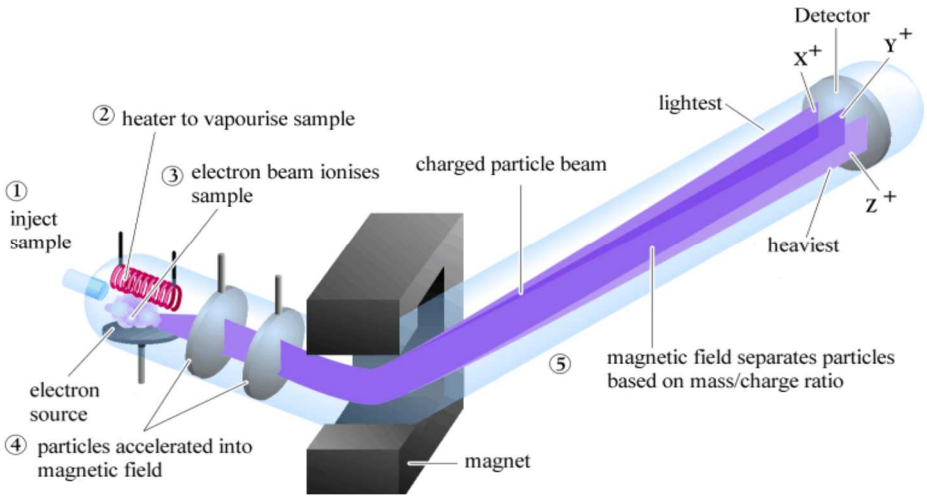


Fig.1.11 The functional aspects of a mass spectrometer.

1.5.3.1 Electrospray and SACI-ESI ionisation

The development of electrospray ionization for the analysis of biological macromolecule was rewarded with the attribution of the Nobel Prize in Chemistry to John Bennett Fenn in 2002. Electrospray ionisation (ESI) is a method of generating ions without causing fragmentation of the ions. ESI techniques have been used to analyse biological molecules extensively(Gaskell S.J., 1997). In ESI, separated analytes are transported in liquid solution into a capillary. The flow of liquid is aided by an inert gas, which passes in the same direction as the liquid. The capillary is either positively charged or negatively charged, depending on the type of ionisation required (positive or negative ionisation mode). The ions emerge as droplets from the column, and are carried by the inert gas towards the counter electrode, which is oppositely charged to the capillary. The gap between the capillary and the counter creates an electrode potential. The sprayed ions which are highly charged, and as they emerge from the capillary and until they reach the counter emerge as gas-ions. These ions are then introduced into the MS where ions separated on the basis of their mass to charge ratio.

ESI can ably ionize across a large mass range, with good levels of sensitivity, preserves the ionised metabolites, and therefore does not cause further fragmentation of the analytes(Gaskell S.J., 1997). Therefore ESI is the most commonly used ionisation method in metabolomics experiments and is appropriate for targeting amino acids, organic acids, sugars, steroids, fatty acids, phospholipids(Allwood & Goodacre, 2010) .

MS systems using an electrospray ion source coupled with mass analysers (LC–ESI–MS) have been

applied to a wide variety of studies in pharmaceutical analysis and life sciences. LC–ESI–MS is now considered the benchmark for the discovery of clinical biomarkers(Brewer & Henion, 1998)(Johanning et al., 2015)(Naldi et al., 2009).
However, due to sensitivity limitations related to in-source ionization yield, many potential biomarkers are not detected by this mass spectrometers. Therefore, more efficient ion-source technologies are needed to improve MS applications in biomarker discovery. Examples of increasing analyser sensitivity include the combination with high-resolution mass analysers, such as the Orbitrap, that are capable of storing and detecting high numbers of ions(Scigelova & Makarov, 2009).

Two strategies can be employed to improve ion source performance: (i) increasing the ion source ionization efficiency and (ii) decreasing in-source production of species (water cluster, contaminants, etc.) that can lead to spectrum chemical noise.
Recently, an interesting approach named Surface-Activated Chemical Ionization (SACI) has been developed in order to reduce the solvent-charged species (solvent charged clusters, etc.) that can lead to an increase in chemical noise(Cristoni, Rubini, & Bernardi, 2007). Basically, a metallic surface at low voltage (50–800 V) is placed inside the ion source. The solvent molecules adsorbed on the metallic surface are not ionized on the surface itself, but polarized, thus modifying their proton affinity. This leads to an increase in the proton transfer reaction efficiency responsible for analyte production. However, the specificity of the ion source for acidic and basic molecules does not facilitate the use of SACI for biomarker discovery purposes. Coupling of the highly sensitive SACI with the well-known ESI technique can overcome this limitation..

The advantage of this combination is the possibility to work at lower ionization potentials with respect to ESI alone. In addition, the electric field of the metallic surface in the SACI source, which is placed at an angle of about 45 degrees relative to the axis of the ESI spray cone can work as an ion deflector, pushing ions towards the entry orifice of the mass analyser. This allows a reduction in the chemical noise due to the extra-charged solvent environment and an increase in analyte ion signal due to the high efficiency of ESI ionization combined with the SACI ion impact evaporation and focalization effects. This technical improvement widens the possibility of potential biomarkers discovery(Sogno, Conti, Consonni, Noonan, & Albini, 2012) .

1.5.3.2 Orbitrap mass analysis

In Orbitrap mass analyser the sample ions produced by ion source are injected tangentially into this ion-trap, where an electric field is set between an outer barrel-like electrode and an inner axial electrode. The ions end up moving in ring-like orbits around the axial electrode. Once their attraction to the inner electrode is exactly balanced by the centrifugal forces, the ions are trapped in their orbits. These rings of ions are now set to move right and left along the axis of the central electrode in harmonic oscillations.

The complex signal from the electrodes is amplified and transformed into a frequency spectrum by fast Fourier transformation. This is finally converted into a mass spectrum, where the oscillation frequency for each ion is used to calculate its mass to charge ratio - being inversely proportional to the square root of m/z(Hu et al., 2005).

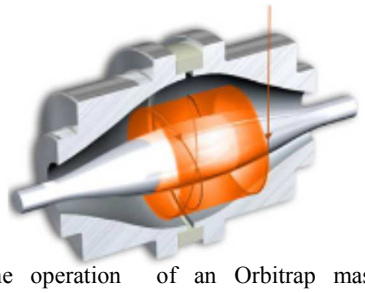


Fig.1.12 The operation of an Orbitrap mass analyser(Http://www.thermoscientific.com, n.d.)

1.5.3.3 Liquid chromatography

Most compounds, which can dissolve in a liquid can be analysed using liquid chromatography. Liquid chromatography separation techniques are achieved by distributing a sample between a liquid (mobile phase) and a column packed with stationary phase substance, by a powerful pump. The end of the column, or outlet is connected to a detector, which records the retention time (RT) for every detected analyte. LC can also be coupled to a MS. The data collected from the analysis of a sample using an LC coupled to an MS, for each separated and detected analyte is retention time, signal response or relative abundance, the molecular mass of the peak and the ionic charge.

The separation and detection of constituent analytes, is achieved on the basis of the affinity of the analyte with the mobile phase and stationary phase(Adlard, 1988). Components that have a higher affinity for the mobile phase compared with the stationary phase migrate more rapidly, while components that have a higher affinity for the stationary phase are eluted from the column later. This difference in migration velocity ultimately leads to physical separation of the components in a sample. The order and resolution of the components emerging from the column depend on the type of selected stationary and mobile phases. A consideration in LC-MS experiments is the time it takes to complete the analysis of a sample. If the assay contains compounds, which require longer retention times, and

this occurs when a sample contains non-polar compounds, such as hydrocarbons and lipids, the separation time required can be greatly lengthened. For this reason a complex aqueous sample such as serum requires sometime extraction processes prior to analysis using LC-MS. In LC-MS the samples have to be extracted in an organic solvent(Beltran et al., 2012).

1.5.3.4 Sample preparation

In any metabolomic study there is a need to ensure that samples collected for analysis are processed in a standardised manner, thus the subsequent storage, and preparation before sample analysis is also performed in a standard manner, in order to reduce the inclusion and influence of external variables which could invalidate the study findings(Ganti & Weiss, 2011)(Dunn, Wilson, Nicholls, & Broadhurst, 2012).

Blood tests are usually performed on plasma or serum. Plasma is the liquid component of blood, containing the protein fibrinogen, which is a clotting factor; when clotting factors are removed the liquid is termed serum. Serum like plasma contains proteins, electrolytes and moreover is home to a large number of metabolites, and is a complex aqueous solution, containing dissolved gases, hormones, metabolic waste and nutrients of the body, and is approximately 95% composed of water(Maxwell et al., 1998)(Marks, Cantor, Mesko, Pullmann, & Nosalova, 2002).

In a biological sample such as human serum there are possibly between 1,000 and 10,000 metabolites, depending on their concentrations(O'Hagan et al., 2007). The total number of metabolites is thought to be dependent on the number of enzymatic reactions. Therefore the metabolite number and concentration remain in a dynamic state. Before the metabolome of a sample can be analysed, the enzymatic activity have to be stopped or quenched. Metabolism is quenched by freezing the sample as quickly as possible. Rapid freezing achieved through the use of liquid nitrogen results in reduction of metabolite concentrations, and causes an irreversible binding of metabolites to cell walls. Therefore storing samples at -80°C is effective at stopping reactions(Dunn, Bailey, & Johnson, 2005).
Once the metabolic activity is stopped, extraction of metabolites by organic solvents prior to analysis can be necessary in order to allow proper analysis. It is essential that the metabolites are separated form large molecules (proteins, lipids and large peptides) and salts. The extraction can be based on their acidic, alkalinic, or ethanolic properties(Goodacre, 2007).

1.5.3.5 Metabolomics experiment work flow

Technologies used in metabolomics produce large amounts of data, and handling such complex metabolomic data sets is an important step that has big impact on extent and quality at which the metabolite identification and quantification can be made. In metabolomics we are primarily interested in biological systems responses, resulting in metabolite level regulation related to genetic variation or to a multitude of environmental changes; the quality of data processing is therefore an essential step for our ability to properly analyse and interpret metabolomic data. The data generated in metabolomics experiments undergoes a number of steps before any statistical analysis can be undertaken.

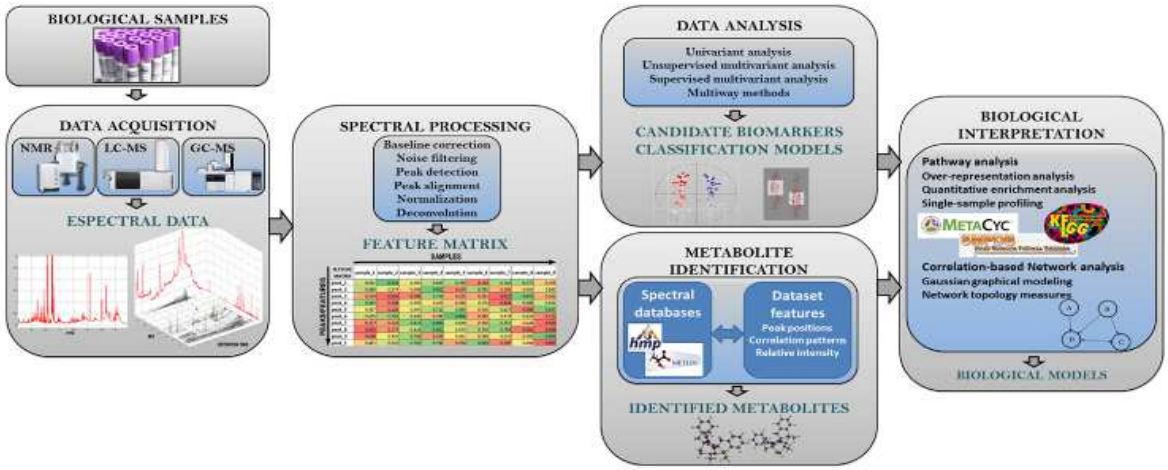


Fig.1.13 Analysis workflow in metabolomic studies(Alonso et al., 2015)].

Data handling tasks in metabolomics can be roughly divided into two steps: data processing and data analysis(Sugimoto, Kawakami, Robert, Soga, & Tomita, 2012)(Katajamaa & Oresic, 2007). The data processing step consists of low-level processing of raw data and combining data between measurements.

These tasks transform the raw data into a format easy to use in the subsequent data analysis steps. The data analysis stage includes tasks for analysis and interpretation of processed data. This typically includes multivariate analyses such as clustering of metabolic profiles or discovering important differences between groups of samples.

1.5.3.6 Data processing

The basic aim of data processing is to transform raw data files into a representation that facilitates

easy access to characteristics of each observed ion.

Since different instrument vendors utilize different proprietary data formats, a preliminary step for data processing requires a software that supports metabolomic data from multiple vendors, and conversion of such raw proprietary data into common raw data format such as netCDF or mzXML(Dettmer, Aronov, & Hammock, 2007)(Pedrioli et al., 2004). Vendor software packages usually contain scripts that can perform data conversion to netCDF or ASCII formats. Converters to more recent mzXML format have been developed both by research groups and companies.

The starting point for data processing is a set of raw data files, each file corresponding to a single biological sample. A single LC–MS data file is a collection of successively recorded peaks, each representing hits of ionized molecules on the detector during a small time frame. A peak consists of a number of m/z and intensity data points. The basic aim of data processing is to transform raw data files into representation that facilitates easy access to characteristics of each observed ion(Sugimoto et al., 2012). These characteristics include m/z and retention time of the ion and an ion intensity measurement from each raw data file.

Typical data processing pipeline usually proceeds through multiple stages(Katajamaa & Oresic, 2007), including:

- Peak detection and filtering, to identify all signals caused by true ions and avoid detection of false positives.
- Peak matching, to match the identified peaks across samples to allow calculation of retention time deviations and relative ion intensity comparison.
- Peak alignment and deconvolution, to correct retention time differences between runs and combining data from different samples. Deconvolution can be used to resolve metabolites whose chromatograms may not be completely resolved, and determine the s/n ratio(O'Hagan et al., 2007).
- Deconvolution is also employed to distinguish co-eluting compounds that have different mass spectra but the same chromatogram.
- Normalization, to remove the unwanted systematic bias in ion intensities between measurements, while retaining the interesting biological variation.

As a result, the data is transformed into a two-dimensional matrix that can be processed with various statistical tools. In this matrix, one index corresponding to the retention time scans, and another to

fixed m/z values, while matrix values represent the ion intensities(Hilario, Kalousis, Pellegrini, & Müller, 2006).

1.5.3.7 Identification of Metabolites

Compound identification is a key element in untargeted metabolomics experiments aimed at understanding the global metabolic changes that can occur in a biological system.
Although the mass-based search alone is inadequate to identify a metabolite, it is an important step in the identification process since it provides putative candidates for subsequent confirmation. Thus, accurate-mass matching is the most commonly used method to identify compounds in metabolomics identification, but mass information alone is not enough to make a confident identification. Combining retention-time matching with accurate-mass matching results in greater confidence in compound identification(Brown et al., 2011).

Multiple metabolite databases have been assembled during the past years for metabolomic identification; databases that include a large number candidate compounds are essential for metabolomic research(Alonso, Marsal, & Julià, 2015)(Wishart, 2009). However, none of the existing databases guarantees a complete coverage of the metabolome(Zhou, Wang, & Ressom, 2012), then the accurate identification of a compound usually requires the ability to match candidate spectra with standard compounds run under the same conditions(Sugimoto et al., 2012).

1.5.3.8 Data analysis

Once a data matrix has been produced from raw data, subsequent steps usually involve different forms of statistical analysis and data mining to allow the identification of samples or variables (metabolites) that capture the bulk of variation between data sets and that may represent candidates for biologically meaningful variables. Typical analyses of metabolomic data consist of two phases; initially an overview of the given data sets is generated using multivariate analysis and individual peaks are subsequently graded by univariable analysis(Sugimoto et al., 2012). Especially commercial software for metabolomic data processing also includes some in-built statistical methods for the downstream data analysis.

Because metabolomics generates data on multiple different metabolites, global overview methods that take into account the possible correlations between variables are the main tools used. However,

when used appropriately, monovariate methods can also provide useful insight and remain widely used, especially for secondary biomarker analyses. Although multivariate classification methods are often used to identify biomarkers, the discrimination of individual metabolites is usually assessed by conventional univariate statistical tests, such as Student's t-test and the Mann-Whitney test for two classes, or ANOVA and Kruskal-Wallis for multiple classes (≤3).

1.5.3.9 SANIST Platform for data analysis

In this project a new bioinformatic tool, termed SANIST, developed in house, was adopted for the first time.

This platform combines LC/SACI/ESI-MS data acquisition of the m/z signal related to potential biomarker candidates, with a sample classification based on an innovative and modified Bayesian mathematical model, adopted for the first time. This novel algorithm is based on the comparison of a selected biomarker fingerprint with those stored in a database using an improved Bayesian mathematical model.

SANIST data elaboration is based on the calculation of the probability that the detected biomarker profile was related to a known disease, for which the system was instructed by analysing and inserting biomarker profiles of samples from patients and controls subjects.

1.6 CARNITINES

1.6.1 L-CARNITINE

L-Carnitine (LC) (laevocarnitine; 3-hydroxy-4-trimethylaminobutyrate) is a naturally occurring compound found in all mammalian species. L-Carnitine was first discovered to be a quantitatively important compound in muscle tissue in 1905(Gulewitsch & Krimberg, 1935) and its chemical structure was established in 1927(Tomita & Sendju, 1927). However, the physiological function of L-carnitine was not established until a series of experiments in the 1950s, which were able to ascertain the importance of L-carnitine in fatty acid oxidation(Fritz, 1955)(Friedtnan & Fraenkel, 1955)(Carter, Bhattacharyya, & Weidman, 1952). Carnitine is a low molecular weight polar molecule that, due to its chiral carbon, exists as two enantiomers: D- and L-carnitine; however, only the L-isomer is physiologically active(Bremer, 1983)(Bahl & Bressler, 1987).

Carnitine is able to form high-energy bonds with carboxylic acids at its hydroxyl group, which results

in the formation of acylcarnitine derivatives. The endogenous carnitine pool is comprised primarily of L-carnitine with short-, medium- and long-chain acylcarnitines forming the remaining component.

1.6.2 CARNITINE FUNCTIONS

The most important biological function of L-carnitine is in the transport of fatty acids into the mitochondria for subsequent ß-oxidation, a process that results in the esterification of L-carnitine to form acylcarnitine derivatives(Bremer, 1983). As such, the endogenous carnitine pool is comprised of L-carnitine and various acylcarnitines ranging from the short-chain acetyl-L-carnitine (2 carbon length acyl moiety) to the long-chain stearoyl-L-carnitine (max 18 carbon length acyl moiety)(Choi, Fogle, Clarke, & Bieber, 1977). L-Carnitine is the major representative in the carnitine pool such that the normal acylcarnitine to L-carnitine ratio is 0.25(Pons & Darryl, 1995).

In addition to its role in facilitating mitochondrial fatty acid metabolism, it has been established that L-carnitine has critical functions in regulating the cellular to mitochondrial ratio of free CoA to acyl-CoA in the transport of short- and medium chain acyl groups from the peroxisome to the mitochondria, and in the removal of excess acyl groups from the body via the preferential renal excretion of acylcarnitines(Bremer, 1983)(Hoppel, 2003)(C J Rebouche & Paulson, 1986).

Human requirements for carnitine are usually met with a combination of diet and endogenous biosynthesis. Supplementation of L-carnitine is ameliorative in various diseases, while some toxic effects are associated with high dose of long-chain carnitine esters. Indeed they have been shown to have detergent effects on cellular membranes and are therefore able to disrupt membrane barriers and produce complete membrane solubilisation(Faergeman & Knudsen, 1997)(Requero, Goni, & Alonso, 1995).

1.6.3 MITOCHONDRIAL FATTY ACID OXIDATION

The mitochondrial metabolism of cytosolic fatty acids begins with activation via acyl-CoA synthase to form acyl-CoA. The resultant thioester is then able to cross the outer mitochondrial membrane via a voltage-gated anion channel into the membrane space where it undergoes transesterification by

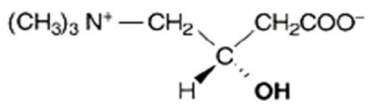


Fig.1.14 Chemical structure of L-carnitine, with the site of esterification illustrated in bold.

carnitine acyltransferase with L-carnitine to form free CoA and the corresponding acylcarnitine(McGarry & Brown, 1997).

