Sveriges lantbruksuniversitet
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Populärvetenskaplig sammanfattning

Prostatacancer är en av de vanligaste cancertyperna i västvärlden och drabbar runt tiotusen män per år i Sverige. Prostatan är en litet organ som ligger under urinblåsan hos män och vars funktion är att producera och utsöndra viktiga komponenter i sädesvätskan. På grund av den stora mängden lidande och de omfattande ekonomiska kostnader som prostatacancer ligger till grund för i samhället så är behovet av forskning inom området mycket stort.

Cancer i prostatan är i sig inte dödligt. När prostatacancer har dödlig utkomst så är det uteslutande i fall där cancern har spridit sig till viktigare organ, så som ben, lungor eller lymfkörtlar. Att cancer sprids kallas metastas och är en komplex biologisk process som består av flera distinkta steg, och som påverkas av många olika faktorer. Tidigare har forskare vid University of Michigan, Ann Arbor visat att metaboliten sarkosin ansamlas i stegvis värre fall av prostatacancer, framför allt då cancern går från att befinna sig lokalt i prostatan till att sprida sig till andra vävnadstyper. I deras artikel beskriver de sarkosin en onkometabolit, det vill säga en metabolit som är kapabel att driva cancers förvärring. Dessutom hävdar de att sarkosin kan användas som analyt i av blod- eller urinprover för att påvisa cancer. Resultaten kring sarkosinets roll inom biomedicinsk analys har ifrågasatts, men resultaten kring dess roll som onkometabolit har inte undersökts i detalj ännu.

I experimentet som ligger till grund för den här rapporten har vi tittat närmare på hur närvaron av sarkosin påverkar prostatacellers metabolism. Prover förbereddes genom att inkubera celler i provrör tillsammans med sarkosin eller med någon av tre närbesläktade metaboliter; glycin, dimetylglycin och alanin. Analyserna utfördes med hjälp av en metod som kallas NMR-spektroskopi (Nuclear Magnetic Resonance-spektroskopi), vilket är en kraftfull analysmetod som används för att analysera kolföreningar. Våra resultat indikerar att sarkosins påverkan på celler har en omfattande effekt på cellens metabolism. Flera viktiga cellulära funktioner, så som energiomsättning, användning av aminosyror samt fettomsättning påverkas då sarkosin tillsatts.

Resultaten som har genererats inom den här studien kan i framtiden ligga till grund för framsteg med klinisk betydelse. Många av de viktigaste etablerade läkemedlen mot cancer angriper cancerns förmåga att ställa om cellens metabolism och det är en måltavla för forskare som avser att hitta nya läkemedel. En ny infallsvinkel på cancermetabolism i en av de kliniskt viktigaste cancertyperna har därför stor betydelse.

Abstract

Prostate cancer is one of the most common form of human cancer in the world and the most common form of cancer in men in the Western world. There are many molecular factors of prostate cancer, both proteins and metabolites. A 2009 study by Sreekumar et al found that the N-methyl form of glycine, also known as sarcosine, is correlated with more advanced prostate cancer and that it could potentially be driving cancer progression. Since then, a transcriptomic analysis has revealed some of the genes that might be involved in sarcosine-driven prostate cancer progression but the exact mechanism is as of yet not known. We set out to examine the effect of treatment with sarcosine, glycine, N,N-dimethylglycine and alanine on the metabolism of cultured prostate cancer cells and non-malignant immortalized prostate cells. Here we demonstrate that sarcosine changes the metabolism of prostate cancer cells and that broad cellular functions, such as energy metabolism, pyrimidine metabolism and amino acid metabolism are affected. Using NMR metabolomics we showed that the cellular concentrations of glucose, choline, O-phosphocholine, creatine, creatine phosphate, proline and other metabolites are altered as a result of the presence of sarcosine. Previous transcriptomic data has pointed towards the cell cycle as being involved in a potential mechanism of sarcosine-induced prostate cancer progression. Our data suggests significant metabolic involvement as well. Metabolism has seen a resurgence recently in cancer research in general and in this research we demonstrate that is worth exploring deeper in prostate cancer specifically.

Introduction

Prostate cancer is the third most common cancer in the world and the most common in the developed world. Every year almost 10,000 new cases are diagnosed and around 2,500 men die from prostate cancer malignancies in Sweden (Cancerfonden, 2013). The prostate is a tiny, excreting gland in the male reproductive system located underneath the bladder. It consists of three main cell types, epithelial luminal cells, basal stem cells and neuroendocrine cells (**Fig. 1**). Cancer in the prostate almost exclusively occur in the prostate epithelial cells, which are responsible for secreting many important component of seminal fluid (Toivanen and Shen, 2017).

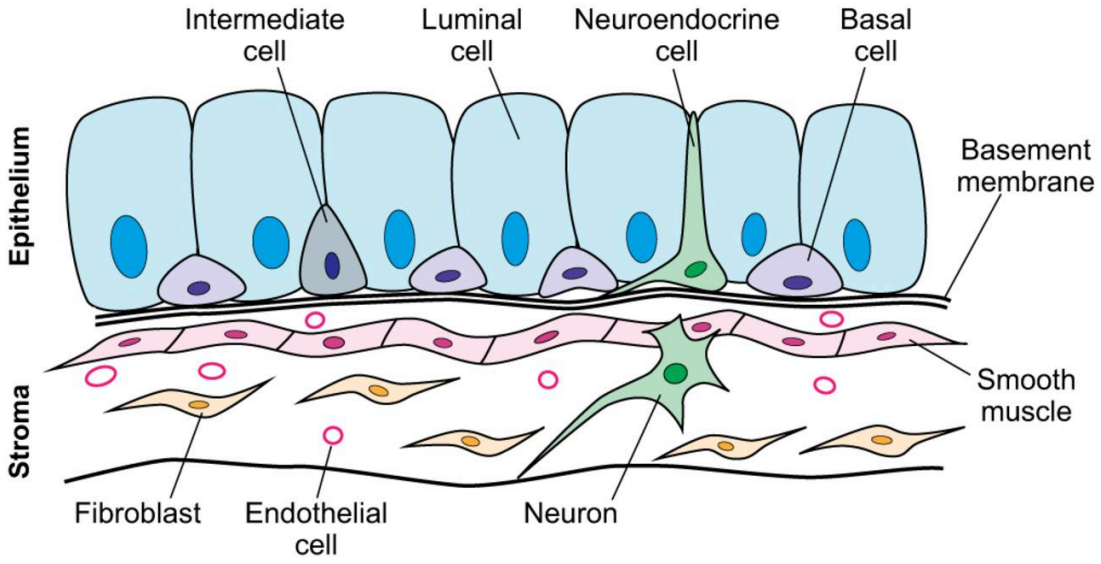

Figure 1: Representative depiction of a prostate epithelium. Large, connected epithelial cells bound together by tight junctions to form a barrier that is difficult to permeate for most molecules. Basal stem cells hold the epithelium anchored to the basement membrane as well divide into new epithelial cells, and nerve cells regulate prostate contraction via smooth muscle fibers encircling gland. Adapted from Toivanen et al, Development, 2017, with permission from the publisher.

Tumors in the prostate are generally not lethal. However, high-grade tumors can eventually become metastatic and invade other tissues such as lung-, bone-, lymph- and liver tissue, which are all potentially lethal sites (Sartor and de Bono, 2018). Because of the human suffering and the high amount of resources that are allocated to fighting prostate cancer, research in this area is desperately needed.

Molecular biology of prostate cancer

The exact mechanism by which cancer occurs is not completely understood, and appears to be diverse, but it involves sequential mutations resulting in similar phenotypic changes. There are different factors governing the rate at which mutations occur, such as the state of DNA repair systems, the presence of carcinogens, and metabolic factors (Hanahan and Weinberg, 2011). Some mutations are more common than others in distinct tissue types. Common genetic factors of prostate cancer are general oncogenes and tumor suppressors, such as phosphatase- and tensin homolog (PTEN), signal transducer and activator of transcription 3 (STAT3) and c-Myc (Shand and Gelmann, 2006; Pourmand *et al.*, 2007; Gandhi *et al.*, 2018). Under healthy physiological conditions, a balance between tumor suppressors and oncogenes exists. This balance is often disturbed in cancer. PTEN, STAT3 and c-Myc are master regulators of cell growth and in cancer their roles are to enable the cells to proliferate endlessly. The role of PTEN under normal physiological conditions is to end the PI3K/Akt/mTOR cascade, which is a downstream response to growth signaling, by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (Pourmand *et al.*, 2007). STAT3 and c-MYC are transcription factors that are activated by growth signaling and which regulate genes involved in cell growth. When Stat3 is knocked out the progression of prostate cancer increases, and a higher expression might have a protective effect (Priolo *et al.*, 2014; Pencik *et al.*, 2015). In some cancers, the activities of these proteins are altered to affect the metabolic state of the cell in important ways, such as increasing the rate of glycolysis and the oxidation of fatty acids through β-oxidation (Eidelman *et al.*, 2017).