This acylcarnitine is then transported across the inner mitochondrial membrane via a membrane-bound L-carnitine/acylcarnitine translocase, in exchange for a free L-carnitine from within the mitochondria(Pande, 1975)(Pande & Parvin, 1980).

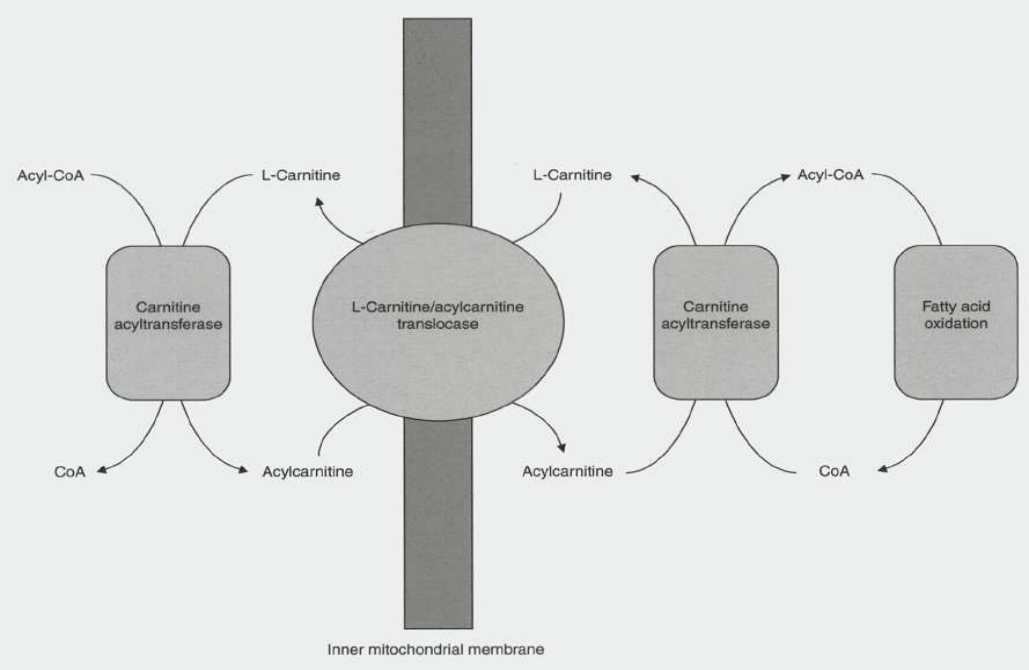


Fig.1.15 Schematic diagram of the function of L-carnitine in the transport of fatty acids across the inner mitochondria membrane. (Stephanie E. Reuter & Evans, 2012)

Once inside the mitochondrial matrix, the acylcarnitine and available free CoA undergo reverse transesterification by carnitine acyltransferase to form L-carnitine and the corresponding acyl-CoA, which enters the fatty acid ß-oxidation pathway (Fig.1.15). Three different groups of carnitine acyltransferases have been described which differ in their substrate specificity: carnitine acetyltransferases mediate the transesterification of short-chain acyl groups; carnitine octanoyltransferases utilize medium-chain acyl groups as substrates; and reactions involving long-chain acyl moieties are facilitated by carnitine palmytoiltransferases(Sharma & Black, 2009) . Given that neither free fatty acids nor acyl-CoA are able to move across the inner mitochondrial membrane alone, the role of L-carnitine in fatty acid metabolism is vital.

1.6.4 MODULATING INTRACELLULAR COENZYME A HOMEOSTASIS

The role of L-carnitine in buffering the ratio of free CoA to acyl-CoA is a function that is particularly important under conditions of stress. Under normal conditions, short- and medium-chain acyl-CoA, formed as a result of various mitochondria pathways, are further metabolized to generate free CoA. However, under abnormal conditions in which excess quantities of these CoA esters are formed within the mitochondria, acyl-CoA can undergo transesterification with L-carnitine to form acylcarnitines, thereby freeing CoA for use in other mitochondria reactions(Bremer, 1983)(Hoppel, 2003)(C J Rebouche & Paulson, 1986). This reversible exchange, in combination with the ability of the resultant acylcarnitine to cross the mitochondrial membrane (via L-carnitine/acylcarnitine translocase), means that the intramitochondrial relationship between free CoA and acyl-CoA is reflected in the extra-mitochondrial ratio of acylcarnitine to L-carnitine(Pons & Darryl, 1995).

1.6.5 PEROXISOMAL FATTY ACID OXIDATION

Peroxisomal fatty acid metabolism involves the incomplete ß-oxidation of very long-chain fatty acids to short- and medium-chain acyl derivatives(Osmundsen, Bremer, & Pedersen, 1991). While the metabolism of very long-chain fatty acids occurs via a carnitine-independent mechanism, activity of carnitine acetyltransferase and carnitine octanoyltransferase have been established within the peroxisomes(Bieber, 1988).

It is thought that chain-shortened acyl-CoA formed via peroxisomes fatty acid oxidation undergoes transesterification by carnitine acyltransferases within the peroxisome to form short- and medium-chain acylcarnitines, which are subsequently transported into the mitochondria for complete ß-oxidation(Ramsay, 1999).

1.6.6 CARNITINE HOMEOSTASIS

Given the fundamental importance of L-carnitine for mammalian survival via its pivotal role in facilitating mitochondrial and peroxisomal fatty acid oxidation, as well as in the modulation of the intracellular relationship between free CoA and acyl-CoA, it is not unexpected that endogenous plasma and tissue concentrations of L-carnitine and its ester derivatives are maintained within relatively narrow limits. L-Carnitine homeostasis is multifaceted because its concentrations are achieved and maintained by a combination of absorption from dietary sources, de novo biosynthesis, carrier-mediated distribution into tissues and extensive, with saturable, renal tubular

reabsorption(Wall & Porter, 2014) .

1.6.7 ABSORPTION

Dietary L-carnitine intake is largely achieved via consumption of animal-based products including red meats, poultry, fish and dairy products, while negligible quantities are available from plant derived foods(Steiber, Kerner, & Hoppel, 2004)(C J Rebouche & Paulson, 1986).

Given the broad range of nutritional choices, dietary L-carnitine intake can vary considerably, with the standard omnivorous diet providing 6-15 μmol/kg/day whereas vegans acquire <1 μmol/kg/day from their diet and semi-vegetarians (i.e. those who eat chicken, fish and dairy products but not red meat) acquire 1-8 μmol/kg/day(Steiber et al., 2004)(Charles J Rebouche, 2004).
However, despite substantial differences in L-carnitine intake, previous research has established that vegetarian diets do not result in a nutritionally significant deficit in endogenous carnitine concentrations(Lombard, Olson, Nelson, & Rebouche, 1989); in fact, on average, endogenous plasma L-carnitine, total carnitine and estimated acylcarnitine concentrations for vegetarian adult subjects were only 10-20% lower than those for adult subjects consuming an omnivorous diet. On the other hand, urinary carnitine excretion for L-carnitine and (estimated) acylcarnitine were, on average, 85-95% and 40-50% lower for vegetarians than for non-vegetarians, respectively(Stephanie E. Reuter & Evans, 2012); These findings indicate that compensatory mechanisms, including renal conservation in conjunction with biosynthesis, are effective in maintaining carnitine homeostasis when dietary L-carnitine intake is low. Orally ingested L-carnitine and its short-chain acyl derivatives are taken up from the small intestinal lumen into the enterocytes via a two-component system: a saturable system mediated via the active, sodium-dependent organic cation/ carnitine transporter OCTN2, and a linear component representing passive diffusion(Charles J Rebouche, 2004)(B. Li, Lloyd, Gudjonsson, Shug, & Olsen, 1992)(Gross & Henderson, 1984). The efficiency of absorption diminishes as the carnitine content of the diet increases, reflecting the saturability of the OCTN2 transporters, even at levels of normal dietary intake(Charles J Rebouche & Chenard, 1991).

1.6.8 SYNTHESIS

L-Carnitine is synthesized from the amino acids L-methionine and L-lysine, after multiple steps involving four enzymes and several cofactors. The entire biosynthetic pathway took a decade of research to be elucidated. In the early sixties it was shown that the injection of ^{14}C-butyrobetaine in

rats resulted in apparition of radiolabeled carnitine in urine and tissues and that methyl-labelled methionine leads to the incorporation of radioactivity into the 4-N-methyl groups of carnitine(Bremer, 1962).The biosynthetic origin of the carbon-chain and 4-nitrogen atom remained unclear for several years and, in 1971, it was shown by different investigators that lysine is the carbon chain and nitrogen atom donor(Vaz & Wanders, 2002)(Tanphaichitr & Broquist, 1973)(Steiber et al., 2004).
The amount of L-carnitine produced endogenously (for an omnivorous adult) is approximately l-2|µmol/kg/day(Charles J Rebouche, 2004)(C. J. Rebouche, 1992)(Steiber et al., 2004).

1.6.9 DISTRIBUTION

L-Carnitine is present in most parts of the body, mainly in skeletal muscle and heart, and this is not unexpected given the critical role of L-carnitine in muscular fatty acid oxidation. Since carnitine biosynthesis in human is restricted to liver and kidney, and two thirds of the carnitine needs are provided by diet, this implies the existence of a mechanism by which carnitine can be distributed from its place of synthesis or absorption to all tissues that rely upon carnitine for their energy supply. The total body L-carnitine content is estimated to be approximately 130 mmoles for a healthy 70 kg adult male(Brass, 1995).
Given that carnitine concentration in tissues is 20 to 50 fold higher than in plasma(Brass, 1995), carnitine uptake from blood into tissues must take place via an active transport process against a concentration gradient.

Tissue compartment	Compartment size	Carnitine concentration	Carnitine content (mmol)	Total body carnitine (%)	Km value (µmol/L)	Turnover time (h)
Plasma	3 L/70 kg	50 µmol/L	0.2	0.1		
Extracellular fluid (including plasma)	13 L/70 kg	50 µmol/L	0.7	0.5		
Skeletal muscle	30 kg/70 kg	4200 µmol/kg ww	126	97	2–60	105
Heart	350 g/70 kg	1300 µmol/kg ww	0.5	0.4	2–60	21
Kidney	350 g/70 kg	600 µmol/kg ww	0.2	0.2	10–200	0.4
Liver	1.5 kg/70 kg	1000 µmol/kg ww	1.5	1	500	1.3
Brain					>1000	223

Km = Michaelis-Menten constant; **ww** = wet weight.

Tab.1.1 Distribution of carnitine into the primary sites of storage in the human body for a 70 Kg adult(Stephanie E. Reuter & Evans, 2012)

A number of transporters have been associated with carnitine distribution. The first ones belong to the organic cation transporter family, that function primarily in the elimination of cationic drugs and other xenobiotics, and include the high affinity transporter OCTN2, the low affinity transporter OCTN1 and the intermediate affinity transporter OCTN3.

OCTN2 is a widely expressed organic cation transporter. It plays a key role in L-carnitine oral absorption, tissue distribution and renal reabsorption. It is very widely expressed in human tissues, in particular, is expressed in kidney, in skeletal muscle, heart, eye, lung and placenta in adult humans. This transporter is a high affinity (3-5 μmol/L) sodium-dependent carnitine transporter, primarily responsible for the uptake of L-carnitine into cells. OCTN2 also operates as polyspecific Na+-independent organic cation transporter, and can transport substrates in both directions across the plasma membrane. (Ikumi Tamai et al., 1998)(Wu et al., 1999).

OCTN1 has a similar tissue expression profile as OCTN2 does(I Tamai et al., 2000)(McBride et al., 2009), but lower affinity for L- carnitine. Indeed the main substrate of this pH-dependent proton antiporter is ergothioneine(Gründemann et al., 2005).

OCTN3 has been identified as peroxisomal carnitine transporter, and it is localized at the peroxisomal membrane (Lamhonwah et al. 2005). It has been suggested that OCTN3 has a higher specificity than OCTNl and OCTN2 for L-carnitine (Tamai et al. 2000) .

$ATB^{0,+}$ is a low-affinity, low-specificity transporter for carnitine which has a distinct tissue distribution with primary expression within the intestinal tract, lung and mammary glands(Nakanishi et al., 2001).

1.6.10 ELIMINATION

As L-carnitine is not systemically metabolized, with the exception of reversible esterification to its acyl derivatives, the primary route of elimination is via renal excretion. Given that L-carnitine is a small, polar molecule that does not undergo protein binding, it is readily filtrated by the renal glomerulus, that is mediated via the active transporter OCTN2(Ohashi et al., 2001)(Flanagan, Simmons, Vehige, Willcox, & Garrett, 2010).

However, in order to reduce L-carnitine loss and maintain adequate endogenous concentrations, more than 95% of filtered L-carnitine is reabsorbed by the proximal tubule and returned into the blood stream (Brass 1995)(Guder & Wagner, 1990). The efficiency of the tubular reabsorption of L-carnitine increases as dietary intake decreases in order to maintain circulating L-carnitine concentrations within a narrow normal range. On the other hand, when L-carnitine concentrations increase, such as with exogenous administration, reabsorption decreases as OCTN2 transporters become partially saturated and greater urinary loss results(Charles J. Rebouche & Mack, 1984)(Charles J Rebouche, Lombard, & Chenard, 1993).

While L-carnitine and its acyl derivatives are both transported via OCTN2, L-carnitine is preferentially reabsorbed over its esters such that renal clearance of acylcarnitine is 4-8 times higher

than that of L-carnitine. As a result, the composition of urine (46% L-carnitine, 29% short-chain acylcarnitines and 16% long-chain acylcarnitines) varies considerably from that of plasma(Ferrari et al. 1992).The preferential reabsorption of L-carnitine by the proximal tubule results in the clearance of acyl compounds that might otherwise lower the rate of fatty acid oxidation and other CoA-dependent reactions(Brady, Ramsay, & Brady, 1993)(Peluso et al., 2005).

1.6.11 CARNITINE POOL COMPOSITION

The endogenous carnitine pool is comprised primarily of L-carnitine with short-, medium- and long-chain acylcarnitines forming the remaining component. The composition of the endogenous carnitine pool varies considerably within the various tissues of the body. Total plasma carnitine concentrations, for example, have generally been reported as 45-60 μmol/L, with a corresponding acylcarnitine concentration of 6-9 μmol/L(Reuter et al. 2008). Therefore, in healthy adults, the plasma carnitine pool comprises 83% L-carnitine and 17% acylcarnitines, of which acetyl-L-carnitine makes up 75%(Stephanie E Reuter, Evans, Chace, & Fornasini, 2008)(S E Reuter, Evans, Faull, Chace, & Fornasini, 2005). The contribution of medium- and long-chain acylcarnitines is minimal(Harper, Wadström, & Cederblad, 1993).

The concentrations of L-carnitine and its esters are maintained within relatively narrow limits for normal biological functioning in their pivotal roles in fatty acid oxidation and maintenance of free coenzyme A (CoA) availability(Evans & Fornasini, 2003). In light of this, acylcarnitines cannot simply be considered by-products of the enzymatic carnitine transfer system, but provide indirect evidence of altered mitochondrial metabolism. Consequently, examination of the contribution of L-carnitine and acylcarnitines to the endogenous carnitine pool (i.e. carnitine pool composition) is critical in order to adequately characterize metabolic status.

Although, for the most part, the relative contribution of L-carnitine and acylcarnitines to the total carnitine pool has been well characterized in the various tissues of the body, there is limited research establishing the specific pattern of acylcarnitine distribution. Given that disorders of metabolic dysfunction are generally associated with altered concentrations of specific acylcarnitines, it is vital to distinguish between L-carnitine and individual acylcarnitines in order to correctly evaluate the state of the carnitine pool.

1.6.12 DISTURBANCES IN CARNITINE HOMEOSTASIS

Aberrations in carnitine regulation are implicated in complications of diabetes mellitus,

haemodialysis, trauma, malnutrition, cardiomyopathy, obesity, fasting, drug interactions, endocrine imbalances and other disorders(Flanagan et al., 2010).

Carnitine deficiency is a metabolic state in which carnitine concentrations in plasma and tissues are less than the levels required for normal function of the organism. Biologic effects of low carnitine levels may not be clinically significant until they reach less than 10-20% of normal(Magoulas & El-Hattab, 2012)(Flanagan et al. 2010).

Carnitine deficiency may be primary or secondary (Pons & Darryl 1995). The first disease is a systemic carnitine deficiency or due to a defect in the OCTN2 transporter, which results in reduced intestinal L-carnitine absorption, limited uptake into the muscles and other tissues, and disturbed renal tubular reabsorption resulting in excessive urinary loss(Nezu et al., 1999)(Wang, Ye, Ganapathy, & Longo, 1999).

The severe carnitine insufficiency in patients with primary carnitine deficiency results in an energy deficit due to the inability of fatty acids to enter the mitochondria and undergo ß-oxidation. The resultant accumulation of fatty acids within the cytosol has a toxic effect on the cell via a detergent action. The 3 areas of involvement include the cardiac muscle, which is affected by progressive cardiomyopathy (by far, the most common form of presentation), the CNS, which is affected by encephalopathy caused by hypoketotic hypoglycaemia, and the skeletal muscle, which is affected by myopathy(Waber, Valle, Neill, DiMauro, & Shug, 1982)(Vielhaber et al., 2004)(Limketkai & Zucker, 2008).

In secondary deficiency, which is caused by other metabolic disorders (eg, fatty acid oxidation disorders, organic acidemias), carnitine depletion may be secondary to the formation of acylcarnitine adducts and the inhibition of carnitine transport in renal cells by acylcarnitines (Pons & Darryl 1995). Although these secondary carnitine deficiencies are less severe than the primary form, their prevalence is more common. Various genetically determined metabolic errors have been associated with carnitine deficiency, the most common of which are defects in fatty acid oxidation with either defects in the enzymes required for the transfer of fatty acids into the mitochondria or those required for the ß-oxidation process (Hoppel 2003). This leads to the accumulation of fatty acids and acylcarnitines which cause clinical symptoms.

Numerous medical conditions have been associated with carnitine disturbances, either via decreased biosynthesis (chronic renal failure(Wanner et al., 1987)(Evans & Fornasini, 2003), chronic liver failure(Rudman, Sewell, & Ansley, 1977)(Krahenbuhl, 1996)) decreased intake (malnutrition(L.

Khan & Bamji, 1977), malabsorption such as in coeliac disease, cystic fibrosis, short-gut syndrome(Pons & Darryl 1995)), decreased body stores or increased requirements (HIV/AIDS(De Simone et al., 1992), pregnancy(Schoderbeck et al., 1995), or increased loss (renal Fanconi syndrome (Bernardini, Rizzo, Dalakas, Bernar, & Gahl, 1985).

Disturbances in carnitine homeostasis have also been attributed to other conditions, including coronary vascular syndrome, peripheral vascular disease, male infertility, autism (Pepine, 1991)(Vitali, Parente, & Melotti, 1995)(Filipek, Juranek, Nguyen, Cummings, & Gargus, 2004).

Secondary carnitine deficiency may also be induced by treatment with certain pharmacological agents, including valproate and pivampicillin(Ahluwalia & Bernad, 2001)(Rose et al., 1992), either via inhibition of OCTN2 or via the increased utilization of L-carnitine for urinary excretion of derivatives as acylcarnitines.

1.6.13 CARNITINE AND CANCER

Anorexia, cachexia and malnutrition occur in many chronic disease states including cancer, heart failure, renal and liver failure, and autoimmune deficiency disease (AIDS).

Cancer cachexia is a paraneoplastic syndrome present in 80% of terminally ill patients(Puccio & Nathanson, 1997)(Nelson, Walsh, & Sheehan, 1994) and is markedly associated with adverse prognosis and shortened survival time(Martignoni, Kunze, & Friess, 2003)(Davis & Dickerson, 2000).

The symptoms include anorexia, weight loss, muscle loss, skeletal muscle atrophy, anaemia, and alterations in carbohydrate, lipid, and protein metabolism(Argilés, Busquets, Stemmler, & López-Soriano, 2014). Patients with cancer are especially at risk for carnitine deficiency. Metabolic alterations caused by cancer cachexia have been attributed to a variety of interactions between the host and the tumour.

A metabolic change that takes place throughout the body is the reduction in endogenous synthesis of carnitine. Low serum levels of carnitine in terminal neoplastic patients are decreased largely due to the decreased dietary intake and impaired endogenous synthesis of this substance(Hoang, Graeme Shaw, Pham, & Levine, 2007). These low serum carnitine levels also contribute to the progression of cachexia in cancer patients(Vinci et al., 2005)(Malaguarnera et al., 2006).

Recent research shows that some anticancer drugs, doxorubicin(Waldner et al., 2006), cisplatin (Chang, Nishikawa, Sato, Utsumi, & Inoue, 2002)(Sue et al., 2014), carboplatin(Sue et al., 2014) and

Ifosfamide (Sayed-Ahmed et al., 2012), interfere with carnitine network. Several experimental and clinical studies have demonstrated that some important anticancer drugs hinder the absorption, synthesis, and excretion of carnitine in non-tumour tissues(Hoang et al., 2007).
Taken together, these data suggest that chemotherapy-induced damage of carnitine system and secondary deficiency of the molecule may cause fatigue due to impaired energy metabolism. Indeed, it is reasonable to hypothesize that carnitine repletion might be an effective strategy for the treatment of fatigue in this population(Waldner et al., 2006).

Furthermore cancer is fundamentally a disorder of cell growth and proliferation, which requires cellular building blocks, such as nucleic acids, proteins, and lipids. Cancer cells often have perturbed metabolism that allows them to accumulate metabolic intermediates as sources of these building blocks. The most understood metabolic perturbation in cancer cells is the Warburg effect, an energetically wasteful alteration to glucose metabolism in which cancer cells use carbon from glucose for biosynthesis of membranes and signalling molecules, instead of completely oxidizing them to carbon dioxide(Currie, Schulze, Zechner, Walther, & Farese, 2013)(Baenke, Peck, Miess, & Schulze, 2013)(Menendez & Lupu, 2007).

Since fatty acids (FAs) are essential for cancer cell proliferation, limiting their availability could provide a therapeutic strategy. From the perspective of lipid metabolism, limiting FAs availability could be achieved increasing blocking FAs synthesis, diverting FAs to storage, decreasing FAs release from storage, or increasing FAs degradation via oxidation(Currie et al., 2013).

FAs degradation via oxidation can be indirectly activated by PPAR activators, AMPK activators or ACC inhibitors, or by exposing cells to high concentrations of fatty acids or carnitine(Carracedo, Cantley, & Pandolfi, 2013).

Carnitine supplementation in several experimental models has been shown to slow down tumour growth(Dionne et al., 2012)(Huang, Liu, Guo, et al., 2012)(Wenzel et al., 2005). Interestingly, normal cells appear to be resistant to the effects of carnitine, and it has been shown to be synergistic with Bortezomib, curcumin and butyrate (Roscilli et al., 2013)(Huang, Liu, Yang, et al., 2012)(Roy et al., 2009). Molecular mechanisms include increased apoptosis of cancer cells under certain conditions. Additional mechanisms may be implicated in the antitumorigenic effects of carnitine. It can exert its inhibitory effects on cancer by virtue of its antioxidant(Gülçin, 2006)(Flanagan et al. 2010)and anti-inflammatory properties(Duranay et al., 2006)(Lee, Lin, Lin, & Lin, 2015), or mediates histone acetylation via inhibiting HDAC which at least partially contributed to its cytotoxicity(Huang, Liu, Guo, et al., 2012). Therefore, L-Carnitine would be promising in cancer therapeutics.

1.7 AIMS OF THE THESIS

Prostate cancer is a disease that affects older males and is associated with significant morbidity and mortality rates. Unfortunately, current diagnostic strategies for the detection of prostate cancer yield a numbers of false and negative results, highlighting the need for a more reliable, non-invasive method. Furthermore, current therapeutic strategies carry considerable side effects. The treatment of this cancer with surgery and /or radiation therapy is often successful in the early stages of the disease; however, these treatments are associated with a high morbidity. Prostate cancer is symptomless in the early stages and therefore many men presenting with symptoms often have metastatic disease and a poor prognosis. Therefore many studies are currently focusing on new prostate cancer biomarkers, in particular liquid biopsy, because blood or urine sampling is minimally invasive and avoids the dangers of biopsies. Metabolomics is becoming an increasingly popular tool in the life sciences since it is a relatively fast and accurate technique that can be applied with either a

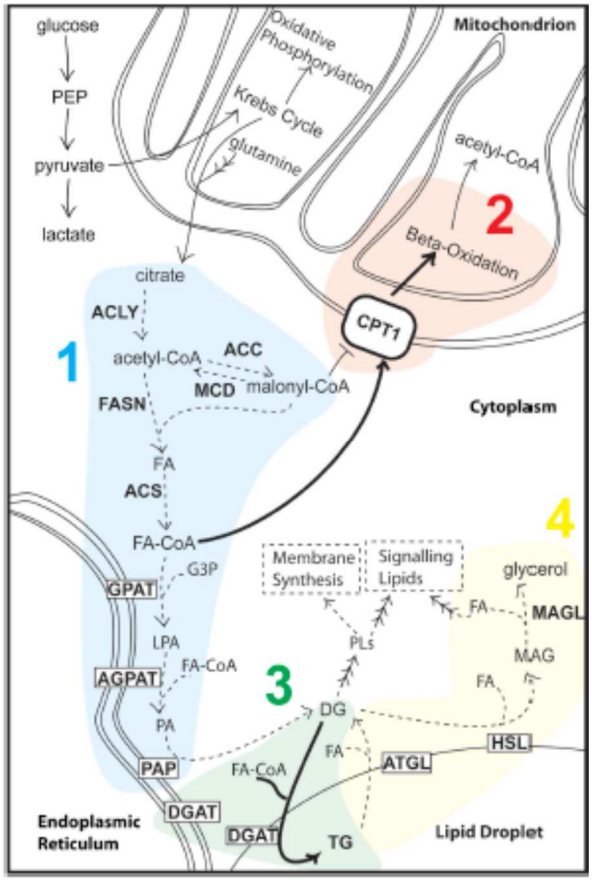


Fig.1.16 Model Showing How Limiting Fatty Acids in the Cell Might Limit Cancer Cell Proliferation. This may be done by (1) blocking the synthesis of FA, (2) increasing the rate of FA degradation, (3) increasing FA storage in neutral

particular focus or in a global manner to reveal new knowledge about biological systems In addition metabolomics has a great potential in the field of oncology, and the analysis of the cancer metabolome to identify biofluid markers is currently undertaken in several research laboratories.

On the other hand, many studies are concentrating on prostate cancer chemoprevention, in order to decrease the morbidity and mortality associated with this disease. Considering the relevance of fatty acid oxidation in prostate cancer, the L-Carnitine role in transporting fatty acids into mitochondria, and its anticancer properties, in this study the effect of L-Carnitine on cytotoxicity in prostate cancer cells PC3 was investigated. L-carnitine is essential for lipid energy metabolism within the mitochondria, and in vitro studies suggest that its antioxidant and anti-inflammatory properties are potentially beneficial toward cancer prevention.

Thus, the broad aims of the project presented in this thesis are to evaluate new potential biomarkers for the diagnosis of prostate cancer by metabolomic analysis, and to assess the prospective effect of L-carnitine evaluating its *in vitro* activity.

SPECIFIC AIMS

The specific aims of this project are:

1. To developed a pilot study, employing a novel dedicated software, named SANIST for both biomarker fingerprint data acquisition and as a diagnostic tool, in metabolomic analysis.
    - Serum samples from patients with positive biopsies were compared to controls (closely matched for age) whose biopsies showed benign prostate hyperplasia (BPH), using an UPLC/SACI-ESI/Orbitrap instrument and the SANIST metabolomic approach.
    - To achieve the aim, SANIST tool was applied to an initial biomarker discovery on a training set of sera from patients and controls.
    - A second test set of sera were then analysed using the SANIST platform to verify the potential specificity and selectivity of the fingerprint identified using the training set.

    In every experiments, the eluted analytes were ionised in the positive mode and analysed by an UPLC/SACI-ESI/Orbitrap.

- To evaluate the effects and the molecular mechanisms of carnitine supplementation on prostate cancer (PC3) cells in vitro. Various cellular mechanisms of tumour progression were

investigated, which include:

- Proliferation/growth rates by cells counting assay
- Apoptosis and cell cycle analysis by flow cytometry.
- Cell cycle arrest and apoptosis were further validated by Western blot and Real time PCR (qPCR), and alterations of key cell cycle regulators were also investigated.

2 MATERIALS AND METHODS

2.1 APPLICATION OF SANIST APPROACH TO DETECT NEW POTENTIAL BIOMARKERS FOR PROSTATE CANCER

2.1.1 STUDY PARTECIPANTS

Following approval by institutional review board ethics committee, the participants recruited in this study were collected at the Urology Unit of the MultiMedica, Castellanza, because of lower urinary tract symptoms (LUTS) and/or elevated PSA. The cancer patients had been diagnosed with PCa through positive findings in TRUS biopsies, and had not yet started cancer treatment. The control group consisted of men with no proven PCa based on negative biopsies when biopsies had been acquired. All men in the control group were diagnosed with BPH. The identification and recruitment of controls was based on matching for age.

All the subjects who were thought to be suitable for the study received a 'Letter of Invitation' and 'Participant Information Sheet'. They were contacted approximately one week later by phone to find out their interest, to arrange a face-to-face interview at the Urology Unit of the MultiMedica, and to sign the written informed consent.

Inclusion criteria

- Patients undergoing prostate biopsy for suspected prostate cancer
- Men aged 50 or more
- Signed informed consent.

If a patient fulfilled any of the below criteria they were excluded from the study from the outset.

Exclusion criteria

- Autoimmune diseases
- Allergies
- Previous neoplastic diseases involving any organ system and apparatus
- Refusal to provide a written consent

2.1.2 CLINICAL CHARATERISATION OF STUDY PARTECIPANTS

The subjects were followed according to current medical practice. In addition to the information reported by the medical history of the Department of Urology, the clinical characterization was achieved by the outcome of some tests already required by the patient: total PSA, free PSA at the time of sampling, Gleason level to identify the tumour aggressiveness and histological evaluation of the biopsy, respectively provided by the Centre for Medicine Laboratory and the Unit of Pathology of MultiMedica, Castellanza.

2.1.2.1 Ethics

The study was approved by IRCCS MulitMedica Sezione del Comitato Etico Centrale IRCCS Lombardia ethics committee.

2.1.2.2 Blood samples

Blood samples of the study participants were collected at the Department of Urology of IRCCS Multimedica on the same day of biopsy. Samples were collected in the morning, and participants observed an overnight fast, prior to blood collection. No additional requests were made to the participants to either restrict their diets or conform to a pre-defined diet leading up to the morning of the blood samples being taken. Samples were collected from peripheral vein. A volume of 7 ml of blood samples was collected and stored immediately at 4°C prior to storage in an, appropriate container for transport to the laboratory, containing cooling packs. All blood samples were collected into a 9 mL serum clot activator Vacuette blood collection tube. Blood samples were allowed to clot for 30 min at 4°C, to minimize variability during sample collection. The samples were subsequently centrifuged at ~3000g for 30 minutes at 4°C and the serum removed from the tube and aliquots of 500 µl collected in 1.8-mL cryotube vials (Thermo Fisher Scientific, Rodano, Milan, Italy). The serum was then stored frozen at -80°C, until required for metabolomic analyses.

2.1.3 CHEMICALS

Methanol (CH_3OH), acetonitrile (CH_3CN), bi-distilled water, acetone (C_3H_6O), reserpine and formic acid (HCOOH) were purchased from Sigma Aldrich (Milan, Italy). The Ultramark mix was bought

from ThermoFisher (San Jose, CA, USA).

2.1.4 METABOLOMIC ANALYSIS DESIGN

The metabolomic analysis of samples had been organised in two steps. A first set of sera, named training set, was used in order to perform an initial biomarker discovery. Sera from 15 patients with histologically confirmed prostate cancer and 13 controls were analysed by SANIST platform. Data obtained were processed by well established statistical software and databases, and a group of metabolites were extracted as differentially expressed metabolites among PCa patients and controls. A second set of 15 PCa patients and 15 BPH subjects were then analysed using the new developed SANIST approach to verify the potential specificity and selectivity of the fingerprint identified using the training set. A total of 58 serum samples were collected from participants for this thesis.

The analysis of samples had been organised as a series of so-called "blocked" experiments. The use of block analysis is common to epidemiological studies(Altman, 1991), also in analytical terms; a block is termed a batch that is the continuous analysis of samples without the need to undertake instrument maintenance. For the purposes of the research detailed in this thesis, in the discovery step the first batch comprised 15 PCa patients and 13 controls, the second 15 patients and 15 controls. In addition there were 2 saline blank samples per batch (1 lead-in blank sample and 1 rear saline blank sample). Subject and saline samples were aliquoted in equal amount.

The final data analysis involved comparing in each step profiles within each block as well as between the block experiments.

2.1.5 SAMPLES PREPARATION

Frozen serum samples collected from PCa patients and controls (which had been stored at -80°C) were thawed on ice at 4°C. For the LC/MS analysis, 290 µL of blood serum samples were spiked with 10 µL of internal standard (reserpine) at known concentration (10 ng/mL) to obtain both a reference for the reproducibility of sample preparation and instrument performance (the stability and performance of commercial instruments are usually checked using this compound). Then 600 µL of CH_3CN (Sigma-Aldrich, Milan Italy) were added to the spiked serum to precipitate proteins, and the sample was vortex-mixed (15 seconds) followed by centrifugation for 20 min at 12,000 g. A volume of 600 µL of supernatant was collected and spiked with 100 µL of Ultramark 1621 polymer solution (10 ng/mL) (Sigma-Aldrich, Milan Italy) to check the LC/MS signal stability during the analysis. Sample was dried using a SpeedVac (ThermoFisher, San Jose, CA, USA). H_2O/CH_3OH (1:1) solution

(100 µL) was used to re-suspend the analyte sample mixture.

2.1.6 RUN ORDER FOR SERUM SAMPLE ANALYSIS

The serum samples were paired such that for each control sample the closest in age were matched With the prostate cancer patient sample. The analysis order of the samples was randomised. Three analytical replicates for each sample were analysed.

2.1.7 UPLC SEPARATION

Serum samples were analysed on Ultimate 3000 UPLC (Thermo_Fisher, San Jose, CA, USA) coupled to an Orbitrap mass spectrometry system (ThermoFisher, San Jose, USA), operating in SACI-ESI ionisation mode.

Serum sample volumes of 10 µl were injected using an autosampler. Each 10µl volume of serum underwent chromatographic separation, using a PFP chromatographic column (50 × 2.1 mm, 1.9 µm; ThermoFisher, San Jose, CA, USA). The mobile phases were: (A) H_2O + 0.2% HCOOH and (B) CH_3OH. A binary gradient was used: 2% of B was maintained for 5 min, in 10 min B% was raised to 30, in a further 20 min B % was brought to 80% and this was maintained for 5 min; 2% of B was reached in 1 min and the column was re-equilibrated at the starting conditions for 9 min. The chromatographic flow was 0.55 mL/min. The run-time for a single sample to be analysed was 50 min.

2.1.8 MASS SPECTROMETRY

Instrument analysis was performed using an Orbitrap mass spectrometer (ThermoFisher, San Jose, USA) coupled to a SACI/ESI source (described previously in the section 1.5.3.1) and operated in positive ion mode. Because carnitines exist as zwitterions (positive and negative charges within the molecule) in solution, their analysis in positive ESI mode is more sensitive if a small amount of acid (0.2% HCOOH in our case) is added to the spray eluent. The Orbitrap parameters for acquisition of mass spectra, mass range and resolution are detailed in Table 2:

Parameters	Value
Capillary voltage	1500 V
SACI surface voltage	47 V
Drying gas flow rate:	2 mL/min
Nebulizer gas pressure	80 psi
Temperature	40°C
Resolution power	15,000
Mass range	40–3500 *m/z*
MS run time	50 min

Table2. Orbitrap-MS key settings for positive ionisation mode.

Full-scan spectra were acquired in the wide range of 40–3500 *m/z* for non-targeted metabolomics analysis to detect all analyte and calibration *m/z* signals. The same *m/z* range was maintained for both discovery and selective biomarker studies in order to standardize the instrument response across the SANIST study mainly in terms of scan velocity.
The instrument stability and reproducibility among the samples was tested by measuring the exact *m/z* value extraction mass chromatogram peak area variance of reserpine internal standard.

The identity of target analytes was supported by means of MS/MS analysis. Tandem mass spectrometry experiments were performed under collision-induced dissociation (CID) conditions using He as the collision gas on a serum sample. An ion trap was employed to isolate and fragment the precursor ions (isolation windows ±0.3 m/z, collision energy 30% of its maximum value (5 V peak to peak)) and the Orbitrap mass analyser was used to obtain the fragments high accurate *m/z* ratio (resolution 15,000, *m/z* error <10 ppm).

2.1.9 DATA PROCESSING AND DATA ANALYSIS IN THE DISCOVERY PHASE

The raw mass spectral data obtained in this phase were converted into the mzXML format before data elaboration analysis, using Bruker Daltonics Data Analysis software. The mzXML format version of the raw spectral data was then analysed using the freely available open-source XCMS software. This data pre-processing is required to deconvolute the raw data into a usable raw data matrix. The raw analytical data was deconvoluted to the output for each sample with associated retention time,

accurate mass and chromatographic peak. XCMS detailed software parameters used for the analysis are reported in Table 3:

Parameters	Value
First scan retention time drift	30 sec
Second scan retention time drift	15 sec
Input file format	mzXML
Output file format	tsv
Metlin database search	activated

Table3. XCMS detailed software parameters used for the data elaboration analysis.

Univariate analysis was then used to identify significantly altered peaks between PCa patients and controls. The discovery samples were therefore matched and stitched together. Matching was based on a mass tolerance of 0.02 m/z. Raw spectra, retention time and accurate mass were used to compare metabolites of interest. The XCMS algorithm in conjunction with an R statistical elaboration package were used in order to obtain both *m/z* discriminant and *p* values (t test). For comparisons of prostate cancer disease and control $p < 0.05$ was considered as significant.

2.1.10 BIOMARKER IDENTIFICATION

Compound identifications were obtained on the basis of a Metlin database search (Tautenhahn, Patti, Rinehart, & Siuzdak, 2012) using the high *m/z* accurate ions. The analyte identities were confirmed on the basis of the fragmentation pathway and also using different database elaboration tools (Metlin (Tautenhahn et al., 2012) mzCloud(Https://www.mzcloud.org/).

2.1.11 SANIST APPROACH

This platform combines LC/SACI/ESI-MS data acquisition of the m/z signal related to potential biomarker candidates, with a sample classification based on an innovative and modified Bayesian mathematical model, developed in-house. This novel algorithm is inspired by NIST library. The National Institute of Standards and Technology (NIST) Mass Spectral search program was conceived as a mass spectral library search system to assist in the identification of unknown compounds from their electron impact spectra in gas chromatography-mass spectrometry analysis.

SANIST approach is similarly based on the comparison of a selected biomarker fingerprint with those

stored in a database, however using an innovative Bayesian mathematical model.

SANIST data analysis was done using software developed with the LabView (National Instruments Corporation, Austin, TX, USA) visual language. Basically, SANIST directly acquires the extracted ion chromatogram of the selected ions of interest, creating an ASCII file containing numeric values (*m/z* value, retention time and extracted ion chromatographic peak area). The data are imported through an IPV4 protocol.

The selected ions found during the discovery phase are used to generate a reference NIST database containing the known PCa and BPH subjects used in the training set, then the potential biomarker profile database identified in the training set was employed to classify the test set, using the SANIST approach. The ion data are elaborated by means of the Bayesian mathematical model: analyte *m/z* value, retention time and their peak chromatographic area are compared to those inserted in the SANIST database and classified as negative or positive prostate cancer subjects.