In prostate cancer specifically, two of the most commonly mutated genes are the androgen receptor (AR) and the ETS-related gene (erythroblast transformation specific-related gene, ERG) (Packer and Maitland, 2016). Under normal physiological conditions these two transcription factors are involved in regulating hundreds of genes related to cell proliferation, differentiation, inflammation and apoptosis. The AR is activated by binding to either testosterone or dihydrotestosterone and this activation results in changes in gene expression that leads to cell proliferation (Massie *et al.*, 2011). ERG is expressed in the entire body but a common gene fusion with the promoter region of the gene TMPRSS2, which results in dramatic overexpression, is found in over 50% of prostate cancer cases (Tomlins *et al.*, 2008; Li and Vederas, 2018). Both of these transcription factors respond to androgen signaling and regulate genes that encode metabolic enzymes (Massie *et al.*, 2011). It has been shown that they cause profound changes in cellular metabolism once overexpressed in cancer. A feature of prostate cancer that generally results in poor patient outcomes is androgen independence. Androgen independence occurs when either of these two genes overcomes the need for the presence of androgen at physiological concentrations for their activity. This can occur through overexpression as a result of a few known biological mechanisms, such as through gene duplications, through point mutations resulting in being constitutively active or through mutations resulting in increased androgen levels (Karantanos, Corn and Thompson, 2013) .

Cancer metabolism

A central aspect of all cellular metabolism that is shared by all cells is the conversion of glucose into energy. The first step of this conversion is through glycolysis, where glucose is converted to pyruvate through a series of ten reactions. Pyruvate then enters the tricarboxylic acid cycle (TCA cycle) via a reaction known as the linking reaction. In most human cells, products of the TCA cycle are used to produce energy through oxidative phosphorylation. Some substrates of the reactions of glycolysis are also used as substrates for a secondary pathway called the pentose phosphate pathway. While glycolysis and the TCA cycle are oxidative pathways that produce energy, the pentose phosphate pathway is a pathway that produces substrates for biosynthetic pathways for many nucleotides and amino acids. These three components together; glycolysis, the TCA cycle, and the pentose phosphate pathway are called central-carbon metabolism and form the backbone of the metabolism of all known forms of life (Noor *et al.*, 2010). Apart from the central carbon metabolism, each distinct cell type has unique aspects to its metabolism. One example in humans is that many cells can sustain their TCA cycle metabolism through the oxidation of fatty acids produced by the liver and stored in adipocytes, as is the case during starvation (Perry *et al.*, 2018). Another example is epithelial cells which are replaced often and therefore need to divide often have a more active nucleotide metabolism than terminally differentiated cells such as neurons or osteocytes (Aird and Zhang, 2015).

It is only recently that metabolism has resurged in cancer research as not only a symptom of cancer but as a phenomenon capable of driving cancer progression and affecting outcomes. Cancer cells rewire their metabolism in order fuel their increased growth rates (Hanahan and Weinberg, 2011). It is believed this rewiring is done partly through changes in the expression or activity of regulator proteins, such as transcription factors or signaling proteins, and partly through changes to metabolic enzymes and transporters which have a direct effect on cellular metabolism. An example of this has been demonstrated in prostate cancer by the metabolism of citrate. Healthy prostate tissue accumulates zinc, which inhibits the TCA cycle enzyme aconitase and results in accumulation of citrate which is subsequently used for lipogenesis or excreted into the seminal fluid. In prostate cancer cells, the intracellular levels of zinc are reduced. This results in an increased aconitase activity, a reduced concentration of citrate and a higher rate of TCA cycle flux (Eidelman *et al.*, 2017). In this regard, prostate cancer stands out compared to most other cancers in which TCA cycle flux is reduced through the so-called Warburg effect (Vander-Heiden *et al.*, 2009).

Another prostate cancer-specific metabolic alteration takes place in development of androgen independence. In healthy prostate epithelial cells, androgen signaling activates the synthesis of vital lipids (Butler *et al,* 2016). Lipids are necessary for growing cancer cells. Loss of androgen signaling can therefore result in growth inhibition of cancers which still rely on androgen signaling. In order for prostate cancer to become androgen independent a significant

shift in the cellular metabolism of lipids needs to take place in which the biosynthesis of lipid molecules can occur without the presence of androgen. Malignant prostate cells overexpress many genes involved in lipogenesis, such as fatty acid synthase (FASN), sterol regulatory element binding protein 1 (SREBP1) and steroyl-CoA desaturase. The activities of these proteins, FASN in particular, have also been shown to be increased in many prostate cancers (Butler *et al*, 2016).

Metabolomics

Metabolomics is an analytical approach in which the concentrations of small, organic molecules are measured in a biological sample. It relies on analytical chemistry techniques, such as mass spectrometry and NMR spectroscopy. While mass spectrometry is and more sensitive, NMR spectroscopy offers one main advantage, which is that it has significantly less instrumental variation. The smaller amount of variation makes it ideal when measuring small differences. Since NMR spectroscopy is not destructive, these two methods can be used together. Traditionally, metabolomics has been used as a method for finding biomarkers of various diseases. However, that has changed lately and it has become a more commonly used method for researchers interested in exploring the cellular metabolism of a given sample (Johnson *et al*, 2016). There are different methods that gather various kinds of biological data about a sample, such as genomics, transcriptomics and proteomics. The foremost advantage of metabolomics as compared with other omics-techniques is that it shows the actual state of the cell. Metabolites are the substrates and the products of most biological processes and other biological changes such as alterations in gene expression or protein activity ultimately result in changes to the pool of metabolites.

One-carbon metabolism and sarcosine

Many of the biosynthetic processes in the cell requires the addition, activation or transfer of single carbon units, which are present in the cell in the form of methyl-groups carried by the enzymatic co-factor folate. Folate-carried methyl-groups are required for methylation of DNA for epigenetic silencing, biosynthesis of amino acids and nucleotides and maintaining the redox balance through $NADP^+$/NADPH cycling. The methyl-groups on folate have slightly different energy-levels depending on to what extent the methyl-groups are oxidized or reduced (Ducker and Rabinowitz, 2017).

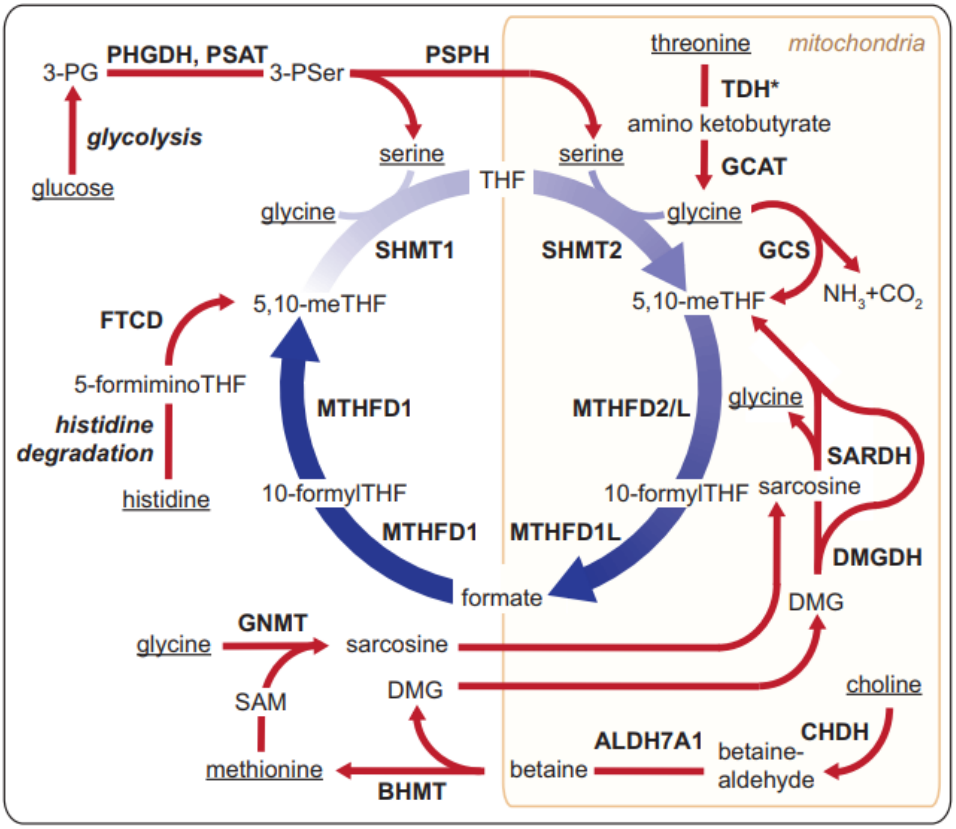

Figure 2: Scheme of human folate metabolism. (Adapted from Rabinowitz, 2017, with permission from publisher).