SANIST data elaboration is based on the calculation of the probability that the detected disease biomarker profile was related to known disease (e.g. prostate cancer), for which the system was instructed by analyzing and inserting biomarker profiles of serum from biopsy positive (PCa) and negative (BPH) subjects. The Bayes' theorem measures the probability in which an event occurs. Under this interpretation, Bayes' theorem is the relationship between P(A) (probability that an acquired profile is a patient subject), P(B) (probability that a 'fingerprint' biomarker profile is a control subject), P(A|B) and P(B|A) for any events A and B in the same event space. In our case A and B represent the biomarker chromatographic peak area and their relative ratios.
The Bayesian interpretation of probability, or uncertainty, measures confidence that something is true. Therefore, Bayes' theorem links uncertainty before and after observing evidence. P(A), the prior, is the initial uncertainty in A. P(A|B), the posterior, is the uncertainty having accounted for evidence B. P(B|A)/P(B) represents the degree of support B provides for A. The relationship among the probabilistic function is shown in Eqn. (1):

$$P(A|B) = \frac{P(B|A)P(A)}{P(B)} \quad (1)$$

We introduced a new coefficient in Eqn. (1) that gives Eqn. (2):

$$P(A|B) = \frac{P(B|A)P(A)}{P(B)} * C(mz, rt, i) \quad (2)$$

C(mz,rt,i) is a correction factor that, on the basis of the number of uncorrected sample classifications, makes possible to progressively increase the disease classification accuracy.

The theorem makes it possible to estimate the probability that the observation observed in the sample is correctly classifiable considering the follow experimental observations: retention time, accurate m/z value and analyte area (counts/s).

Two scores are provided by the SANIST software:

(a) The absolute identity score (expressed in ‰). This value range is between 1 and 1000 where the score 1000 indicate a complete similarity between the vector of the unknown sample profile and the most similar vector in the database.

(b) The per cent identity score (expressed in %). This coefficient reports the classification specificity of the subject state (e.g. BPH or unhealthy) identified by the best match score.

2.2 L-CARNITINE SUPPLEMENTATION ON PROSTATE CANCER CELL LINE PC3

2.2.1 CHEMICALS AND ANTIBODIES

L-Carnitine was purchased from Sigma-Aldrich (Milan, Italy). GIBCO^{TM} RPMI 1640 medium and Foetal bovine serum (FBS) were purchased from Life Technologies (Monza, Italy). Mouse polyclonal antibodies anti-active plus pro Caspase 3 was purchased from Abcam (Cambridge, UK), mouse monoclonal antibodies against p21 $^{Waf1/Cip1}$ from Millipore (Darmstadt, Germany). Mouse antibodies against FAK, anti Phospho-specific FAK (PY397) were obtained from BD Biosciences (Milan, Italy). Mouse antibody anti β-actin was purchased from Sigma Aldrich (Milan, Italy) and horseradish peroxidase (HRP)-labelled secondary anti mouse and anti rabbit antibodies from GE Healthcare (Milano, Italy).

2.2.2 CELL CULTURE

Human prostate cancer PC3 cell line was purchased from ATCC (Milan, Italy) and preserved in liquid nitrogen. Each ampoule of cryo-preserved cells was resuscitated by rapidly thawing at 37°C, and diluted in 1 ml of pre-warmed to 37°C RPMI 1640 medium. Medium shall refer to RPMI 1640 supplemented with 10% heat inactivated FBS, 100 U/mL benzyl penicillin and 100 U/mL of

streptomycin (Euroclone,; Milan, Italy), 1% L-Glutamine (Euroclone,; Milan, Italy),, and pre-warmed to 37°C. Cells were then pellet by centrifugation at 250 g for 5 min at room temperature. The surnatant was aspirated and the cells resuspended in 1 ml of medium and transferred to a 25 cm^2 tissue culture flask (Corning; New York, USA). Cell line was cultured in an atmosphere of 95% air, 5% CO_2 at 37°C in a Series 8000 DH CO_2 incubator (Thermo Scientific; USA)

2.2.2.1 Routine passage of cells

Cells were preserved in 75 cm^2 flask and passaged at approximately 80% confluence. Spent medium was aspirated from the cells and the cell monolayer was washed in Phosphate Buffered Saline (PBS), followed by addition of 1 ml of 0.05% trypsin 0.5-EDTA in PBS. The flask was then incubated at 37°C for approximately 3 min. An appropriate volume of medium was added to the flask, in order to deactivate the trypsin. The cells were then pellet by centrifugation at 250 g for 5 min. The surnatant was aspirated and the cells pellet was resuspended in 3 ml of medium and aliquoted at the desired split (1:3). The cells were transferred to a 75 cm^2 flask (Corning; New York, USA) containing 15 ml of medium and incubated at 37°C.

2.2.3 PREPARATION OF CRYO-PRESERVED STOCKS

When stocks of preserved cell line were used, new aliquots of cells were prepared to replenish those resuscitated. This involved passaging cells in the usual manner but rather then aliquoting cells in medium after trypsin-treatment, they were resuspended in a inactivated serum containing 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich; Milan ,Italy) and transferred in a cryovial. The cells were then frozen slowly at -80°C over several hours in a isopropanol-containing-cryovessel. The frozen cells were then transferred to liquid nitrogen for long-term storage.

2.2.4 CELLS COUNTING USING TRYPAN BLUE STAIN:

Viable PC3 cells cultured were counted using the Trypan blue staining procedure. Cultured cells were trypsinised and after cell detachment the trypsin was neutralised with 1 ml of culture medium. The number of viable cells was determined by staining them with 10 µl of 0.4% Trypan blue (NanoEntek; Seoul, Korea) added to an equal volume of cells suspension. Viable cells were counted using a Countess™ Automated Counter (Invitrogen; Monza, Italy). 10 µl of cell suspension/trypan blue stain was pipetted at one edge of the cover slip, so that it ran under the cover-slip, and inserted in the Countess. Both viable and dead cells were counted, referring to 1 ml of cells suspension.

2.2.5 CELLS VIABILITY ASSAY

PC3 cells were plated in triplicate in 96-well flat bottom plates Costar® (Corning Incorporated, USA) at a density of $3 \cdot 10^3$ cells/well, and incubated overnight. The cells were treated with various concentrations of L-carnitine (0-10 mM) for 24,48 and 72 h. Cell survival was determined by cell counting using Trypan Blue stain (Section 2.2.4). Cell viability was calculated by the following formula: cell viability (%) = (count of treated group)/(count of untreated group-)x100%.

2.2.6 CELLS DEATH ASSAY

This experiment was performed using PE Annexin V Apotosis detection Kit I (BD Biosciences; Milan, Italy), following the manufacturer's instructions. PC3 cells were plated in 6-well flat bottom plates Costar® (Corning Incorporated, USA) at a density of $1.5 \cdot 10^6$ cells/well, and incubated overnight. The cells were treated with various concentrations of L-carnitine (0-10 mM) for 24, 48 and 72 h.

Cultured PC3 cells were trypsinised and diluted with 1 ml of culture medium and counted. Cells are resuspended with the Binding Buffer diluted 1:10, at a concentration of $5 \cdot 10^5$ cells/ml. 100 µl of the solution was transferred to a 5 ml culture tube, centrifuged and washed twice with PBS. PE Annexin and 7-aminoactinomycin D (7-AAD) were then added. Double staining is used to distinguish between viable, early apoptotic, and necrotic or late apoptotic cells. After incubation for 15 min at room temperature in the dark, 400 µl of diluted Binding Buffer was added. The stained cells were analysed by flow cytometry within 1 hour, using a FACSCantoII flow cytometer (BD Bioscience),taking a minimum $1 \cdot 10^5$ cells in each sample. According to this method, the lower left quadrant shows the viable cells, the upper left quadrant shows the early apoptotic cells, the lower right quadrant shows necrotic cells and the upper right quadrant shows the late apoptotic cells.

2.2.7 CELL CYCLE ANALYSIS

Cell cycle analysis was performed using propidium iodide (PI). PC3 cells were seeded in 6 well flat bottom plates at a density of $1.5 \cdot 10^6$ cells/well,, and incubated overnight. The cells were treated with various concentrations of L-carnitine (0-10 mM) for 24, 48 and 72 h.

Cultured PC3 cells were trypsinised and diluted with 1 ml of culture medium and counted. $5 \cdot 10^5$ cells/ml was transferred to a 5 ml culture tube, centrifuged and washed twice with PBS. Samples were

thus stained with 500µl of a solution containing PI (20mg/ml), Triton 0,1%, RNAse (20µ/ml) in deionised water. After incubation for 15 min at 4°C in the dark, samples were immediately analysed by flow cytometry, using a FACSCantoII flow cytometer (BD Bioscience) Data were analysed based on the distribution of cell populations in the different phases of cell cycle.

2.2.8 WESTERN BLOT

2.2.8.1 Intracellular Protein Extraction

PC3 cells were seeded in 6-well plates at a density of $1.5 \cdot 10^5$cells/well overnight. The cells were then treated with various concentrations of L-carnitine (0-10 mM) for 24, 48 and 72 h. For every time, spent medium was aspirated from well and the cell monolayer was washed in PBS. 50 µL of ice-cold mixed solution, containing cell lysis buffer (Cell Signalling; Danvers, USA), protease inhibitor (Roche; Milan, Italy) and water, was added to the well plates placed on ice. Cells were scraped off the well using a plastic cell scraper. The cell suspension was transferred into a pre-cooled tube, then the cell solution was drawn through a 26-gauge needle to lyse the cells. Lysates were centrifuged at 14,000 g for 15 min at 4°C and stored at -80°C.

2.2.8.2 Protein Quantitation

The Lowry ^{DCTM} Protein Assay Kit (Biorad; CA,USA) was used following the manufacturer's instructions to determine protein concentration. Increasing concentrations of BSA (0-1.2 mg/ml) were used to generate a standard curve. 4 µl of samples of unknown concentration were diluted to a ratio of 1:5 in water in a total volume of 20 µl. 100 µl of a solution of Reagent A plus Reagent S(1 ml Reagent A per 20 µl Reagent S) was added, followed by 800 µl of Reagent B. Diluted samples were then incubated 15 min at room temperature in the dark. Following this incubation, samples were transferred in cuvettes and colourimetric absorbances were determined spectrophotometrically at 550nm using the Gene Quant 1300 reader (GE Healthcare; Milano, Italy). Log-log curves were generated by Gene Quant 1300 reader software and concentrations of each unknown sample were calculated.

2.2.8.3 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were separated using a Mini Protean® TGX Stain Free™ Gels (Biorad, Milan, Italy).

Protein samples (20 µg made up to a total volume of 15µl) were added to 4.75 µl of 4x Laemmli buffer (Biorad, Milan, Italy) and 0.25 µl of β-mercaptoethanol (Sigma Aldrich; Milan ,Italy), and boiled for 5 min. A running buffer containing 100 ml 10X Running buffer TGS (Biorad; Milan, Italy) and 900 ml of deionized water was used. Samples were electrophoresed, using a Mini Protean Tetra Cell (BioRad; Milan, Italy), through the separating gel at 150 mV. When the dye front reached the desired position, the gels were removed from the apparatus.

2.2.8.4 Western Blotting

Proteins separated by SDS-PAGE were transferred to a Trans-Blot® Turbo™ Nitrocellulose membrane (Biorad; Milan, Italy) using a Trans-Blot® Turbo™ Transfer System (Biorad; Milan, Italy). Electrophoresis was performed in the Transfer Buffer, containing 200 ml of Trans-Blot® Turbo™ Transfer Buffer, 200 ml of ethanol and 600 ml of deionized water, for 30 min at 1 A and 25 V. After the protein transfer, the membrane was stained in Ponceau Red (Sigma Aldrich; Milan, Italy) for 1 min to ensure transfer and equal loading had occurred. The membrane was rinsed in PBS to remove the stain and immediately placed in 5% blocking solution, composed of 5% non-fat milk powder (wt/vol) in Tris-buffered saline (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20), to block non-specific sites for 1 h at room temperature. Primary antibodies were prepared in 5% blocking solution to the required concentration and then incubated at 4°C overnight. The membranes were incubated with primary antibodies directed against the following human antigens: caspase 3 pro+ active, p21, FAK and phospho FAK and LC3. The blot was washed 5x10 min washes in PBS/Tween. The blots were then incubated in horseradish peroxidase (HRP)-conjugated appropriate secondary antibodies to detect the designated proteins. The bounded secondary antibodies on the nitrocellulose membrane were reacted to the Clarity™ Western ECL Blotting Substrate (Biorad; Milan, Italy) and exposed in the ChemiDoc™ MP Imaging System (Biorad; Milan, Italy). The density of desired bands was calculated with the ImageJ software.

Several blots were re-probed with other antibodies. In these cases, the membrane was re-blocked in blocking solution and the incubation method repeated as above.

2.2.9 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

2.2.9.1 RNA Extraction

PC3 cells were seeded in 6-well plates at a density of $1.5 \cdot 10^5$cells/well overnight. The cells were then

treated with various concentrations of L-carnitine (0-10 mM) for 24, 48 and 72 h. For every time, spent medium was aspirated from well and the cell monolayer was washed in PBS. Cultured cells were trypsinised and the trypsin was then neutralised with culture medium. Cells were pellet by centrifugation at 250 g for 5 min at room temperature. The surnatant was aspirated and the pellet was wash twice in PBS and stored at -80°C.

Total RNA was extracted by an automated nucleic acid purification system, the Maxwell® RSC Instrument (Promega; Milan, Italy). The kits Maxwell® RSC simply RNA Cells Kit (Promega; Milan, Italy) was used to process the samples, according to the manufacturer's instructions. Briefly, the samples were prepared using a solution of Homogenization Solution containing 1-Thioglycerol (20µl of 1-Thioglycerol per millilitre of Homogenization Solution) was prepared. 200 µl of the 1-Thioglycerol/Homogenization Solution was added to RNA samples placed on ice, subsequently vortexed until pellet was dispersed and cells appear lysed. The lysates were diluted with 200µl of Lysis Buffer (Part# MC501C) and vortexed vigorously for 15 seconds. The samples were transferred in the Maxwell RSC® Cartridge, that were rinsed with blue DNase I Solution and Nuclease-Free Water for the dilution of extracted RNA. The automated extraction took place in 50 min.

The RNA was analysed for purity (A_{260}/A_{280}: ratio = 2.0) and concentration (A_{260}: O.D of 1 ≈40 µg/ml RNA) using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, USA). Samples were then stored at -80°C until needed.

2.2.10 REAL TIME POLYMERASE CHAIN REACTION (RT-Q-PCR)

2.2.10.1.1 Reverse transcription

cDNA synthesis was carried out using 500 ng of DNAse I treated RNA. First strand synthesis was achieved using I Script™ cDNA Synthesis Kit (Biorad; Milan, Italy) according to the manufacturer's instructions. RNA was diluted with Nuclease-free water to 15 µl, then 5x iScript reaction mix and iScript reverse transcriptase were added, reaching a total volume of 20 µl. The following reaction protocol was carried out in a T 100™ Thermal cycler (Biorad, USA,CA): 5 minute at 25°C, 1 h at 42°C, 5 minutes at 85°C.

2.2.10.1.2 RT-q-PCR

Real time PCR was performed using primers specific for the gene of interest and carried out using SsoFast™ EvaGreen Supermix (Biorad; Milan, Italy)), according to the manufacturer's instructions. Reaction mixtures contained 5 µl of SsoFast EvaGreen Supermix, 2.5 µM each of forward and reverse

primer and 3 µl of cDNA made up to 10 µl with RNase/DNase-free water. The following RT-q-PCR protocol was carried out in a CFX96^{TM} Real Time System (Biorad; Milan, Italy): 2 min at 98°C (enzyme activation), 40 cycles at: 98°C for 2 sec (denaturation), 30°C for 5 sec (annealing); then from 70°C to 95°C in 0.5°C/sec min (melt curve). The primer sets for amplification are listed below: p21-F:5'CCTGCCCAAGCTCTACCTTCC3';p21R:5'GGTCCACATGGTCTTCCTCTGC3'; p27-F: 5'TCTTGCAGAAGCTGACCTGG3'; p27-R: 5'CCACACAGCTGGATGCTAGT3'; Ciclofiline A-F:5'GACCCAACACAAATGGTT3'; Ciclofiline A-R: 5'TTTCACTTTGCCAAACACCA3'. Relative expression values with standard errors and statistical comparisons (unpaired two-tailed *t*-test) were obtained using Qgene software.

2.2.10.1.3 Statistical methods

Data were analysed using Graph Pad Prism software version 5.0. Unless indicated otherwise, Mean ±SD are presented where applicable. Unpaired Student's t-test is used for comparisons between two groups . *P* value less than 0.05 is considered significant. All statistical tests were two-sided. IC_{50} value was extrapolated from three independent experiments using both nonlinear regression (curve fit) analysis and the sigmoidal dose–response equation with Prism software.

3 RESULTS

3.1 APPLICATION OF SANIST APPROACH TO DETECT NEW POTENTIAL BIOMARKERS FOR PROSTATE CANCER

An ideal biomarker for a disease as complex as prostate cancer should be obtained quickly with minimal invasive procedures and be relatively inexpensive to test. Further the biomarker of the disease must have a high specificity and sensitivity for the disease. Blood based biomarkers offer a convenient biological fluid to access, and serves as a middle ground for the body's physiological activity. Although gene and protein expression have been extensively profiled in human tumours, little is known about the global metabolomic alterations that characterize neoplastic progression. However, technological advances in instrumentation, including the recent introduction of UPLC and the Orbitrap mass spectrometer have increased the speed, resolution and the degree of sensitivity in analysing biological fluids. In recent years several metabolomic studies have been carried out on serum(Osl et al., 2008), urine(Gamagedara et al., 2012)(Sreekumar et al., 2009), biopsy tissue(McDunn et al., 2013) and cell lines(Teahan, Bevan, Waxman, & Keun, 2011), with the purpose of identifying promising targets for the early detection of PCa. Unfortunately, the majority of the candidate biomarkers are still awaiting validation or validation has failed to reproduce these findings(Jentzmik et al., 2010)(Gamagedara et al., 2012), and therefore, their utility is still under discussion(A. P. Khan et al., 2013).

The experiments detailed in this section used the liquid chromatography LC/SACI/ESI-MS coupled with the novel SANIST software to analyse serum samples obtained from 30 patients with histologically confirmed prostate cancer prostate cancer patients and 28 subjects whose biopsies showed BHP. An initial SANIST biomarker discovery on a training set of sera from 15 PCa and 13 controls (Training set) were performed by SANIST platform. Data obtained were processed by well established statistical softwares and databases, and a group of metabolites were extracted as differentially expressed between the PCa patients and controls. A second set of 15 PCa patients and 15 BPH subjects (Test set) were then analysed using the new developed SANIST approach to verify the potential specificity and selectivity of the fingerprint identified using the training set.

3.1.1 CLINICAL DATA

Patients were classified according to clinical parameters, including total and free PSA at the time of sampling, Gleason level to identify the tumour aggressiveness and histological evaluation of the biopsy. Patients were not undergoing any therapy for PCa at the time of sampling. The age and the clinical data relative to subjects included in this study are summarised in the following tables:

Training Set	ID	AGE AT BIOPSY	PSA total (ng/ml)	PSA free (ng/ml)	free/total (%)	Gleason/ Histology
BIOPSY POSITIVE	P1	74	14.94	nd	nd	3+4
	P2	72	3.82	1.12	29.32	4+3
	P3	75	9.10	2.20	24.18	4+3
	P4	70	11.26	nd	nd	3+4
	P5	65	3.97	1.23	30.98	3+3
	P7	81	5.15	0.49	9.51	3+3
	P8	62	3.54	0.61	17.23	3+3
	P9	81	5.38	1.68	31.23	4+3
	P10	67	5.10	0.53	10.39	4+3
	P14	69	3.75	0.55	14.67	3+3
	P16	74	4.62	0.68	14.72	3+3
	P17	81	4.46	0.48	10.76	3+3
	P18	61	0.28	nd	nd	3+3
	P19	82	57.49	nd	nd	4+4
	P29	54	77.96	nd	nd	3+3
Mean ± SD		71.2±8.4	14.1±22.4	1.0±0.6	17.5±10.2	
	C21	59	5.06	0.90	17.79	BPH
	C23	66	5.58	1.28	22.94	BPH
	C29	72	4.42	0.97	21.95	BPH
	C4	58	1.17	0.34	29.05	BPH
	C31	72	4.18	1.64	39.23	BPH
	C32	57	7.59	1.91	25.16	BPH
	C33	66	11.62	nd	nd	BPH
	C34	67	6.11	1.58	25.86	BPH
	C35	71	9.73	1.78	18.29	BPH
	C36	52	3.99	0.89	22.31	BPH
	C38	77	1.58	0.53	33.54	BPH
	C39	65	5.10	1.03	20.19	BPH
	C40	54	14.31	1.82	12.73	BPH
Mean ± SD		64.3±7.7	6.2±3.8	1.2±0.5	18.8±12.0	

Fig.3.1 Table showing the age and the clinical parameters and their relatives means of the subjects whose serum was used in the discovery phase (Training set).