Folate gets its high-energy methyl groups primarily from the metabolism of the non-essential amino acid serine (**Fig. 2**). Serine is metabolized into glycine by the enzyme serine hydroxymethyltransferase (SHMT), giving one hydroxymethyl-group to THF. The methyl-group can then be used to methylate S-adenosylhomocysteine to make S-adenosylmethionine (SAM), which can be used as a co-factor by the enzyme glycine N-methyltransferase (GNMT) to methylate glycine into sarcosine. SHMT is present in mitochondria, but the mitochondrial need for single carbon units is small. Most of the use of single carbon units is taking place in the cytoplasm, making the localization of their production in the mitochondria counterintuitive. The reason this division is taking place is not known. One hypothesis is that it decouples serine-dependent production of single-carbon from glycolysis (Amelio *et al.*, 2014; Ducker and Rabinowitz, 2017).

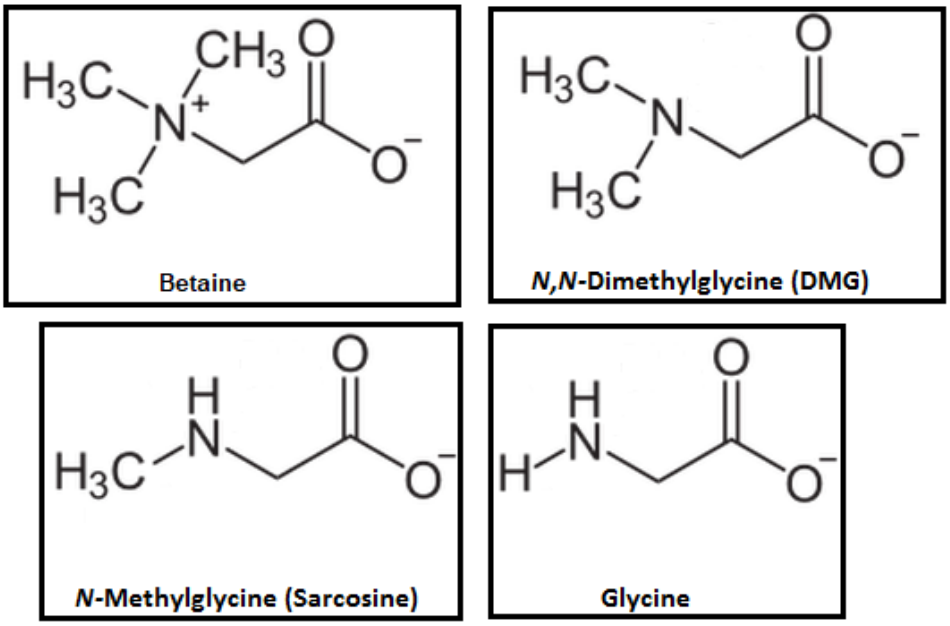

Figure 3: Metabolites in the pathway breaking catabolizing betaine through serial demethylation into glycine.

The enzymes regulating the concentration of sarcosine present in the cell are GNMT, which methylates glycine to form sarcosine, sarcosine dehydrogenase, which demethylates sarcosine to form glycine, and dimethylglycine dehydrogenase, which demethylates N,N-dimethylglycine to form sarcosine (**Fig. 3**). GNMT is present in the cytosol, whereas sarcosine dehydrogenase is present in the matrix of mitochondria. Glycine can be methylated in the mitochondria or in the cytoplasm, transported across the mitochondrial membrane as sarcosine and subsequently be demethylated to regenerate glycine. Both GNMT and sarcosine dehydrogenase are regularly mutated in cancers (Tibbetts and Appling, 2010; Ducker and Rabinowitz, 2017) GNMT is also regulated largely by the androgen receptor, which makes it more likely to be overexpressed in prostate cancer. This results in increased sarcosine concentrations and also increased mitochondrial one-carbon metabolism (DebRoy *et al.*, 2013; Khan *et al.*, 2013; Ottaviani *et al.*, 2013).

Description of the problem

In 2009 a paper was published in Nature by Sreekumar et al suggesting a role of sarcosine in prostate cancer progression (Sreekumar *et al.*, 2009). Using an untargeted metabolomics approach the authors had discovered six metabolites that had increasing concentrations from benign prostate tissue to localized prostate cancer tissue, and from localized prostate cancer tissue to metastasized cancer tissue with origins in the prostate. One of these metabolites was sarcosine so they hypothesized that sarcosine had a role in the progression of prostate cancer. By treating cells with sarcosine they could show that sarcosine induced an invasive phenotype in benign prostate epithelial cells. They could also demonstrate that glycine could induce an invasive phenotype but to a lesser extent. The effect of glycine disappeared when the enzyme converting glycine to sarcosine, GNMT, was knocked down, concomitant with a decrease in the concentration of sarcosine below the limit of detection. This suggested that the effect came from sarcosine specifically.

In their paper Sreekumar et al suggested that sarcosine could be used as a biomarker for aggressive metastatic prostate cancer progression, which has been questioned in other papers (Jentzmik *et al.*, 2011; Khan *et al.*, 2013; Ankerst *et al.*, 2015). Other groups have attempted to find epidemiological data supporting the use of sarcosine as a biomarker but have failed to do so, however it should be noted that they only looked for correlation between the levels of sarcosine and cancer prevalence in general. In 2016 the first paper came out that looked deeper into the mechanistic aspects of sarcosine-induced invasiveness. Heger et al examined the transcriptional profiles of prostate cancer xenografts grown with sarcosine and could demonstrate profound alterations in gene expression compared to xenografts treated with alanine. The genes with the biggest change in expression were genes involved in cell cycle progression (Heger *et al.*, 2016). While this transcriptomic analysis generated intriguing clues to a mechanism for sarcosine-induced invasiveness, it did not give a clear answer.

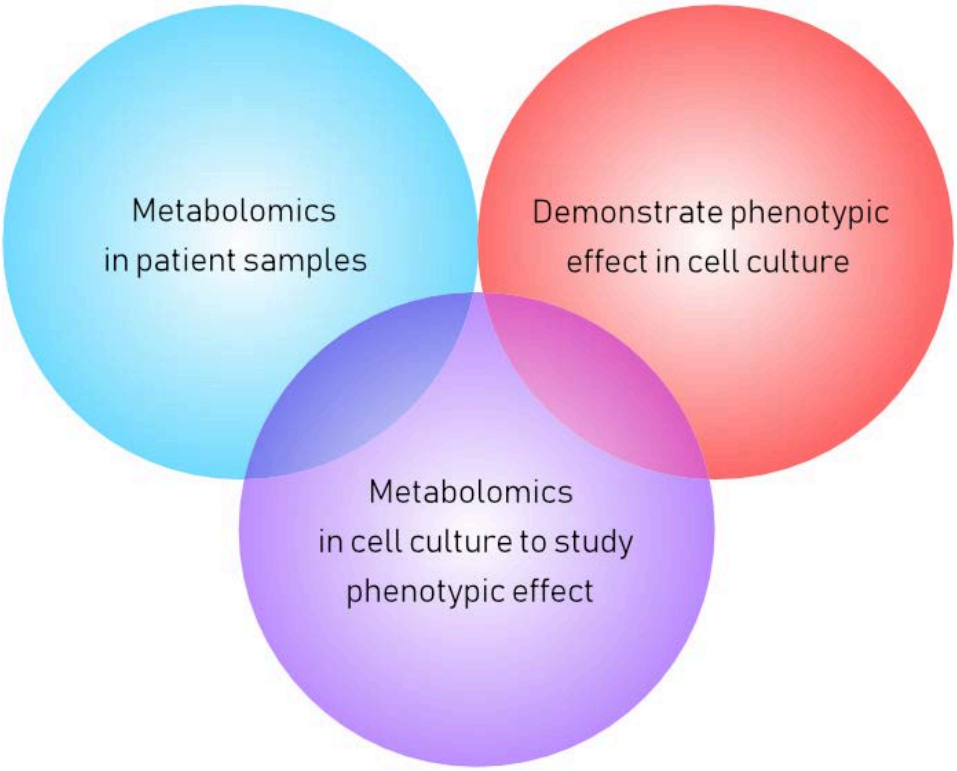

Figure 4: The missing piece (purple) from past experiments (blue and red). In their original publication Sreekumar et al conducted metabolomics experiments on patient samples and both Sreekumar et al and Heger et al found phenotypic alterations as a result of incubation with sarcosine. The purpose of the experiment underlying this report was to conduct metabolomics analyses on cell cultures to explore metabolic effects of sarcosine treatment.

The purpose of this experiment was to examine whether or not sarcosine had a metabolic effect on prostate epithelial cell cultures (**Fig. 4**). We hypothesized that a metabolic effect

brought on by sarcosine treatment might be required to support the change in phenotype reported by Sreekumar et al and the physiological alterations reported by Heger et al. Therefore, we decided to probe the effects of sarcosine on the metabolism of two prostate cancer cell lines, one androgen sensitive and one androgen insensitive, and one benign prostate cell line as a control.

Materials and methods

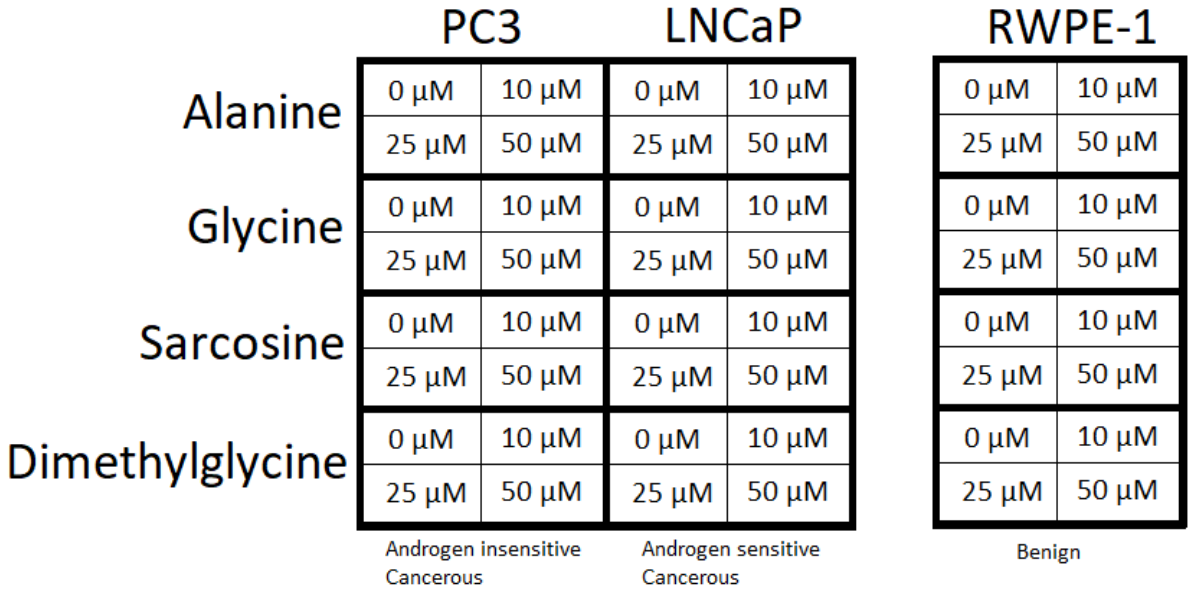

Figure 5: The experimental set up for the cell growth and subsequent metabolomics analysis of prostate cancer cell cultures treated with increasing concentrations of amino acids.

Cell growth

A non-malignant immortalized prostate epithelial cell model RWPE-1 and two human metastatic cancer cells, PC3 (androgen independent) and LNCaP (androgen dependent), were confirmed to be free for mycoplasma contamination. RWPE-1 cells were cultured in Keratinocyte-SFM (Gibco, Life Technologies) medium supplemented with recombinant human Epidermal Growth Factor and Bovine Pituitary Extract (Gibco, Life Technologies). LNCaP and PC3 cells were cultured in RPMI 1640 (Gibco, Life Technologies) medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Cells were seeded in 6-well plates in 2 ml of respective medium and incubated at 37^{o}C to reach 60-70% confluency.

The growth medium was aspirated and cells were incubated in 2 ml growth medium with 0 µM, 10 µM, 25 µM or 50 µM of glycine (Sigma Aldrich), L-alanine (Sigma Aldrich), Sarcosine (Sigma Aldrich) or N,N-Dimethylglycine (Sigma Aldrich) separately for 48 hours at 37^{o}C and 5% CO_2 (**Fig. 5**). To harvest the cells, the conditioned medium was collected from the plates and the cells were washed with PBS. The cells were then scraped off the bottom of the wells, transferred into 1.5 ml centrifuge tubes, centrifuged and flash frozen in liquid

nitrogen. All samples were stored at -80°C until they were sent from Vienna, Austria to Uppsala, Sweden on dry ice. In total 284 samples were generated.

Sample preparation

The samples were stored in -80°C and consisted of 1.5 ml centrifuge tubes with a solid pellet in the bottom. Extraction of cell pellets was done in batches of 24 samples per batch. The order of the samples was randomized using the random-generator at http://random.org. Before the extraction was started methanol was cooled to -20°C and taken out immediately before being used. A batch of samples were removed from -80°C and placed on ice. 500 µl of cool methanol was added on top of the pellet and the pellet was physically broken up by the pipette tip. Once the pellet was broken up into small pieces the methanol suspension was transferred to a 10 ml glass tube. The centrifuge tube was cleaned by another 500 µl- and then 1000 µl twice of cool methanol being added to the tube and then transferred to the same glass tube to a total of 3 ml of methanol suspension. The tubes were sealed with a rubber stopper and covered with Parafilm.