Test Set	ID	AGE AT BIOPSY	PSA total (ng/ml)	PSA free (ng/ml)	free/total (%)	Gleason/ Histology
BIOPSY POSITIVE	P101	58	13.33	2.46	18.45	4+5
	P201	70	138.05	19.84	14.37	4+5
	P301	71	0.86	0.16	18.60	4+4
	P401	67	5.95	1.83	30.75	4+4
	P501	65	35.41	7.69	21.71	4+3
	P601	62	2.65	0.42	16.40	3+4
	P701	72	4.47	0.62	13.87	3+4
	P801	61	28.28	4.63	16.37	3+4
	P901	69	4.81	0.47	9.77	3+3
	P1001	78	4.59	0.26	5.66	2+3
	P1101	62	6.23	0.75	12.03	3+3
	P1201	80	6.31	1.13	17.90	3+3
	P1301	49	6.60	0.48	7.27	3+3
	P1401	71	7.00	1.73	24.71	3+3
	P1501	76	8.59	1.07	12.45	3+3
Mean ± SD		67.4±8.2	18.2±34.5	2.9±5.1	16.0±6.6	
BIOPSY NEGATIVE	C101	68	2.46	0.49	19.91	BPH
	C201	57	3.30	0.71	21.51	BPH
	C301	65	0.82	0.35	42.68	BPH
	C401	50	4.00	0.75	18.75	BPH
	C501	61	3.80	0.83	21.84	BPH
	C601	50	6.24	0.69	11.05	BPH
	C701	68	5.89	1.17	19.86	BPH
	C801	62	5.23	1.54	29.44	BPH
	C901	61	4.12	1.07	25.97	BPH
	C1001	77	4.94	2.53	51.21	BPH
	C1101	62	4.66	0.79	16.95	BPH
	C1201	55	8.02	1.52	19.00	BPH
	C1301	56	4.67	0.79	16.82	BPH
	C1401	63	0.92	0.25	27.17	BPH
	C1501	58	4.93	1.77	35.90	BPH
Mean ± SD		60.9±7.1	4.3±1.9	1.0±0.6	25.2±10.8	

Table 3.2 Table showing the age and the clinical parameters and their relatives means of the subjects whose serum was used in the test set.

3.1.2 METABOLIC PROFILING IN THE DISCOVERY PHASE USING SANIST PLATFORM

The training set of sera composed of 15 PCa patients and 13 controls were analysed by SANIST platform. All samples were produced in accordance to the standard preparation protocol described in Section 2.1.2.2. The analysis was performed in accordance to the procedure illustrated in Section 2.1. Three analytical replicates for each sample were analysed.

Typical representative full scan SACI/ESI mass chromatograms of BPH and prostate cancer subjects extracted under base peak conditions are shown in Figure 3.3 and 3.4, respectively:

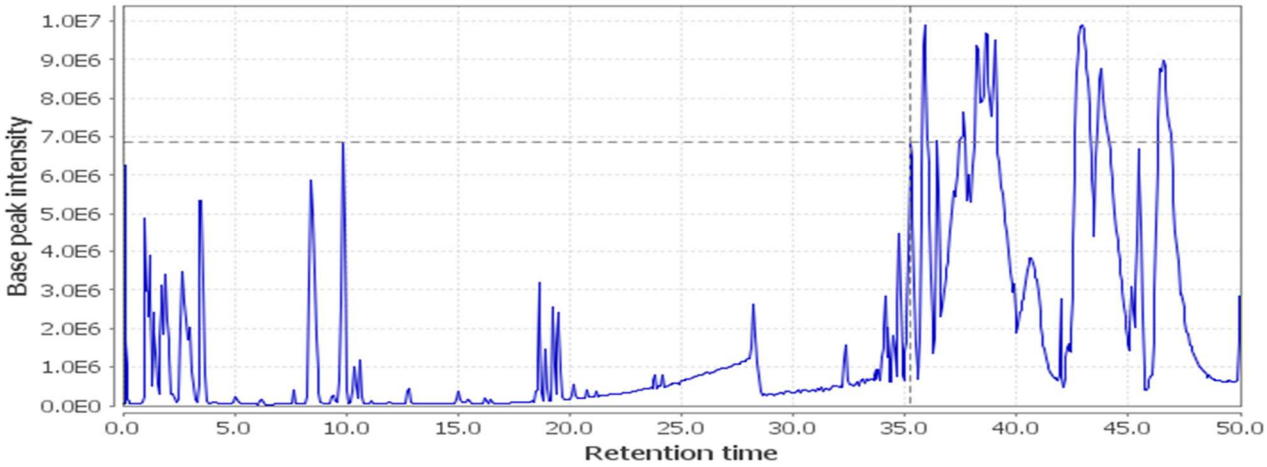


Figura 3.1 Representative full scan (base peak) extracted ion 'SANIST' mass chromatograms of biopsy negative (BPH) subject.

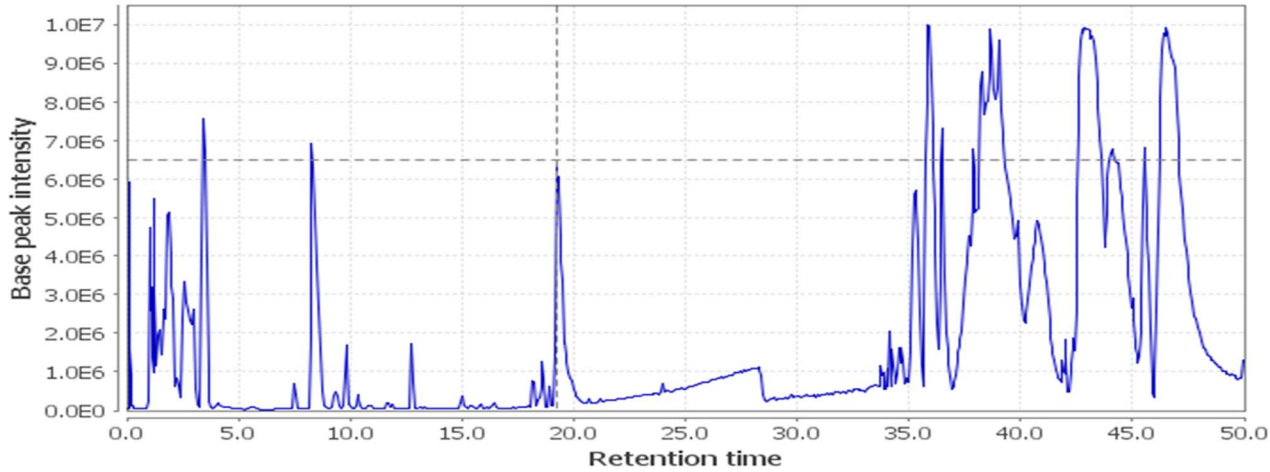


Figura 3.2 Representative full scan (base peak) extracted ion 'SANIST' mass chromatograms of biopsy positive (PCa) subject.

The total number of metabolite peaks detected was 1070.

The instrument stability among the samples was tested by measuring the exact *m/z* value extraction mass chromatogram peak area variance (10%) of the reserpine internal standard, indicating that no

analytical bias was present during the experiment. The sample instrument average analyte area variation was used to check the reproducibility, and was from 73 to 90%. Therefore all the 1070 peaks were selected for statistical analysis.

Data files were transformed into mzXML format and deconvolved using XCMS software (Section 2.1.9). These data were then analysed using the R bioinformatic statistical environment allowing a comparison of all the *m/z* values of the chromatographic peak areas between BPH and prostate cancer subjects. In particular, the XCMS/R approach was useful to obtain a simple t-test or assessing the statistical validity in difference of levels of each *m/z* value.

Of the 1070 peaks detected, 3 were the most significantly altered across the PCa patients and controls. The signals at *m/z* values of 316.256, 288.217 and 370.295 acquired in positive ion mode were all significantly differentially expressed (p value <0.001) at lower levels in the prostate cancer subjects (Table 2) using the reserpine and Ultramark spiked in references.

m/z value	Ion species	Metabolite name	Fold (Patient Area/Control Area)	P value
317.256	[M+H]+	Decanoyl-L-Carnitine	0.76	0.0001
288.217	[M+H]+	Octanoyl-L-Carnitine	0.85	0.0003
370.295	[M+H]+	5-cis-Tetradecenoyl carnitine	0.72	0.0004

Table 3.3 Ions that were differentially expressed between serum samples from BPH and positive prostate cancer (PCa positive) subjects in the discovery study and their corresponding metabolites

The identity of target analytes was also supported by means of MS/MS analysis and structural studies. Figures 3.3 (a), 3.3(b) and 3.3(c) report their MS/MS spectra, obtained by CID. The high accurate fragment *m/z* ratio and the hypothetical structure are reported.

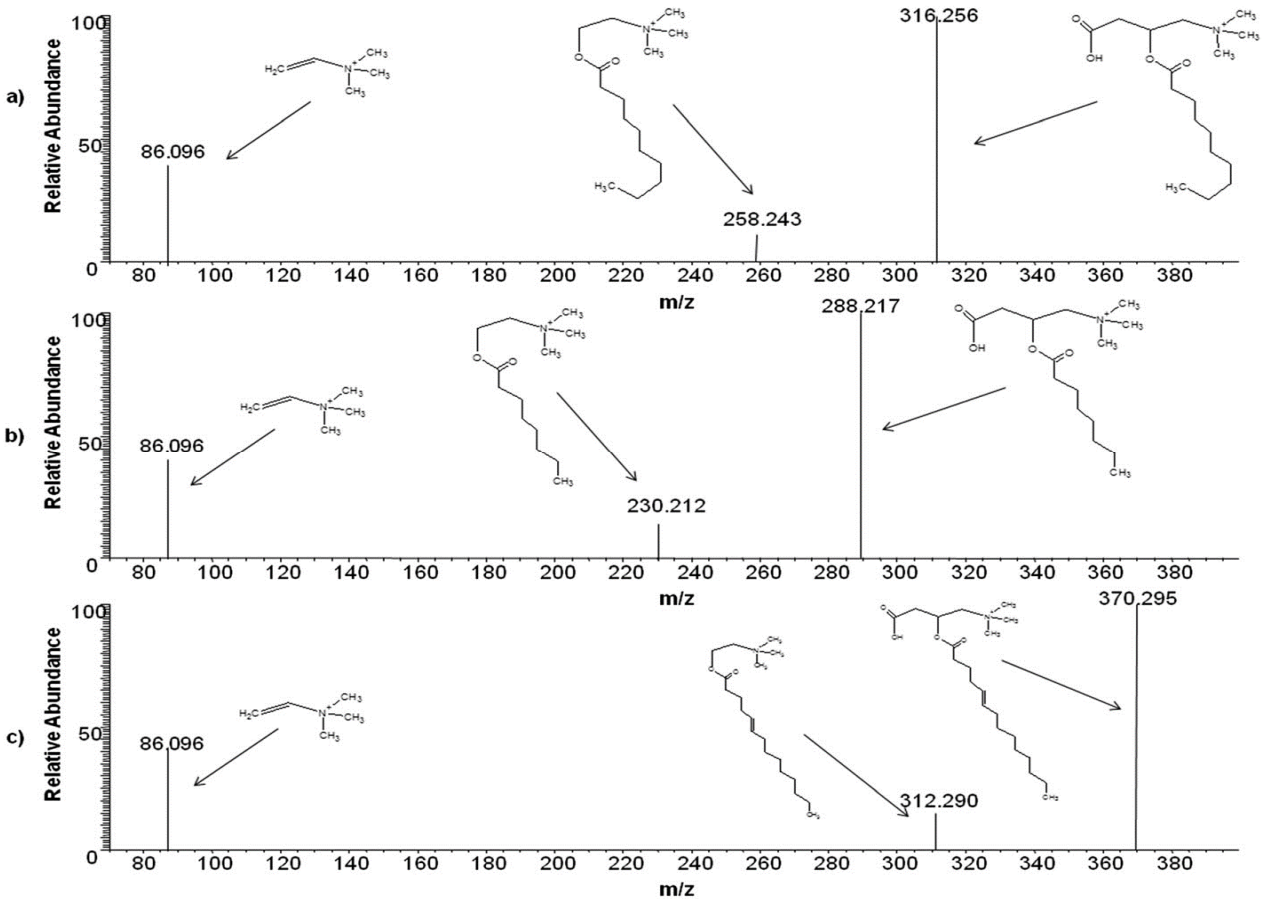


Fig. 3.3 MS/MS spectra, obtained by collision-induced dissociation (CID), of (a)decanoyl-L-Carnitine(b) octanoyl-L-carnitine, and (c) 5-cis-tetradecenoyl carnitine

After data acquisition, SANIST data elaboration was performed using the Bayesian elaboration model developed in house in order to statistically evaluate the classification efficiency of the carnitine metabolite biomarker 'fingerprint'. A schematic representation of the SANIST platform is shown in Figure 3.4:

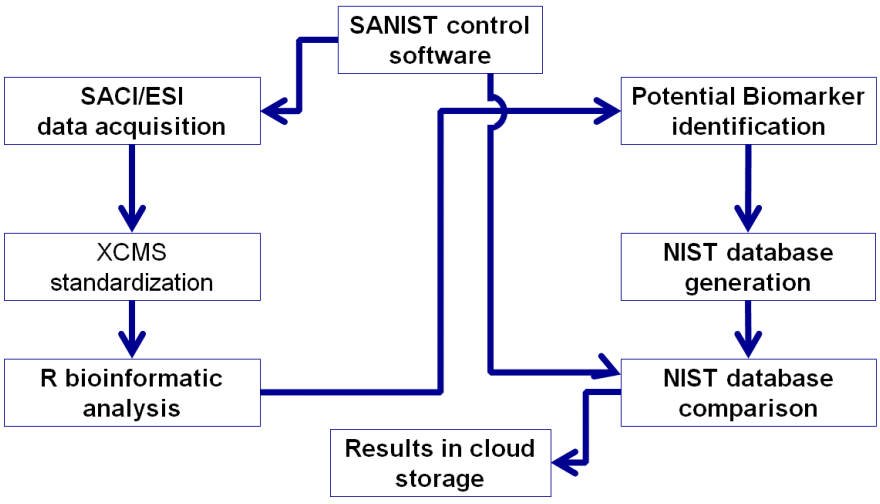


Fig. 3.4 'SANIST' bioinformatics platform scheme based on LabView software: The evoluted Bayesian comparisons to a developed database makes it possible to statistically evaluate biomarker panel classification efficiency.

Generation of NIST database were obtained basically acquiring the extracted ion chromatogram of the selected ions of interest found during the discovery phase, creating an ascii file containing numeric values (*m/z* value, retention time and extracted ion chromatographic peak area). The potential biomarker profiles of all control prostate cancer in the training set in the training set were inserted into the NIST database.

As an initial test, the biomarker fingerprint of each subject in the training set was compared with the same inserted in the database, excluding the identical match to itself. The samples were then characterized as BPH or prostate cancer on the basis of their similarity with the best match: A complete discrimination (100%) was achieved among BPH and prostate cancer patients with a recognition identity % match between 90 and 94% (Table 3.4).

Sample	**Database Sample match**	**Match**	**Identity %**
P1	P2	904	91
P2	P1	911	92
P3	P7	903	90
P4	P5	905	94
P5	P10	912	92
P7	P9	920	91
P8	P16	903	91
P9	P1	907	92
P10	P16	904	93
P14	P19	903	92
P16	P29	906	92
P17	P3	905	93
P18	P29	901	94
P19	P10	902	93
P29	P3	913	91
C21	C31	923	90
C23	C21	908	91
C24	C31	909	91
C29	C31	905	93
C31	C24	904	94
C32	C31	914	93
C33	C29	905	90
C34	C32	914	91
C35	C31	903	91
C36	C40	906	90
C38	C21	907	92
C39	C36	903	92
C40	C38	903	93

Tab3.4 Classification per cent identity score and absolute identity score obtained on the basis of the SANIST platform and the three identified carnitine biomarkers. All prostate cancer patient (PCa positive) profiles are similar to another prostate cancer, while benign prostate hyperplasia (BPH) are closest to other BPH subjects

3.1.3 ANALYSIS OF THE TEST SET BY SANIST PLATFORM TO VERIFY THE IDENTIFIED FINGERPRINT.

A second test set of 15 patients and 15 BPH subjects (indicated with 01 as a suffix) were then analysed using the SANIST platform to verify the potential specificity and selectivity of the fingerprint identified using the training set

All samples were produced in accordance to the standard preparation protocol described in Section 2.1.2.2. The analysis was performed in accordance to the procedure illustrated in Section 2.1. Three analytical replicates for each sample were analysed.

The signals at *m/z* values of the three represented metabolites (Table 3.5) in this test set were comparable to those shown in Table 3.3 and highly statistically significant between the patients and BPH subjects, confirming the same potential biomarker panel found in the training set.

m/z value	**Ion species**	**Metabolite name**	**Fold (Patient Area/Control Area)**	***P* value**
317.256	[M+H]+	Decanoyl-L-Carnitine	0.56	0.0021
288.217	[M+H]+	Octanoyl-L-Carnitine	0.47	0.0024
370.295	[M+H]+	5-cis-Tetradecenoyl carnitine	0.41	0.0075

Tab3.5 Ions of the differentially expressed metabolites discovered in the training set in serum samples applied to the test set of BPH and PCa positive subjects.

Using this approach, all test set sera with BPH biopsies were matched to training set BPH biopsy samples, while all test set prostate cancer positive patients were matched to training set prostate cancer patients (Table 4). Moreover, a reverse search approach was used by employing the test set as the training set and the training set as the test set; very similar results were obtained, confirming the discriminating power of the SANIST approach.

Sample	SANIST sample classification	Database Sample match	Match	Identity %
P101	PCa Positive	P16	804	90
P201	PCa Positive	P16	811	89
P301	PCa Positive	P9	807	85
P401	PCa Positive	P9	815	91
P501	PCa Positive	P16	824	86
P601	PCa Positive	P16	800	87
P701	PCa Positive	P16	781	87
P801	PCa Positive	P29	815	85
P901	PCa Positive	P16	831	84
P1001	PCa Positive	P29	829	83
P1101	PCa Positive	P29	825	89
P1201	PCa Positive	P5	915	91
P1301	PCa Positive	P5	905	84
P1401	PCa Positive	P5	905	87
P1501	PCa Positive	P5	913	88
C101	BPH	C32	909	88
C201	BPH	C31	907	81
C301	BPH	C32	907	86
C401	BPH	C24	911	85
C501	BPH	C40	913	84
C601	BPH	C32	912	84
C701	BPH	C29	917	86
C801	BPH	C38	918	86
C901	BPH	C21	914	83
C1001	BPH	C40	914	82
C1101	BPH	C13	918	82
C1201	BPH	C36	917	83
C1301	BPH	C36	914	91
C1401	BPH	C32	908	87
C1501	BPH	C31	908	89

Tab3.6 All the second samples set (01 suffix) match corresponding training set samples within the respective benign prostate hyperplasia (BPH) and prostate cancer patient (PCa positive) groups. No samples were misclassified.

Based on these experiments we may conclude that the carnitines panel identified could be a useful tool for supervised diagnostic purposes in prostate cancer. Moreover, the SANIST bioinformatic application is a dedicated SACI/ESI tool that can identify and analyse biomarker panels. However a more extensive validation of carnitines panel using a much larger cohort is needed to confirm its clinical usefulness.

3.2 EFFECT OF L-CARNITINE SUPPLEMENTATION ON PROSTATE CANCER CELL LINE PC3.

The metabolomic analysis showed significant differences in the metabolic profiles of blood samples from PCa patients and BPH controls. Based on relevance of fatty acid oxidation in prostate cancer, and based in part upon L-Carnitine role in transporting fatty acids into mitochondria, in part its anticancer properties, in this study the effect of LC on cytotoxicity in prostate cancer cells PC3 was investigated. The mechanism of LC-mediated cytotoxicity was further considered.

3.2.1 L-CARNITINE TREATMENT INHIBITS PC3 CELL GROWTH

The anti-proliferative effects of L-Carnitine were assessed in PC3 cells in vitro. Proliferation study were performed by cell counting using Trypan Blue stain. All samples were prepared in accordance to the standard protocol described in Section 2.2.5. The cells were treated with 0, 1, 2, 5, 10 mM of LC for 24, 48 and 72 h. Three analytical replicates for each experiment were analysed and three independent experiments were conducted.

The growth curves ware shown in Fig. 3.5, and the results showed that LC inhibits PC3 cell proliferation in a dose dependent manner with a maximun.at 10mM.

The exact concentration at which LC caused 50% growth inhibition (IC_{50}) compared to control cells (not treated cells, NT) was also determined, and the greatest inhibitory effect is at 72h with a IC_{50} =8.8 mM.

An unpaired t-test on the cell count percentage at 72 h in LC treated compared to control cells was performed to compare each treatment with the control and calculate *p* value (Table 3.7).

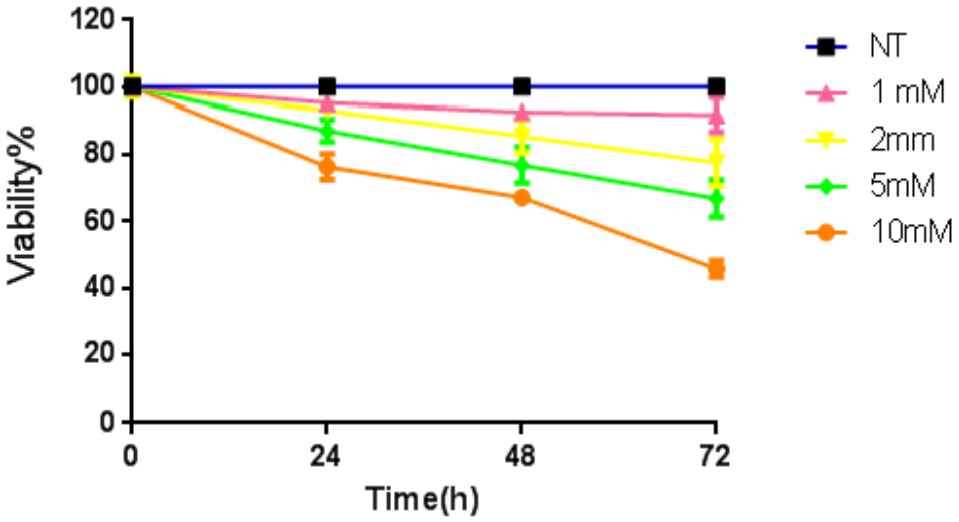


Fig.3.5 Curve dose–response of PC3 cells treated with various doses of LC (1, 2, 5, 10 mM) for 24 h, 48 h, and 72h. Cell proliferation was detected by cell counting (n = 3).

L-Carnitine (mM)	Viability %	*P* value
1	91,836±5,107	>0.05
2	77,723±7,013	>0.05
5	66,970±5,540	0.01
10	45,843±2,505	0.01

Tab.3.7 The percentage of PC3 cells viability (± standard deviation), mean of 3 experiments, in control and LC treated cells at 72 hours and the corresponding *p* values.

The cytotoxic effect of LC on PC3 was also visible at morphologic level cells (Fig.3.6). In addition to a dramatic decrease in cells number at 10 mM, an increase in cells detachment was observed. However the detached cells did not show markers of apoptosis, as cell shrinkage or apoptotic bodies, but they acquired a rounded shape and appeared living.

In order to evaluate the mechanism of L-Carnitine-mediated cytotoxicity, therefore apoptosis and cell cycle assay were analysed.

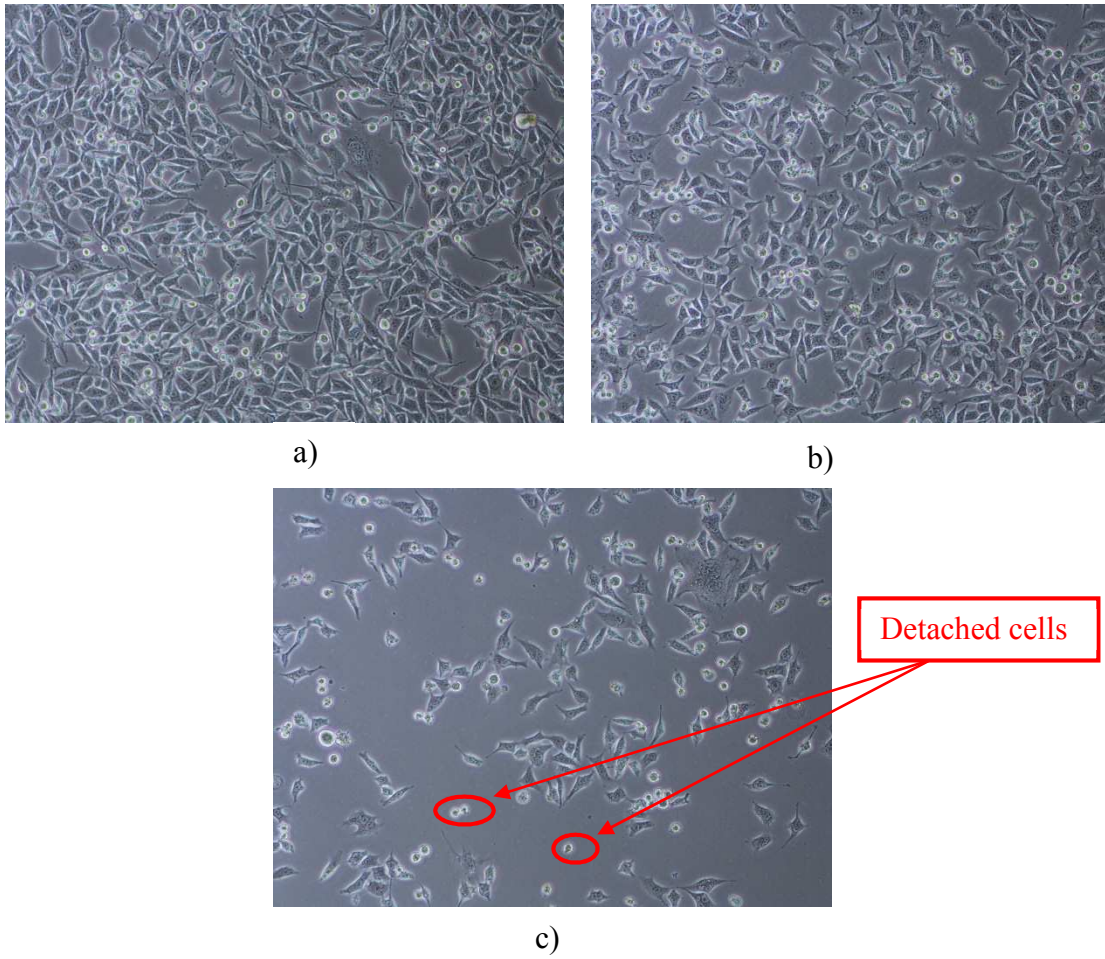


Fig.3.6 Microscope images of PC3 at 72 h: a) untreated PC3, b) PC3 treated with LC 5 mM and c) treated cells with LC 10 mM. The images confirmed the cell density decrease in dose dependent manner.

3.2.2 L-CARNITINE SUPPLEMENTATION DOES NOT INIBHIT PC3 CELLS GROWTH BY APOPTOSIS

Cell death studies were performed using PE Annexin V and 7-AAD double staining, followed by flow cytometry. All samples were prepared in accordance to the standard protocol procedure described in Section 2.2.6. The cells were treated with 0, 5, 10 mM of L-Carnitine for 24 and 48 h. Three analytical independent experiments were analysed.

The results did not showed any significant increase in the percentage of early and late apoptotic cells upon treatment with LC, even though a trend was observed in late apoptosis, both at 24 and 48 h (Fig. 3.7)

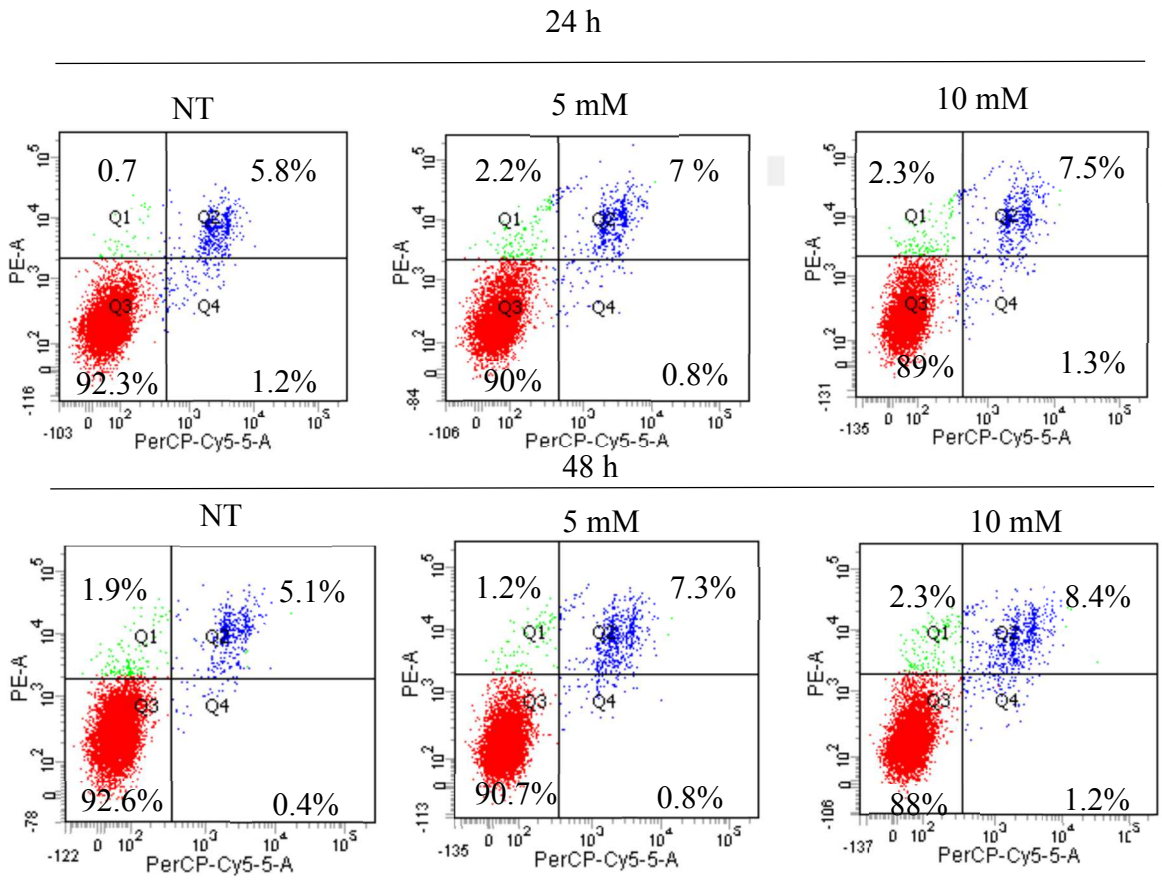
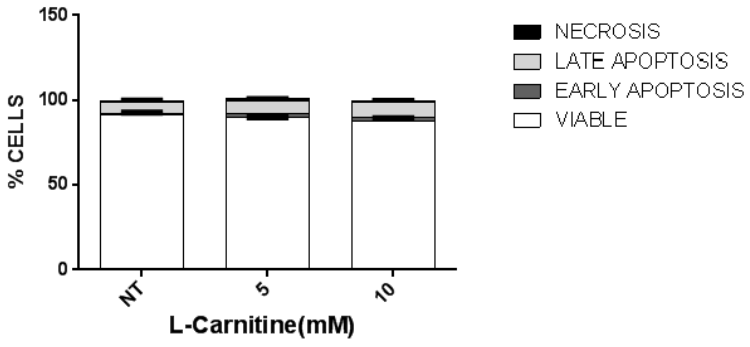


Fig.3.7 Apoptosis analysis of PC3 cells treated with LC 0, 5 10 mM for 24 and 48 h, harvested, stained with annexin V/7-AAD and analysed by flow cytometry. PE Annexin V signals are recorded in PE-A channel and 7-AAD in PerCP-Cy5-5-A. Cells in the bottom left quadrant (annexin V-negative, 7-AAD-negative) are viable, whereas cells in the top left quadrant (annexin V-positive, 7-AAD -negative) are in the early stages of apoptosis, the cells in the top right quadrant (annexin V-positive, 7-AAD- positive) are in later stages of apoptosis, and the cells in the bottom right quadrant are in necrosis. Three analytical replicates for each experiment were analysed.

a)



b)

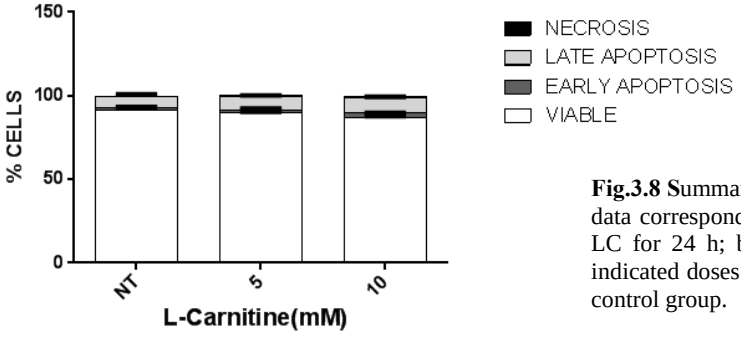


Fig.3.8 Summary of the cell death data shown in Fig.3.7. a) data corresponding to PC3 treated with indicated doses of LC for 24 h; b) data corresponding to PC3 treated with indicated doses of LC for 48 h. P >0.05 compared with the control group.

Apoptosis is a coordinated and often energy-dependent process that involves the activation caspases and a complex cascade of events that link the initiating stimuli to the final demise of the cell. Thus, a western blot for the executioner caspases-3 was conducted.

Western blot was performed in accordance to the standard protocol procedure described in Section 2.2.6. The cells were treated with 0, 5, 10 mM of L-Carnitine for 24, 48 and 72 h. Three independent experiments were analysed.

As shown in Fig. 3.9 and Fig. 3.10, there was no significant difference in expression of inactive caspase3 protein (casp3 pro) between treated cells and control. The caspase3 active form was not expressed at 24 and 48 h, whereas it was slightly expressed a 72 h, but no significant increase after LC treatment was observed. These results confirmed the LC treatment did not induce apoptosis activation, supporting the previous data obtained by flow cytometry analysis.

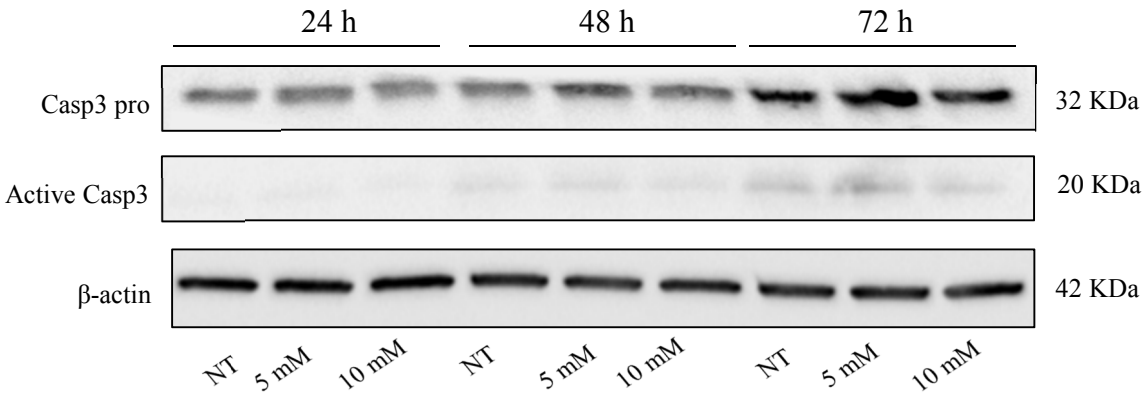


Fig.3.9 Western blot of Caspase 3 pro and active and β actin proteins. PC3 cells were treated with various doses of L-Carnitine for 24, 48 and 72 h.

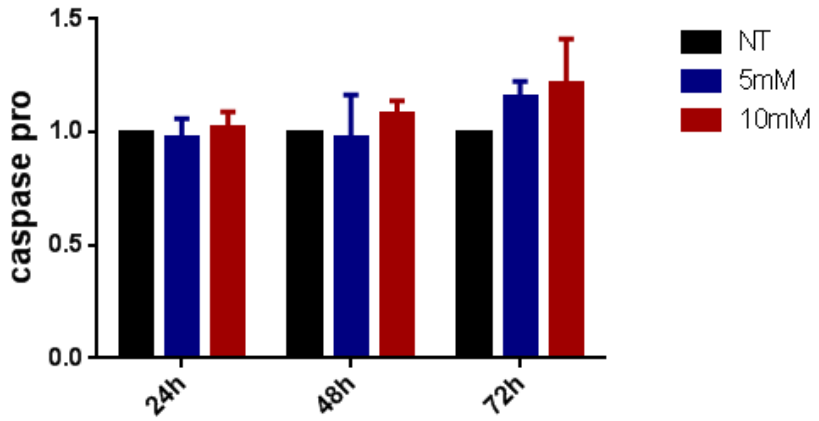


Fig.3.10 Quantification of caspase pro bands corresponding to Fig 3.9. Fold increase of the LC-treated PC3 cells versus control (NT) was shown. Mean+SD (n = 3). *P*>0.05, compared with control group.

3.2.3 L-CARNITINE IS NOT A CELL CYCLE INHIBITOR IN PC3 CELLS

Cell cycle studies were performed using PI staining, followed by flow cytometry. All samples were prepared in accordance to the standard protocol procedure described in Section 2.2.7. The cells were treated with 0, 5, 10 mM of L-Carnitine for 24 and 48 h. Three analytical independent experiments were analysed.

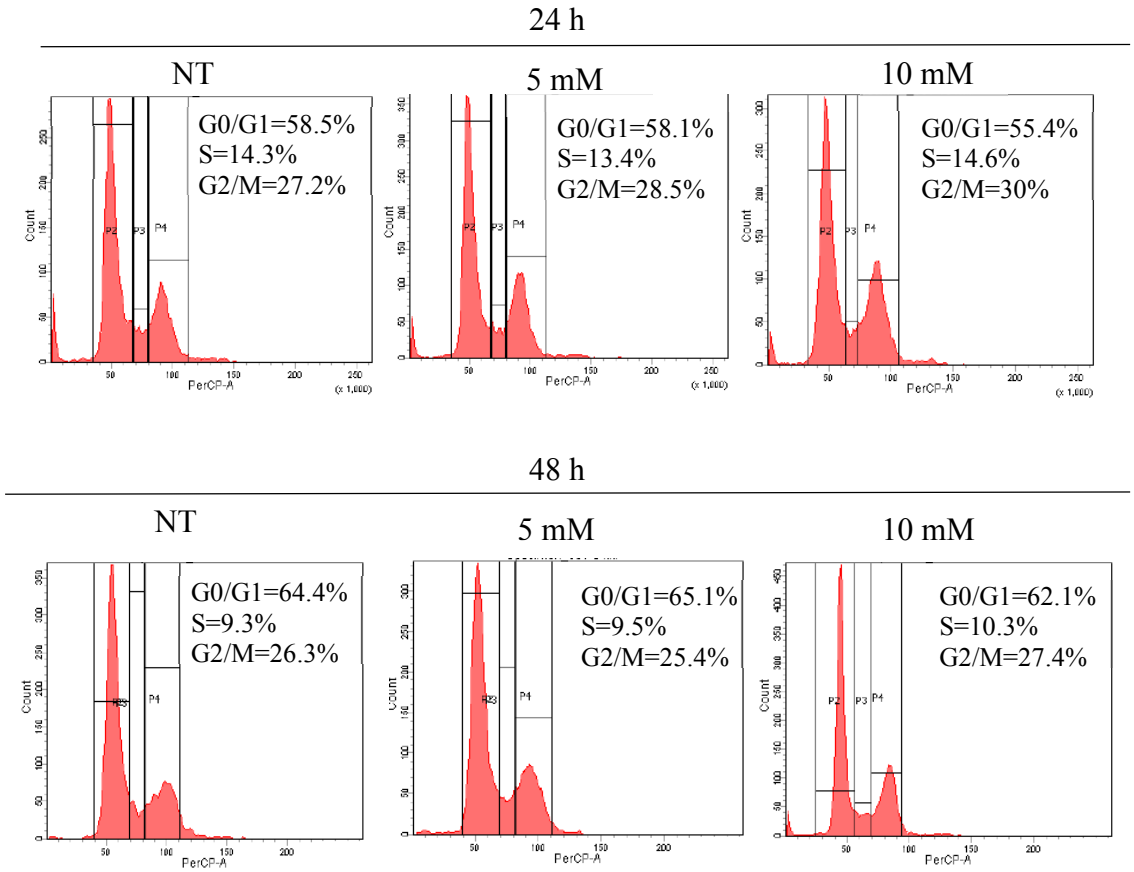


Fig.3.11 Cell cycle analysis of PC3 cells treated with LC 0, 5 10 mM for 24 and 48 h, harvested, stained with PI and analysed by flow cytometry. P1 = G0/G1 phase, P2 = S phase, P3 = G2/M phase. Three analytical replicates for each experiment were analysed.