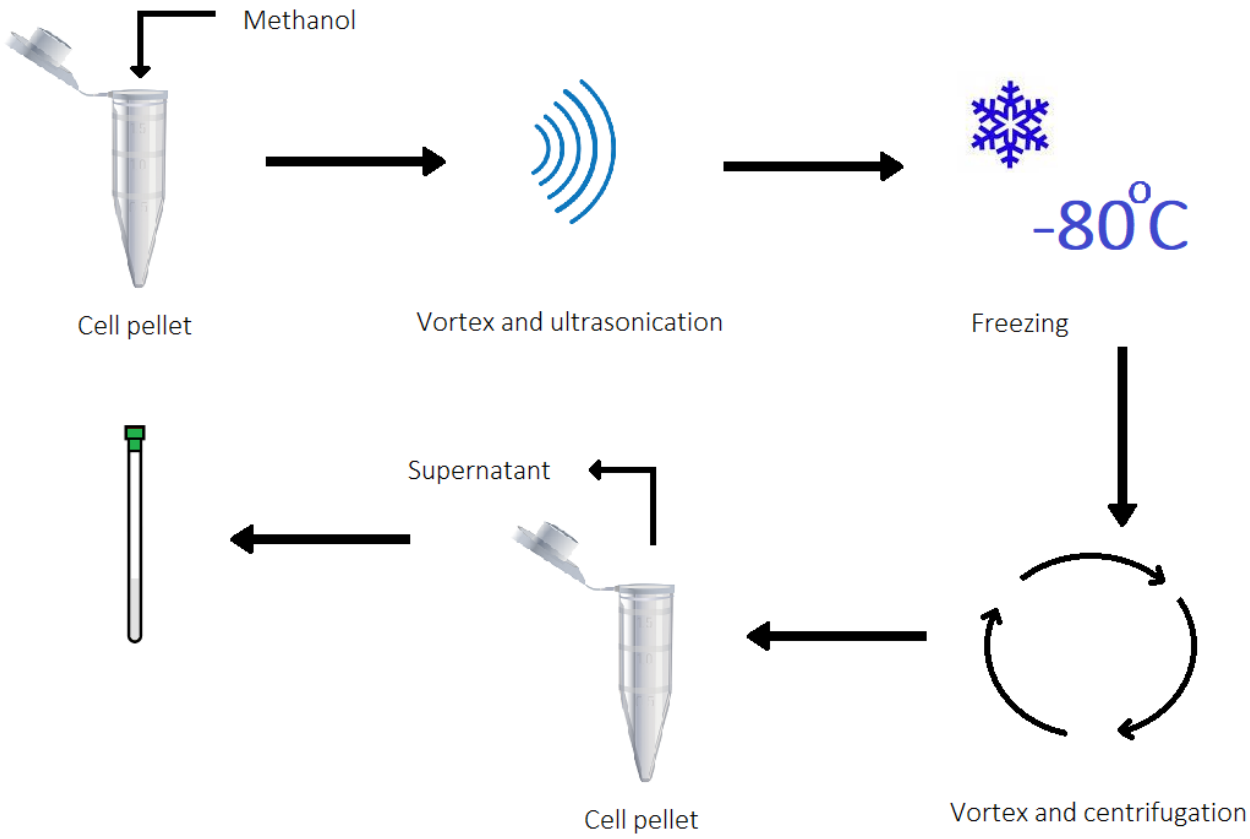

Figure 6: Schematic overview of the extraction protocol used to extract metabolites from cell pellets.

The cells were lysed by placing the glass tubes in a cooled ultrasonic bath for 30 minutes. After the ultrasonic bath the tubes were vortexed briefly and then placed at -80°C for 2 hours and then placed at room temperature for 15 hours. After the lysis procedure the 3 ml of

methanol suspension was transferred to 1.5 ml centrifuge tubes and centrifuged at 10,000 x g for 10 minutes in 1 ml aliquots. The supernatants were moved to a new glass tube and the methanol was dried off under continuous flow of N_2 for 4-5 hours (until all the liquid was gone and then one extra hour). Left in the glass tube was a white precipitate on the side of the glass wall. The precipitate was suspended in 570 µl of 0.135M of phosphate buffer at pH 7, 50 µl of D_2O and 30 µl of 5.8 mM TSP internal standard (Trimethylsilylpropanoic acid, Larodan).

NMR spectroscopy and statistical analysis

Analysis was performed using Bruker 600 mHz (Karlsruhe, Germany) with a 5-mm cooled probe. The NMR experiment was performed at 25ºC with 400 scans and 65536 data points over a spectral width of 17942.58 Hz. The acquisition time was 1.83 s. The spectral baseline and phase were corrected manually using the built in features in Topspin 3.5.7.

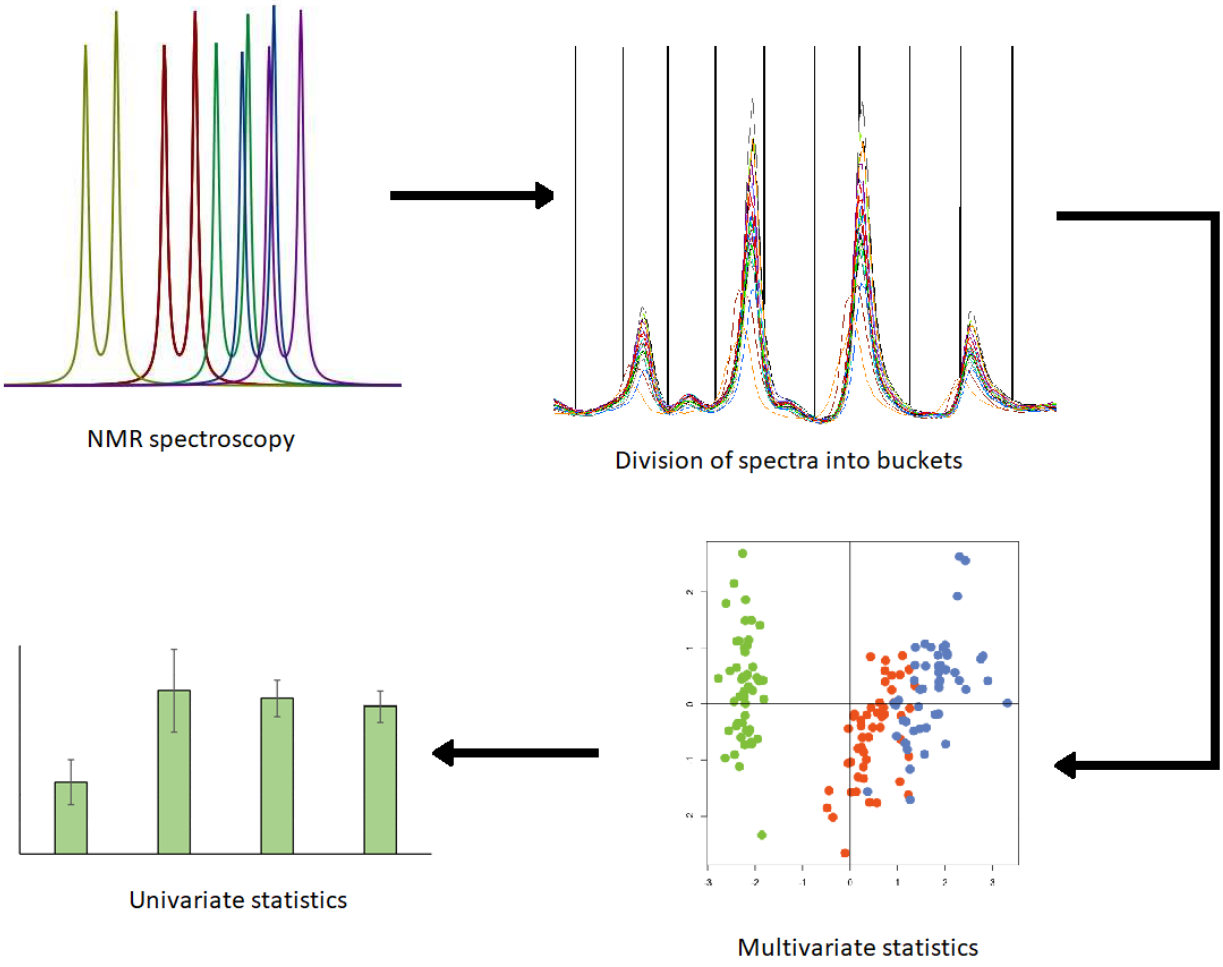

Figure 7: Scheme of the data analysis pipeline utilized to explore the data generated in the experiment presented in this report.

Spectral data was analyzed statistically using primarily SIMCA 14 (UMETRICS, Umeå, Sweden) and MiniTab. The NMR spectra were divided into 0.01 ppm wide sections (buckets) which were integrated and then divided by the integral of the entire spectra in Amix 3.9.7 (Bruker Biospin GmbH). The areas between 2.93-2.97 ppm, 3.04-3.08 ppm, 3.14-3.19 ppm and 3.85-3.89 ppm, containing the peaks from the buffering agent HEPES, as well as 4.50-5.20, which corresponds to the water peak, were excluded. Once the bucket table had been extracted multivariate statistical modeling in SIMCA 14 was done to find individual buckets that differed between the different groups. A high threshold of VIP – cv > 1 was set to make sure that buckets were meaningful. Once a bucket had been identified to cause separation in a partial least squares-discriminant analysis (PLS-DA) model the next step was to determine what metabolite that bucket corresponded to. This was done using a combination of Amix 3.9.7, Chenomx Profiler 8.31 (Chenomx Inc.) and the ^{1}H-spectral database at the Human Metabolome Database (www.hmdb.ca). Once a metabolite had been suggested to be different in the multivariate models it was confirmed using Minitab 17.3.1 (Minitab Inc.). ANOVA-tests followed by post-hoc Tukey-tests were used to confirm the statistical significance of the finding. Prior to the univariate statistical analysis an Anderson-Darling normality test was done on the data to ensure normal distribution, which was the case in all statistically significant analyses.

Results

Comparisons were made within each cell line individually. Within each cell line, each treatment was analyzed individually to assess the metabolic effects of the treatment with regards to the concentration of amino acid added. Principal component analyses (PCA) were conducted to eliminate outliers and partial least squared discriminant analyses (PLS-DA) were used to discover buckets corresponding to metabolites that differentiated between the samples.