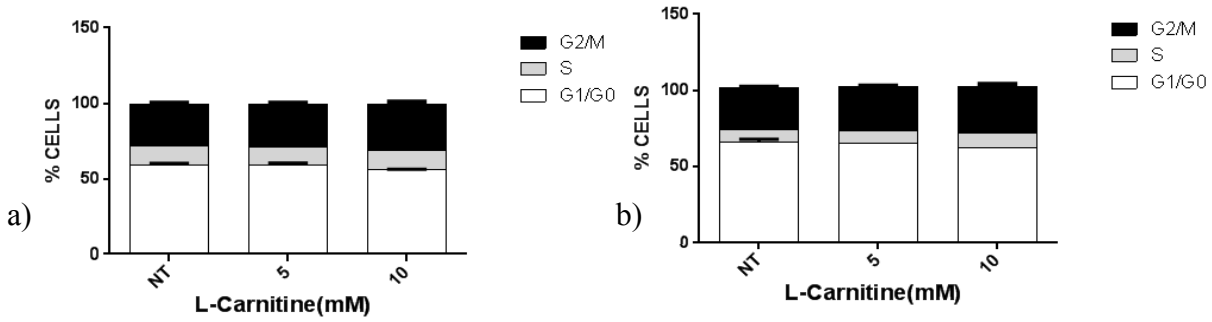


Fig.3.12 Summary of the cell cycle assay data shown in Fig.3.11.7.a) data corresponding to PC3 treated with indicated doses of LC for 24 h; b) data corresponding to PC3 treated with indicated doses of LC for 48 h. P >0.05 compared with the control group.

The results did not showed any significant increase in the percentage of PC3 cells in a particular phase of cell cycle after treatment with LC, both at 24 and 48 h (Fig. 3.11 and Fig. 3.12), suggesting that LC did not induce cell cycle arrest in PC3 cells.

3.2.3.1 LC treatment induces the expression of $p21^{Cip1}$ gene mRNA and protein in PC3 cells

Cell division relies on the activation of cyclins, which bind to cyclin-dependent kinases (CDKs) to induce cell-cycle progression towards S phase and later to initiate mitosis. Since uncontrolled cyclin-dependent kinase activity is often the cause of human cancer, their function is tightly regulated by cell-cycle inhibitors such as the $p21^{Cip}$ and $p27^{Kip}$ proteins.

To confirm the results of cell cycle analysis by flow cytometry, the transcriptional profile of two genes, $p21^{Cip}$ and $p27^{Kip}$, was investigated in PC3 cells after treatment with LC treatment (0, 5, and 10 mM) for 24 and 48 h. The effect of LC treatment on mRNA levels was determined by real-time PCR.

All samples were prepared and real-time PCR was performed in accordance to the standard protocol procedure described in Section 2.2.9.

Unexpectedly, after treatment with LC, the $p21^{Cip}$ mRNA level dose dependently increased both at 24 h and 48 h time points, and $p21^{Cip}$ mRNA level is relatively higher after 48 treatment than 24 h treatment, while $p27^{Kip}$ mRNA did not show a significant change at these two time points(Fig-3.14).

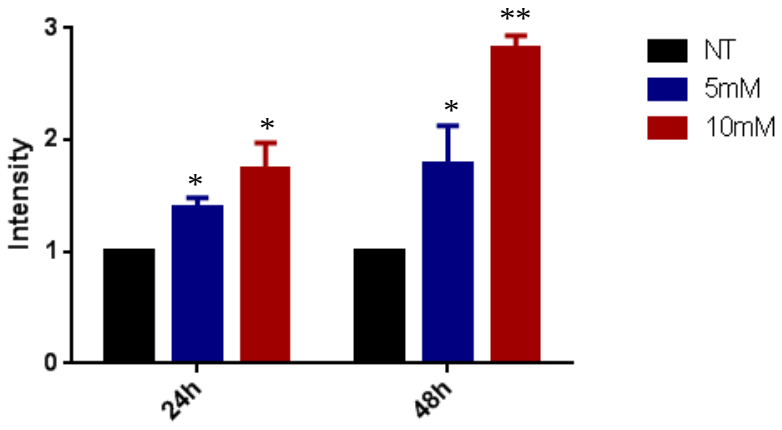


Fig.3.131 LC dose dependently induced $p21^{Cip1}$ mRNA expression. PC3 cells were incubated with different concentrations of LC (0, 5, 10 mM) for either 24 h or 48 h; the cells were collected for mRNA assay of $p21^{Cip1}$ by real-time PCR. Fold increase of the LC-treated PC3 cells versus control was shown. Mean+SD (n = 3). $^{**}P<0.01$,$^{*}P<0.05$, compared with control group(NT).

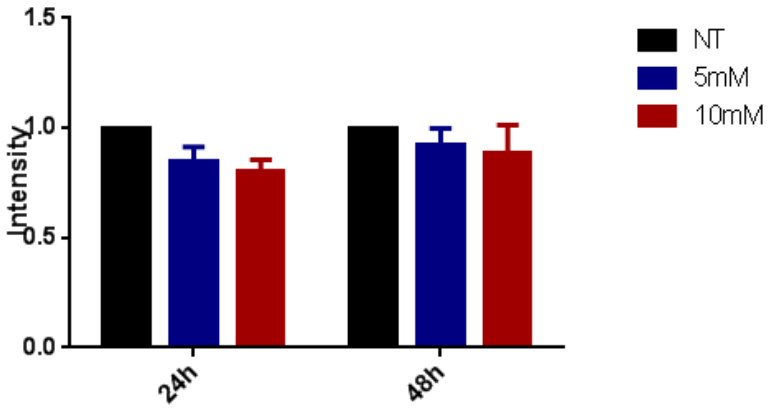


Fig.3.14 LC dose dependently did not induce p27 ^{Kip} mRNA expression. PC3 cells were incubated with different concentrations of LC (0, 5, 10 mM) for either 24 h or 48 h; the cells were collected for mRNA assay of p27 ^{Kip} by real-time PCR. Fold increase of the LC-treated PC3 cells versus control was shown. Mean+SD (n = 3). *P*>0.05 compared with control group(NT).

To further investigate the effect of LC on p21^{Cip1}, protein levels were determined by Western blot assay. The analysis was performed in accordance to the standard protocol procedure described in Section 2.2.8. The cells were treated with 0, 5, 10 mM of L-Carnitine for 24, 48 and 72 h. Three analytical replicates for each experiment were analysed.

It was found that treatment with LC increased p21^{Cip1} protein levels in a dose-dependent manner and time-dependent manner in PC3 cancer cell lines (Fig. 3.15 and Fig.3.16). This agrees with the observed p21^{Cip1} mRNA expression.

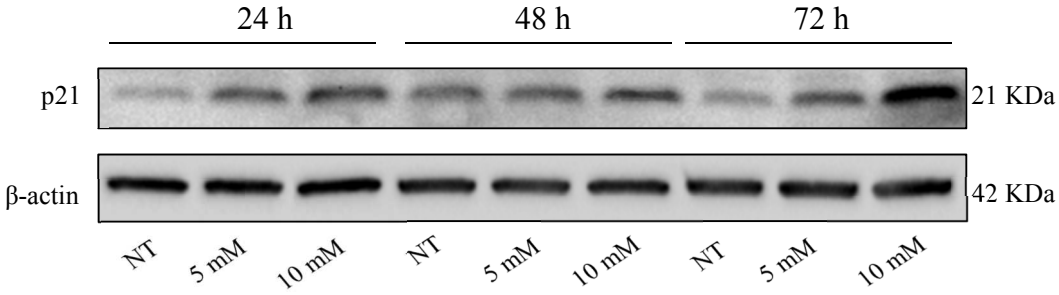


Fig.3.15 LC dose-dependently induces p21^{Cip1} protein accumulation in PC3 cancer cells. PC3 cells were treated with various doses of LC for, 24, 48 and 72 h; p21 and β-actin proteins were detected by Western blot.

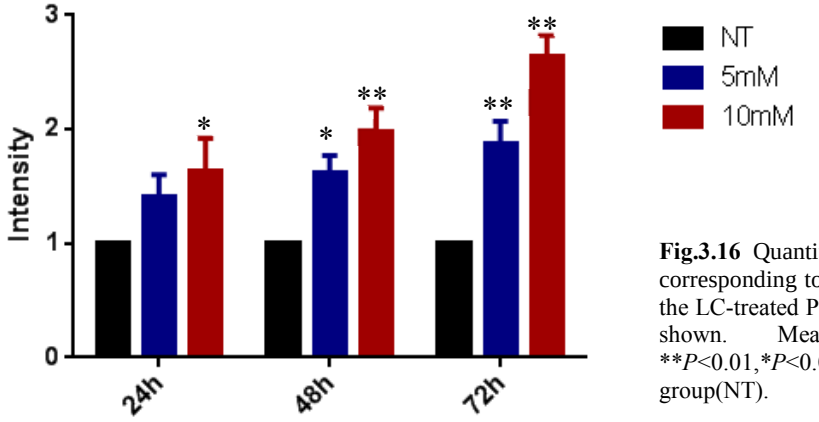


Fig.3.16 Quantification of Western bands corresponding to Fig 3.13. Fold increase of the LC-treated PC3 cells versus control was shown. Mean+SD (n = 3). **P<0.01,*P<0.05, compared with control group(NT).

The results showed that LC cell induced a significant increase in $p21^{Cip}$ both mRNA and protein in PC3 cells, even though LC did not associated with cell cycle arrest. In addition, $P21^{Cip1}$ levels increased in a dose and time-dependent manner.

3.2.3.2 LC treatment induces the downregulation of activated FAK protein in PC3 cells

LC treatment did not induce a significant apoptosis or cell cycle inhibition in PC3 cells, but a decrease of cells number, an increase of cells detachment (Fig. 3.6) and $p21^{Cip1}$ expression. To investigate and analyse these aspects, levels of FAK and phospo-FAK(p-FAK) were evaluated. Besides clustered integrins themselves, multiple structural and signalling molecules have been localized to focal adhesions, which highlight the importance of focal adhesions in the regulation of cellular structure and functions. Chief among these proteins is FAK, that is the earliest identified and one of the most prominent signalling molecules in focal adhesions. The activated FAK triggers multiple downstream pathways to regulate cell survival, apoptosis, cell cycle progression and proliferation, and focal adhesion dynamics and cell migration. In addition integrin-FAK signalling has been shown to activate a number of signalling pathways through phosphorylation and protein-protein interactions to promote tumorigenesis(Guan & Shalloway, 1992).

In particular FAK activation can promote cell survival and progression via interactions with p53. This interaction suppresses transcriptional activation of a number genes including $p21^{Cip}$ (Jihe Zhao & Guan, 2009)(Mitra, Hanson, & Schlaepfer, 2005). Therefore, the effects of LC treatment on FAK and p-FAK protein expression were determined by Western blot.

The analysis was performed in accordance to the standard protocol procedure described in Section 2.2.8. The cells were treated with 0, 5, 10 mM of L-Carnitine for 24, 48 and 72 h. Three analytical independent experiments were analysed.

The results showed that LC treatment leaded to a significant decrease in the levels of active p-FAK, in a dose-dependent manner, without altering the overall amount of the total FAK (Fig.3.17 and Fig.3.18). Thus, LC supplementation induced $p21^{Cip}$ expression, and this induction could be related to the downregulation of FAK activation. These results are also in accordance with the morphological changes observed on PC3 upon LC treatment (Fig. 3.6)

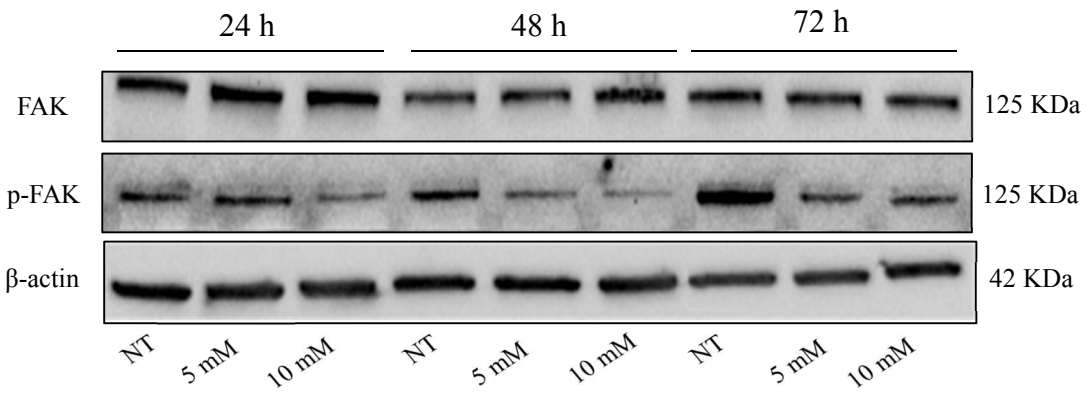


Fig.3.17 LC treatment decreased p-FAK protein accumulation in PC3 cancer cells. PC3 cells were treated with various doses of LC for, 24, 48 and 72 h; FAK, pFAK and β-actin proteins were detected by Western blot.

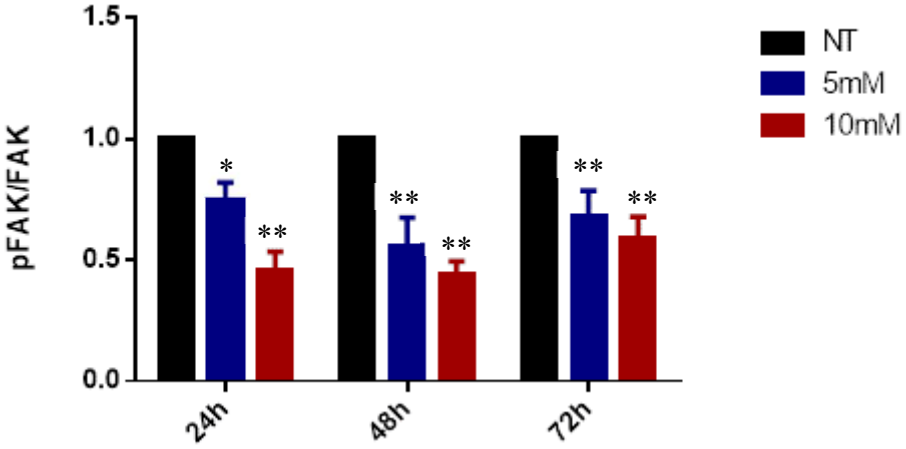


Fig.3.18 Quantification of p-FAK/FAK western bands corresponding to Fig 3.15. Fold increase of the LC-treated PC3 cells versus control was shown. Mean+SD (n = 3). $^{**}P<0.01$,$^{*}P<0.05$, compared with control group (NT).

3.2.3.3 LC treatment induces the expression of LC3 protein in PC3 cells

The p21 gene is regulated directly by the p53 gene, and involved in the p53-mediated DNA-damaging response. Tasdemir et al. have suggested the role of P53 in the regulation of autophagy (Tasdemir et al., 2008). However, the relationship between P21 and autophagy is still not understood.

Previous studies have reported that the autophagic capacity of cancer cell lines is lower than their normal counter-parts. In fact, studies of carcinogen-induced pancreatic cancer in rats have shown that pancreatic adeno-carcinoma cells have lower autophagic activity during tumour progression(Toth, Nagy, & Palfia, 2002).

Microtubule-associated protein light chain 3 (MAP-LC3) is the main marker of autophagic process. The amount of LC3-II and the ratio of LC3-II to LC3-I reflects the autophagic activity.

During autophagy, LC3 proteins is lipidated to trigger autophagosome formation. The LC3 lipidation

is preceded by a proteolitic cut of LC3, generating a shorter and active LC3 II isoform.

Expression of the LC3 protein was therefore determined in PC3 cell line, after treatment of LC, in order to evaluate the LC role as an autophagy promoter, by activation of LC3.

The analysis was performed in accordance to the standard protocol procedure described in Section 2.2.7. The cells were treated with 0, 5, 10 mM of L-Carnitine for 24,48 and 72 h. Three analytical independent experiments were analysed.

The results showed that LC treatment noticeably increased levels of LC3-II, promoting the conversion of water-soluble LC3-I to lipidated LC3-II, suggesting that LC induced autophagy. Besides, it is evident that the LC-induced autophagy reached its peak at 24 h after treatment, but began to decline thereafter (Fig 3.19 and Fig. 3.20).

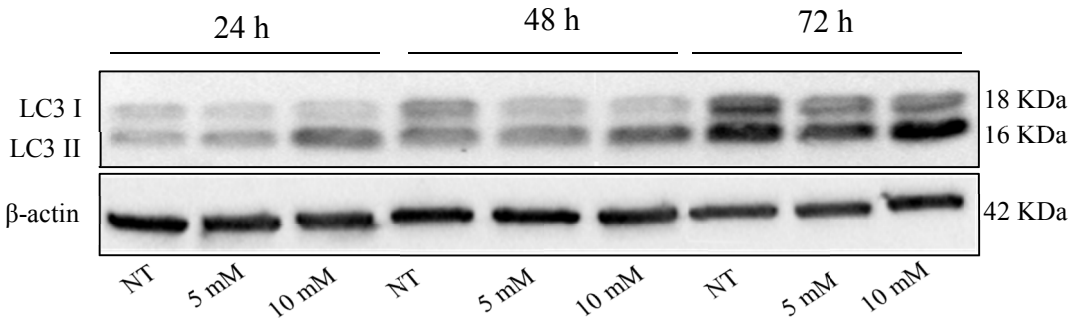


Fig.3.19 LC treatment increase LC3 II protein accumulation in PC3 cancer cells. PC3 cells were treated with various doses of LC for, 24, 48 and 72 h; p21 protein were detected by Western blot.

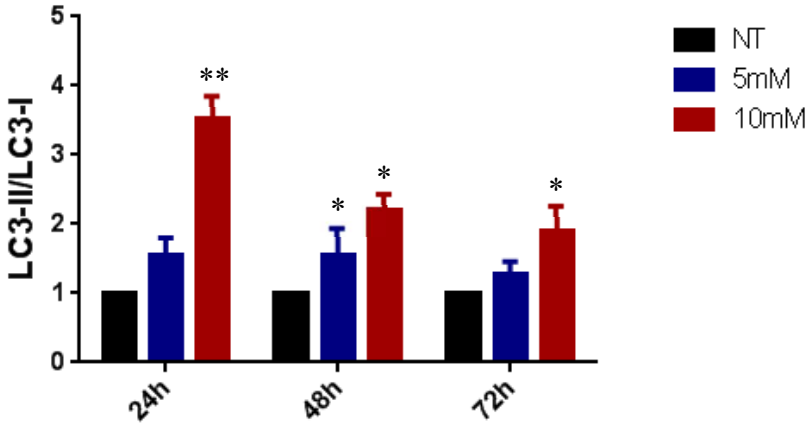


Fig.3.20 Quantification of LC3 II/LC3 I western bands corresponding to Fig 3.17. Fold increase of the LC-treated versus control was shown. Mean+SD (n = 3). $^{**}P<0.01$,$^{*}P<0.05$, compared with control group (NT).

L-Carnitine treatment, therefore, induced autophagy and $p21^{Cip}$ expression. However, whether $p21^{Cip}$ mediate autophagy or is merely a result of autophagy downstream remains to be unclear.

Based of these experiments, we may conclude that LC inhibits proliferation of PC3 cells by inducing

autophagy. We also observed that $p21^{Cip}$ is a crucial mediator of LC3 activity.

Further analysis are needed to define how the downregulation of FAK activation is linked to $p21^{Cip}$ activation and how p21 is connected with autophagy, in order to fully characterise the molecular mechanism controlled by L-carnitine in PC3 cells and the contribution to its anti cancer effect.

4 DISCUSSION

Systems biology research is focused on the study of biological components and, more importantly, their complex interactions to define the emergent properties of biological systems. Metabolomics constitutes a core area of System Biology research, focused on the study of low-molecular-weight organic and inorganic (typically < 1,500 Da) metabolites. Metabolites have an important role in biological systems. They are the building blocks for many other biological components (e.g., proteins, RNA, DNA and cell walls), they are central in intermediary metabolism, they provide many necessities for life (e.g., ATP for energy release) and they have an active role in regulation and signalling. Primary and rapid responses to environmental perturbations are generally, but not exclusively, metabolically focused and are followed by changes at the transcriptional and translational levels.

Metabolomics, like the other omics technologies, is currently being used for the identification of biomarkers and metabolic pathways altered in cancer, Differences in serum or plasma metabolite concentrations between patients and controls have been detected in several cancers, including colorectal cancer(Nishiumi et al., 2012) pancreatic cancer(Kobayashi et al., 2013) and oral cancer(Tiziani, Lopes, & Günther, 2009).

In PCa, a few studies proved evidence of metabolic differences between PCa patients and healthy controls in plasma(Lokhov, Dashtiev, Moshkovskii, & Archakov, 2010), serum samples(Zang et al., 2014) and prostatic secretions(Serkova et al., 2008). Citrate, myo-inositol and the polyamine spermine have previously been suggested as metabolic markers of PCa in prostatic secretions.

In this study prostate cancer was used as the disease model in order to test a new developed in-house bioinformatic tool for metabolomic analysis, termed SANIST. The approach combines single ion monitoring LC/SACI/ESI-MS data acquisition of the *m/z* signal related to potential biomarker candidates with a sample classification based on an innovative and modified Bayesian mathematical model. SANIST algorithm is based on the comparison of a selected biomarker fingerprint with those stored in a database using an improved Bayesian mathematical model. By using a non-targeted metabolomics analysis for the cancer detection, it is sometimes difficult to propose a limited number of candidate molecules as specific biomarkers for the diagnosis of cancer, due to the large number of differentially expressed metabolites. This hurdle may be overcome by: (i) considering the total number of peaks from the chromatographic spectra at a fixed m/z range (i.e., the entire m/z set of the molecular species ionized by MS for each sample, and (ii) comparing their profiles with known

spectra of a specific library to search for the most similar ones. For the above reasons, SANIST was tested as an alternative approach to discriminate the MS-generated spectra of plasma samples from PCa and BHP subjects.
Moreover, SANIST software was coupled to a LC/SACI/ESI-MS instrument, making up a novel analytical platform for metabolic profiling.

SACI-ESI technology is characterized by highly sensitive detection of low molecular weight compounds, in particular when applied for biomarker discovery(Sogno et al., 2012). SACI/ESI makes it possible to efficiently detect potential disease-related biomarker candidate metabolite signals(Sogno et al., 2012)(Conti, Motta, Puggioli, & Brambilla, 2013)(Conti, Tazzari, Bertona, Brambilla, & Brambilla, 2011)and represents a new frontier in the medical diagnostic field, providing relevant information beyond conventional analysis of blood and urine. Among the different approaches developed for mass spectrometry (MS) biomarker discovery over the last few years(Chapman, Goodlett, & Masselon, 2013)(Flatley, Malone, & Cramer, 2014)(Liebler & Zimmerman, 2013), SACI/ESI has been shown to be more sensitive and accurate with respect to classic mass spectrometric technologies based on ESI alone and Atmospheric Pressure Chemical Ionization(Sogno et al., 2012)(Conti et al., 2013)(Conti et al., 2011). A strong reduction in matrix effect together with improved quantification performance was obtained with SACI when matched with ESI(Sogno et al., 2012)(Conti et al., 2013)(Conti et al., 2011).

In the study presented herein, SANIST platform was applied to serum samples from PCa patients and BPH subjects, thus the study represents a highly relevant clinical setting where PSA levels can be misleading. Samples were obtained from subjects undergoing a prostate biopsy for PCa diagnosis, which represents a typical clinical situation and challenging biomarker *scenario* where the samples must discriminate between PCa positive and patients with other potential prostate conditions.
Samples were subjected to LC/MS analysis using water and CH_3OH gradients, solvents often used in carnitine studies(Fingerhut et al., 2009)(Morand, Donzelli, Haschke, & Krähenbühl, 2013). A first initial SANIST biomarker discovery on a training set of sera from patients with histologically confirmed prostate cancer and subjects whose biopsies showed BPH. In order to obtain potential biomarker candidates for a prostate cancer panel to be used for the SANIST approach, the SACI/ESI data were converted into the mzXML standardised format and elaborated using the XCMS software. These data were then analysed using the R bioinformatic statistical software allowing a comparison of all the *m/z* values of the chromatographic peak areas between BPH and prostate cancer subjects.

The results showed that three of the acylcarnitines, namely Octanoyl-L-carnitine, Decanoyl-L-carnitine, and 5-cis-Tetradecenoyl carnitine, had decreased levels in plasma samples from PCa patients compared with BPH controls. The identity of target analytes was also supported by means of MS/MS analysis and structural studies.

SANIST software was used to acquire the specific *m/z* values from the extracted mass chromatograms of the selected biomarker signatures, using the high accuracy Orbitrap mass analyser. These biomarkers are used to generate a reference NIST database and as an initial test, biomarker fingerprint of each subject in the training set was compared with those inserted in the database, excluding the identical match to itself. SANIST automatically proceeds to data elaboration using the Bayesian elaboration model developed in order to statistically evaluate the classification efficiency of the carnitine metabolite biomarker 'fingerprint'. A complete discrimination (100%) was achieved among BPH and prostate cancer patients with a recognition identity % match between 90 and 94%.
A second test set of PCa patients and BPH subjects were then analysed using the SANIST platform and the potential biomarker profile database identified in the training set was employed to classify the test set. This analysis confirmed the same potential biomarker panel found in the training set. Using this approach, all test set sera with BPH biopsies were matched to training set BPH biopsy samples, while all test set prostate cancer positive patients were matched to training set prostate cancer patients.
These data suggest that the detected acyl carnitines are biologically relevant candidate biomarkers likely generated by possible altered mitochondrial metabolism of prostate cancer subjects and could represent potentially useful markers for diagnosis.

Carnitines and their derivatives are involved in transport of activated long-chain fatty acids from cytoplasm into mitochondrial compartment where the enzymes involved in β-oxidation are located. Inside the mitochondria the fatty acids are catabolised to generate acetyl-CoA, NADH and $FADH_2$. Therefore, carnitines are responsible for maintenance of normal mitochondrial functions, thus disturbances in carnitine metabolism result in mitochondrial dysfunction. I
In cancer cells carbon must be diverted from energy production to FAs for biosynthesis of membranes and signalling molecules. The bulk of cell membrane lipids are phospholipids (PLs), such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), in addition to other lipids, such as sterols, sphingolipids, and lysophospholipids. All of these lipids are derived in part from acetyl CoA, and many contain FAs.

Mutations of mitochondrial DNA were also identified in prostate cancer(Chen, Gokden, Greene, Mukunyadzi, & Kadlubar, 2002)(Jerónimo et al., 2001)(Lindberg et al., 2013) and deregulated mitochondrial metabolism has been suggested to play a relevant role in prostate carcinogenesis (Leav et al., 2010)(Altieri et al., 2009)(De Bari, Moro, & Passarella, 2013).

In contrast to most cancers, however, increased glucose consumption is not as characteristic for PCa as for other cancers, as evidenced by low fluorodeoxyglucose uptake on PET imaging(Hofer et al., 1999)(Yu, Desai, Ji, Groshen, & Jadvar, 2014). Experiments in three different PCa cell lines showed a higher cellular uptake of fatty acids over glucose, suggesting that fatty acid metabolism is a vital energy source for PCa cells (Liu, Zuckier, & Ghesani, 2010). As rapid cell proliferation in malignant tumours is associated with increased energy supply, if glucose consumption is not elevated in prostate cancer, alterative metabolic approach, especially fatty acid oxidation may exist dominantly to provide bioenergy for abnormal cells proliferation and growth.

Decreased levels of acylcarnitines found in this study in the blood of PCa patients, confirmed, therefore, an increased biosynthesis of membranes and signalling molecules and a higher degree of mitochondrial fatty acid metabolism in PCa.

Based on relevance of fatty acid oxidation in prostate cancer, and based in part upon carnitine role in transporting fatty acids into mitochondria, it could be postulated that L-carnitine supplementation further increase lipid metabolism in PCa.

The majority of the research in healthy subjects has shown no effect on fatty acid oxidation or muscle concentrations of L-Carnitine despite increases in plasma carnitine (Spriet, Perry, & Talanian, 2008). Some studies indicate that endogenous carnitine is sufficient for fatty acid oxidation(Vukovich, Costill, & Fink, 1994) (Oyono-Enguelle et al., 1988)(10,25). Other studies have shown declines in fatty acid oxidation(Roepstorff et al., 2005).

L-Carnitine (3-hydroxy-4-N-trimethylaminobutyric acid) has an essential role in the metabolism of lipids and in the production of cellular energy. Carnitine has numerous critical physiological roles, including β-oxidation of fatty acids, facilitating transport of fatty acids across the mitochondrial inner membrane as acylcarnitine esters and modulating intracellular CoA homeostasis (Tein, 2003) (Lahjouji, Mitchell, & Qureshi, 2001). Carnitine is involved in the transfer of the products of peroxisomal β-oxidation to the mitochondria.

Carnitine is a potent antioxidant and has been shown to possess anticancer properties, usually in combination with others anti tumour substances. In combination of butyrate or curcumin treatment

on HT29 cells, L-carnitine had beneficial effects on colon cancer prevention, inhibiting neoplastic cell proliferation, inducing colon cancer cell apoptosis via downregulating antiapoptotic and upregulating proapoptotic genes (Roy et al., 2009)(Roscilli et al., 2013). In these cases it was supposed that L-carnitine may help to maintain preserved levels of fatty acids β-oxidation, and when colon cancer cells were forced to use fatty acids as substrates for energy metabolism, they undergo apoptosis by the increased burden of ROS produced in the respiratory chain (Wenzel et al., 2005). In prostate cancer L-carnitine was never studied, probably because fatty acid metabolism is not impaired in this type of tumour. However, L carnitine selectively inhibited hepatoma cancer cell growth both in vitro and in vivo in mice by another approach, directly inducing $p21^{Cip}$ expression and inhibiting HDAC activities (Huang, Liu, Guo, et al., 2012).

Thus, because L-carnitine may acts as an anticancer agent activating different mechanism, in the second study presented here the effects of L-carnitine cytotoxicity on prostate cancer cells PC3 was investigated for the first time.

The experiments confirmed that L-carnitine inhibited PC3 cell proliferation, but not modulating cell cycle or exerting apoptotic effects, as demonstrated by flow cytometry analysis. Interesting, the antiproliferative effect of L-carnitine has been associated to an increased expression of $p21^{Cip}$ mRNA but not $p27^{Kip}$ mRNA. It is well known that $p21^{Cip}$ and $p27^{Kip}$ are cyclin-dependent kinase inhibitors that directly inhibit the activity of cyclin E/CDK2 and cyclin D/CDK4/6 complexes. Thus, to further investigate the effect of L-carnitine on $p21^{Cip1}$, its protein levels were determined, and the results showed that $p21^{Cip}$ protein level increased dose and time-dependently with L-carnitine treatment. The increased $p21^{Cip}$ protein level and, on the other side, the negative results of flow cytometry analysis about cell cycle appeared, at a glance, conflicting.

Considering that L-Carnitine treatment caused both increased $p21^{Cip}$ protein level and PC3 cells detachment, the behaviour of focal adhesion kinase protein was evaluated.

Focal adhesion kinase is, effectively, a non-receptor tyrosine kinase that is localized at focal adhesion sites, and is a signal integrator capable of relaying signals from soluble growth factors and cytokines mechanical stimuli, as well as integrin engagement. FAK is, moreover, a multifunctional regulator of cell signalling within the tumour microenvironment. During development and in various tumours, FAK promotes cell motility, survival and proliferation through kinase-dependent and kinase-independent mechanisms. In the past few years, Phase I and II clinical trials have been initiated with FAK inhibitors(Jean et al., 2014)(Stokes et al., 2011)(Ward et al., 2013); however, some of the

functions of FAK in tumorigenesis remain under investigation. The most well-characterised mechanism that promotes FAK activation involves FAK dimerization(Brami-Cherrier et al., 2014). This leads to FAK autophosphorylation at Y397, binding of SRC-family kinases to the phosphorylated site, SRC-mediated phosphorylation of the FAK kinase domain activation loop (Y576 and Y577), and formation of an activated FAK–SRC complex(Jihe Zhao & Guan, 2009)(Schaller, 2010). The activated FAK/SRC complex then trigger a cascade of phosphorylation events and new protein-protein interactions to activate several signalling pathways. These FAK signalling pathways have been shown to regulate a variety of cellular functions both in normal and cancer cells.

Many studies demonstrated a link between FAK and $p21^{Cip}$, involving different mechanisms(Cheng et al., 2014)(Lim et al., 2008)(J Zhao, Pestell, & Guan, 2001). For example FAK deletion have promoted p53-mediated induction of $p21^{Cip}$ in advanced squamous cancer cells, exposed to γ irradiation(Graham, Moran-Jones, Sansom, Brunton, & Frame, 2011). Another study have suggested that in endothelial cells the ability of cells to degrade $p21^{Cip}$ in response to many diverse stimuli likely converge at the level of FAK signalling.

In the present study PC3 cells treated with L-Carnitine showed a marked reduction of p-FAK protein levels. Thus, there was a possible connection between downregulation of FAK activity and activation of $p21^{Cip}$, in agreement with previous publications.

On one side on the downregulation of FAK activity could explain activation of $p21^{Cip}$, on the other side they didn't clarify the mechanism by which L-Carnitine inhibits PC3 cells proliferation and increases PC3 detachment. The cytotoxic effect of L-Carnitine was not associated with induction of apoptosis at the doses used in the study, as shown by absence of caspase 3 and co-staining of PC3 cells with 7-aminoactinomycin D and annexin V, or with block of cell cycle, as confirmed by staining of cells with propyl iodide.

P21 was originally identified as a universal inhibitor of cyclin-dependent kinases (CDK). But, accumulating evidence has shown that $p21^{Cip}$ has multiple functions in addition to CDK inhibition including cell cycle regulation, inhibition or mediator of autophagic signalling pathway(Fujiwara et al., 2008)

LC3 is recognized as the most common autophagy marker, and LC3-II is typically used as a marker for autophagosome formation. The detection of expression of the LC3 protein in PC3 cells after treatment showed that L-Carnitine significantly increased the level of LC3-II, promoting the conversion of water-soluble LC3-I to lipidated LC3-II, which was recruited to autophagosomsal membranes. In recent years, the autophagic process has been observed in response to various anti

cancer compounds(Yang, Chee, Huang, & Sinicrope, 2011). Some studies regarding the effect of anti tumour agents on prostate cancer cells have reported that autophagy was correlated to an up regulation of $p21^{Cip}$(Peng et al., 2013)(X. Li, Li, Wang, Ye, & Li, 2012). For this reason the inhibition of proliferation and induction of autophagy by L-Carnitine in PC3 prostate cancer cells possibly confirmed the association with activation of $p21^{Cip}$.

5 CONCLUSIONS

In the present study, a new strategy to perform metabolomic analysis was proposed, using a LC/SACI/ESI-MS system. A novel, dedicated SACI/ESI software, named SANIST, was developed in house for both biomarker fingerprint data acquisition and as a diagnostic tool. Prostate cancer (PCa) was used as the disease of interest. Prostate cancer was used as the disease model in order to test the SANIST platform, because PCa is a leading cause of mortality and morbidity in males worldwide. New effective diagnostic approaches are urgently needed as prostate-specific antigen (PSA), the best-known prostate cancer biomarker candidate, is prostate but not cancer specific, thus resulting in false positives. The newly developed method was used to analysed the samples of PCa patients and BHP subjects. Serum biomarkers identified using the SANIST platform demonstrated that patients with PCa had lower medium-chain acylcarnitines (Decanoyl-L-carnitine, Octanoyl-L-carnitine 5-cis-Tetradecenoyl carnitine) than BHP controls. Using these biomarkers, the SANIST algorithm allowed separation of patients with PCa from biopsy negative subjects with high accuracy and sensitivity. These results proved the applicability of SANIST approach in biomarker discovery for disease research.

Moreover, this pilot study showed significant differences in the metabolic profiles of blood samples from PCa patients and BPH controls. The analysis of serum gave successful discrimination of patients and controls with BPH, and provided an insight into the metabolic processes characteristic of PCa, with data suggesting changes in fatty acid metabolism.

A more extensive validation of this panel using a much larger cohort is needed to confirm the clinical usefulness of this panel. So blood samples from a larger study cohort are currently being collected for further validation of the metabolic differences between PCa patients and controls with clinically relevant BPH, and to perform correlations between the metabolic data and the clinical parameters as PSA and Gleason score.

Based on relevance of fatty acid oxidation in prostate cancer, and based in part upon L-Carnitine role in transporting fatty acids into mitochondria, and in part upon its anticancer properties, the second project of this thesis focused on effect of L-carnitine supplementation on the prevention of cancer development in PC3 cells.

L-carnitine plays a variety of important metabolic functions: are involved in the oxidation of long chain fatty acids, regulate acyl-CoA l. In cancer cells L-carnitine levels and influence protein activity and stability by modifying the extent of protein acetylation. In addition L-carnitine seems to possess

anticancer properties.

Treating PC3 with L-carnitine, cells proliferation was inhibited, but nor modulating the expression of cell cycle regulatory genes or triggering apoptosis. The antiproliferative effect was dose and time-dependent and associated with an increase of $p21^{Cip}$, as demonstrated by mRNA and protein levels. $p21^{Cip}$ is a key molecule in the regulation of the cell cycle and autophagy/cell death. In effect L-carnitine treatment induced autophagy in PC3 cells, as demonstrated by increased expression of LC3-II by Western Blot.

Moreover to further investigate the effect of L-Carnitine on the upstream pathway of $p21^{cip1}$, FAK modulation was evaluated, considering that inhibition of PC3 proliferation showed an increase of cells detachment also. L-Carnitine treatment decreased level of p-FAK protein, downregulating FAK activity. This result on one side did not clarify the cause of cells detachment, on the other side it could be in relation with activation of $p21^{Cip}$.

The present studies have demonstrated that L-Carnitine has antiproliferative and pro-autophagic activities on PC3 human prostate cancer cells. The link between up-induced suppression of PC3 cell proliferation and induction of autophagy is $p21^{Cip}$, that is the crucial mediator of LC3 activity.

However, additional analysis are needed to verify these results and uncover the signalling mechanisms by which FAK modulate $p21^{Cip}$ activity, and how $p21^{Cip}$ is connected with autophagy, in order to fully characterise the molecular mechanism controlled by L-Carnitine in PC3 cells and the contribution to its anti cancer effect.

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