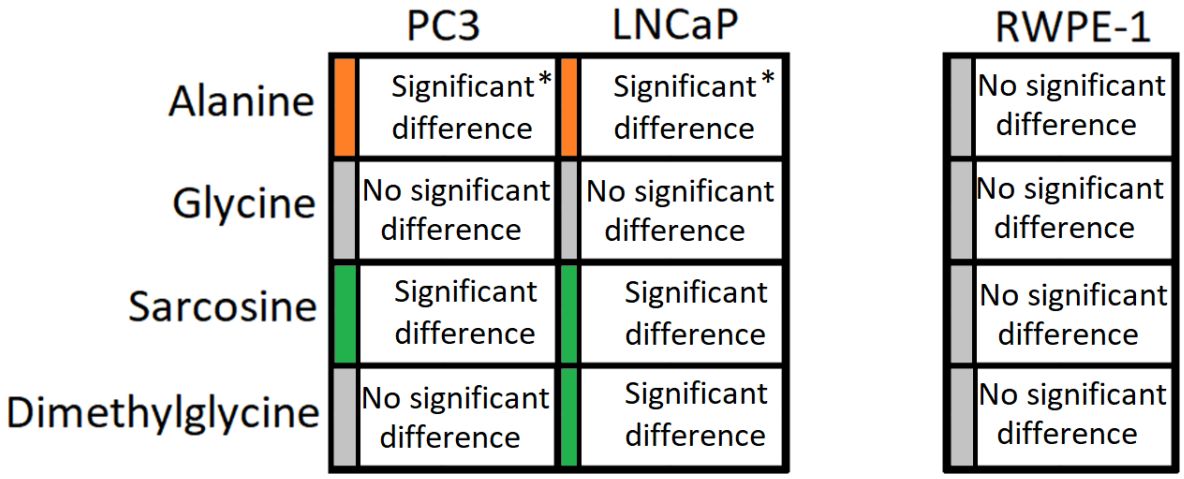

Figure 8: Summary of the results from the comparisons of metabolic profiles with 0 µM, 10 µM, 25 µM and 50 µM of metabolite. For PC3 grown with sarcosine, and for LNCaP grown with sarcosine and N,N-dimethylglycine, differential concentrations of individual metabolites could be detected (green). For both PC3 and LNCaP grown with alanine, significant models could be produced with multivariate statistics but no individual metabolites differed significantly between the groups (orange). For all other parts of the experiment, no statistically significant effects were detected (grey).

A summary of the results can be found in figure 8. Metabolic shifts were detected in PC3 and LNCaP, but not in RWPE-1.

RWPE-1

To see the metabolic effects of treatment with sarcosine, glycine, N,N-dimethylglycine an alanine on non-malignant prostate cells, the buckets from the RWPE-1 cells were compared with multivariate statistics. The area under the spectrum for each bucket was used in multivariate analysis to compare the metabolic effects of the different concentrations of treatment metabolite. No components were fitted in PLS-DA models of the data from RWPE-1 cells. The bucket data corresponding to metabolites found to be differential in the other cell lines were also examined in RWPE-1 but no significant changes were found.

LNCaP

No significant differences in the metabolic profiles were detected in LNCaP cells grown with glycine. No discernible pattern was present in the plots of PLS-DA models from the spectra coming from cells grown in glycine or alanine, but distinct groupings could be seen in the spectra coming from cells grown in sarcosine and N,N-dimethylglycine. In the case of sarcosine, three components were generated, where the first two components explained 46.3% of the variation, the third of which was not statistically significant (**Fig. 9**) (Supplementary table 1).

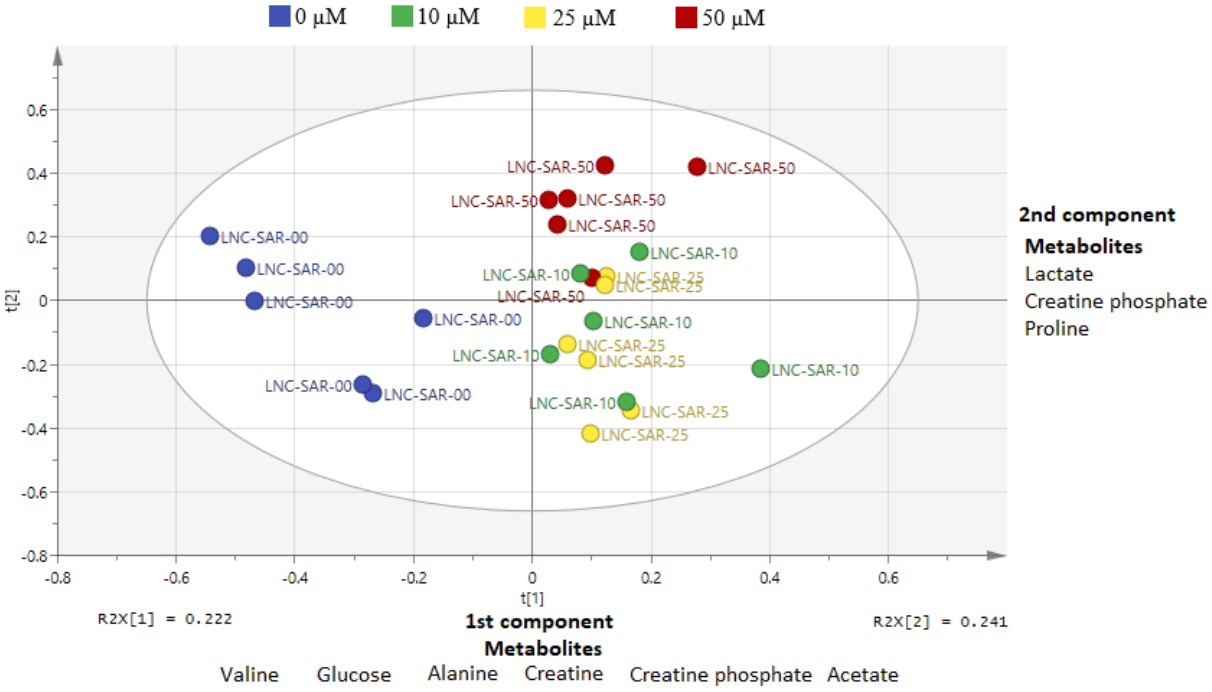

Figure 9: PLS-DA model of bucket values from LNCaP cells grown with increasing concentrations of sarcosine. The metabolites corresponding to the bucket values with the highest contributions are listed by their variable importance from left-to-right in the 1st component and from top-to-bottom in the 2nd component.

Univariate statistical analysis was conducted on the individual metabolites identified using the multivariate statistics. These roughly corresponded to two groups; energy metabolites (**Fig. 10**) and amino acid metabolites (**Fig. 11**).

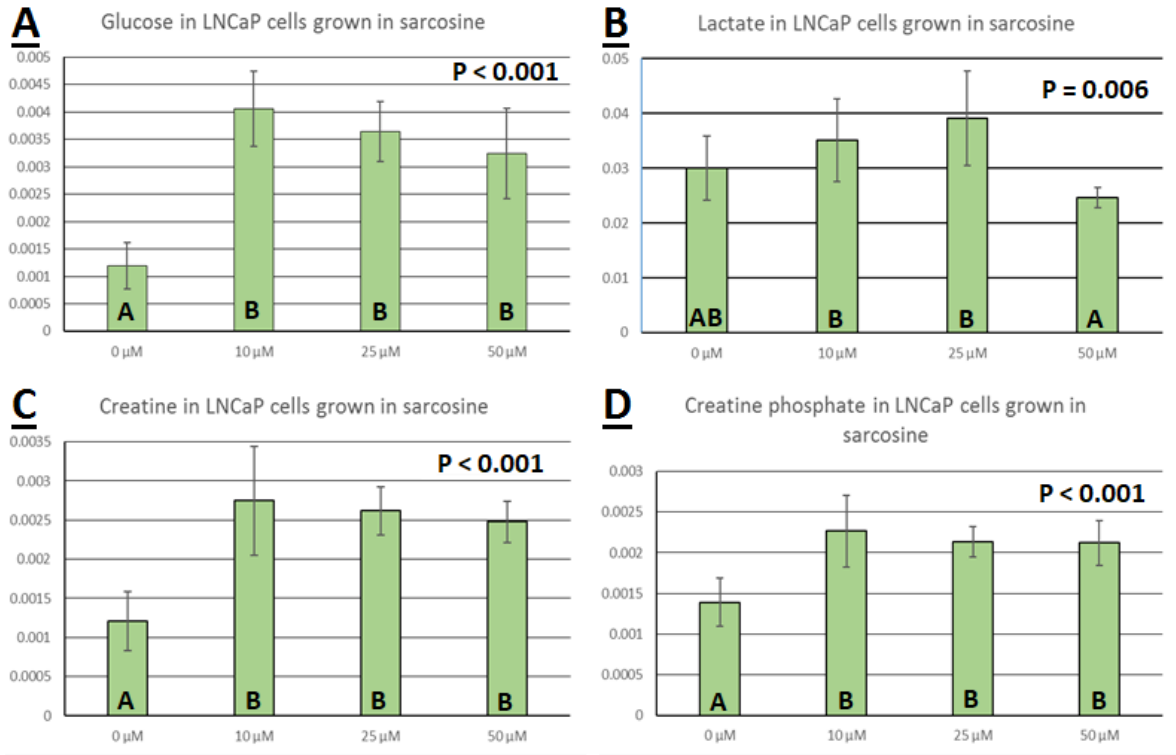

Figure 10: Statistically significant differential energy metabolites in LNCaP cells grown with sarcosine. Glucose (A), creatine (C) and creatine phosphate (D) have increased concentrations in cells grown with sarcosine. Lactate (B) has increased concentration in cells grown with 10 µM and 25 µM of sarcosine, but not in cells grown with 50 µM of sarcosine.

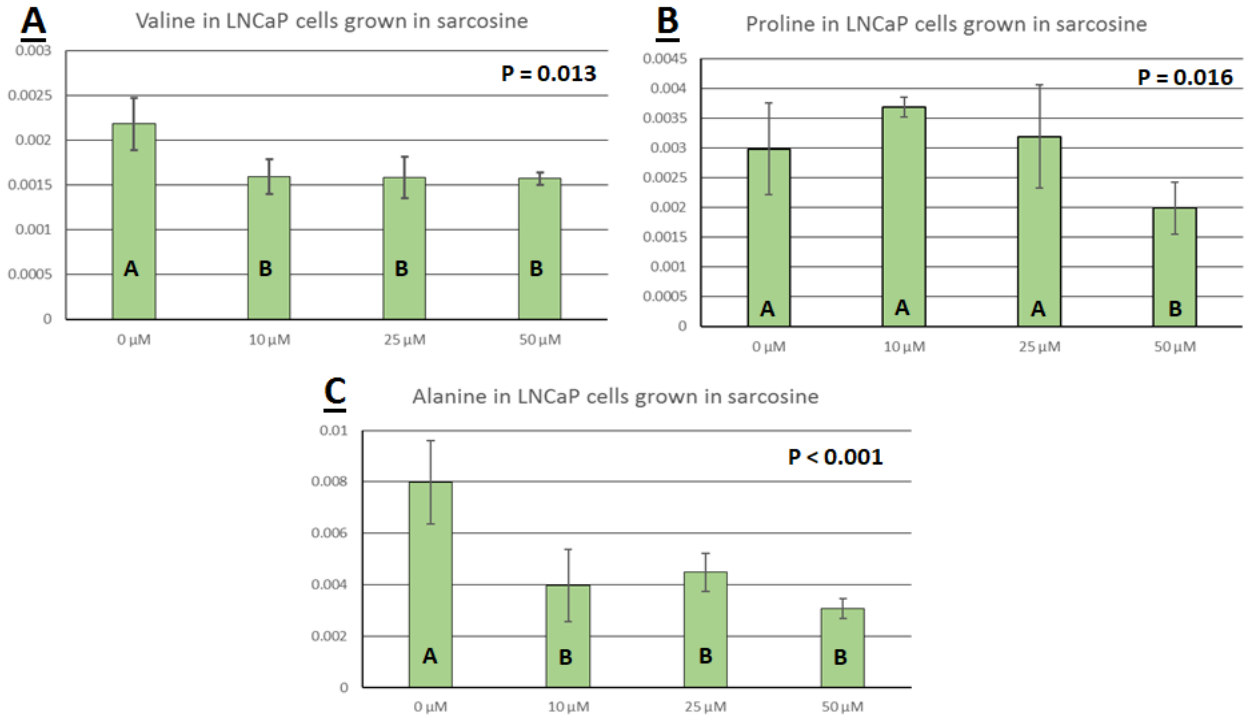

Figure 11: Statistically significant differential amino acid metabolites in LNCaP cells grown with sarcosine. A decrease in concentration of valine (A) and alanine (C) was detected in

cells grown with sarcosine. Proline (B) had lower concentration in cells grown with 50 µM of sarcosine.

In the case of LNCaP cells grown in N,N-dimethylglycine four metabolites had VIP scores over 1: short-chain fatty acids, creatine, creatine phosphate and uridine (**Fig. 12**). Out of those four, only the values from the buckets corresponding to short-chain fatty acids differed significantly (**Fig. 13**).

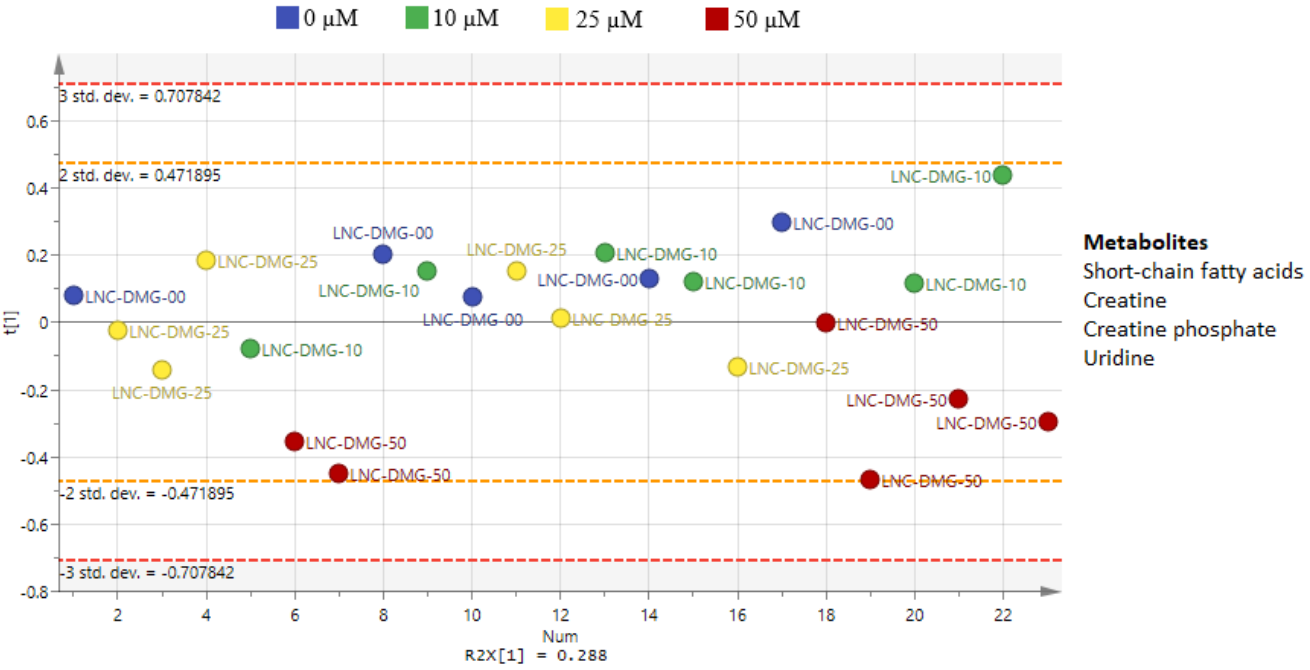

Figure 12: PLS-DA model of bucket values from LNCaP cells grown with increasing concentrations of N,N-dimethylglycine. The metabolites corresponding to the bucket values with the highest contributions are listed by their variable importance from top-to-bottom.

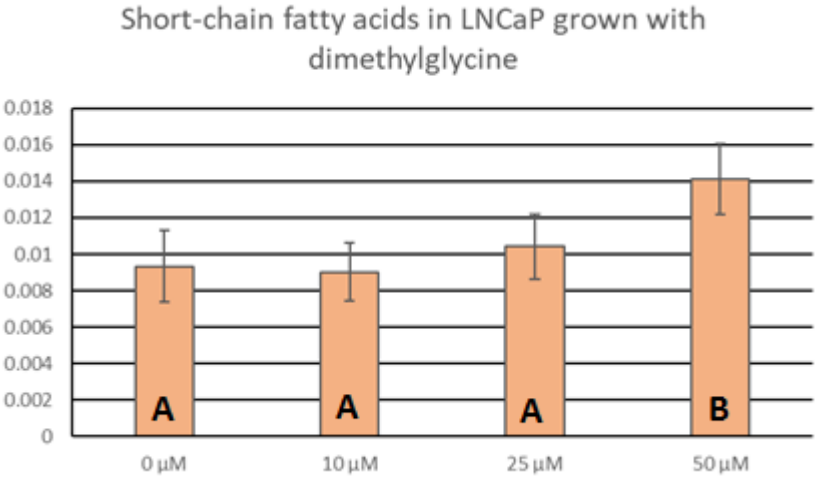

Figure 13: The concentrations of short-chain fatty acids increased in cells grown with higher levels of N,N-dimethylglycine.

PC3

No significant differences in the metabolic profiles were detected in PC3 cells grown with glycine or N,N-dimethylglycine. No discernible pattern was present in the plots of principal component analyses from the spectra coming from cells grown in glycine, N,N-dimethylglycine or alanine, but distinct groupings could be seen in the spectra coming from cells grown in sarcosine. Two components were generated (**Fig. 14**) (Supplementary table 1).

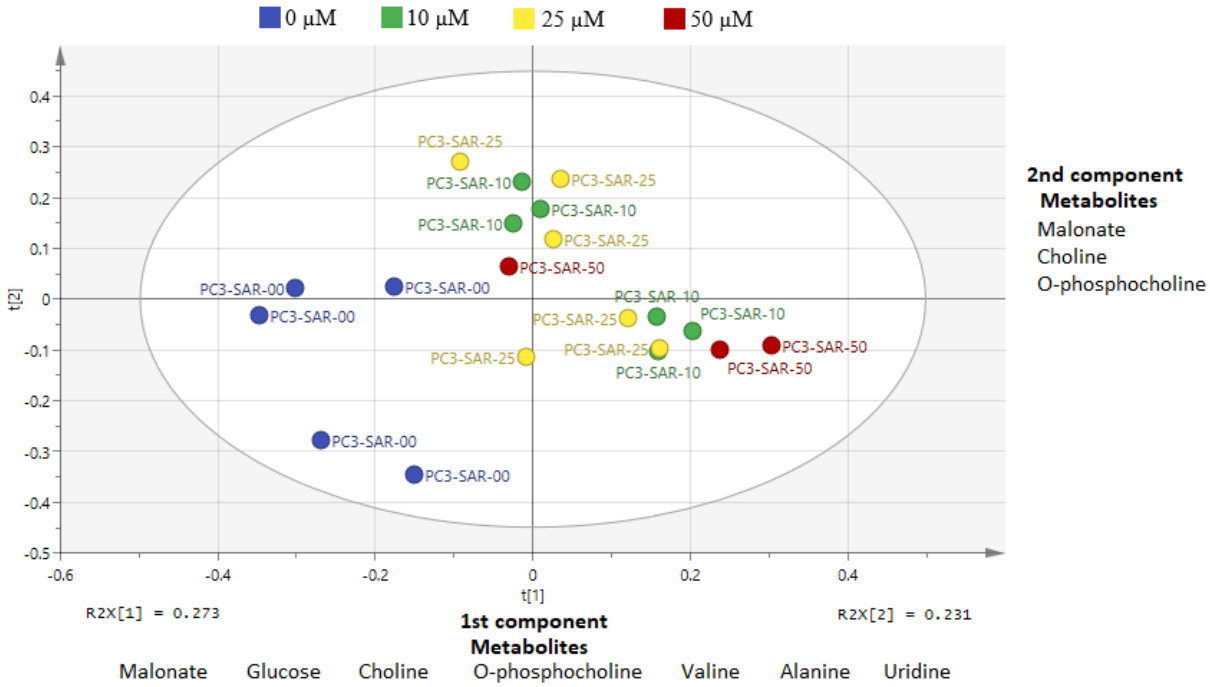

Figure 14: PLS-DA model of bucket values from PC3 cells grown with increasing concentrations of sarcosine. The metabolites corresponding to the bucket values with the highest contributions are listed by their variable importance from left to right in the 1st component and from top to bottom in the 2nd component.

Univariate statistical analysis was conducted on the individual metabolites identified using the multivariate statistics (**Fig. 15**).

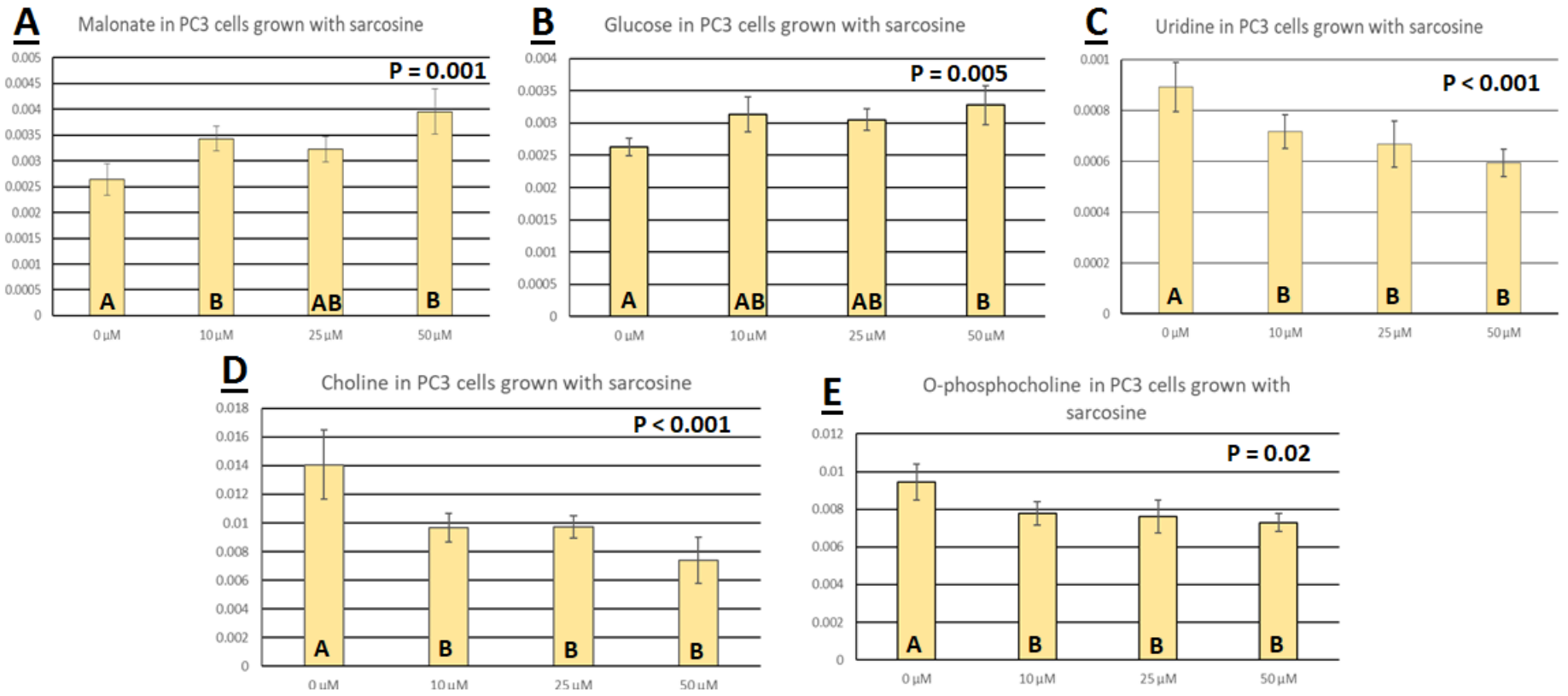

Figure 15: Metabolites found to be different in PC3 cells incubated with increasing concentrations sarcosine. Malonate (A) and glucose (B) have increased concentrations in cells grown with sarcosine, whereas uridine (C), choline (D) and O-phosphocholine (E) have decreased concentrations in cells grown with sarcosine.

Intracellular levels of sarcosine

The intracellular levels of sarcosine were briefly compared. In the cancer cell samples with increased concentrations of sarcosine an increase in intracellular sarcosine could be demonstrated. However, in benign cells there was no significant change in intracellular sarcosine (**Fig. 16**).

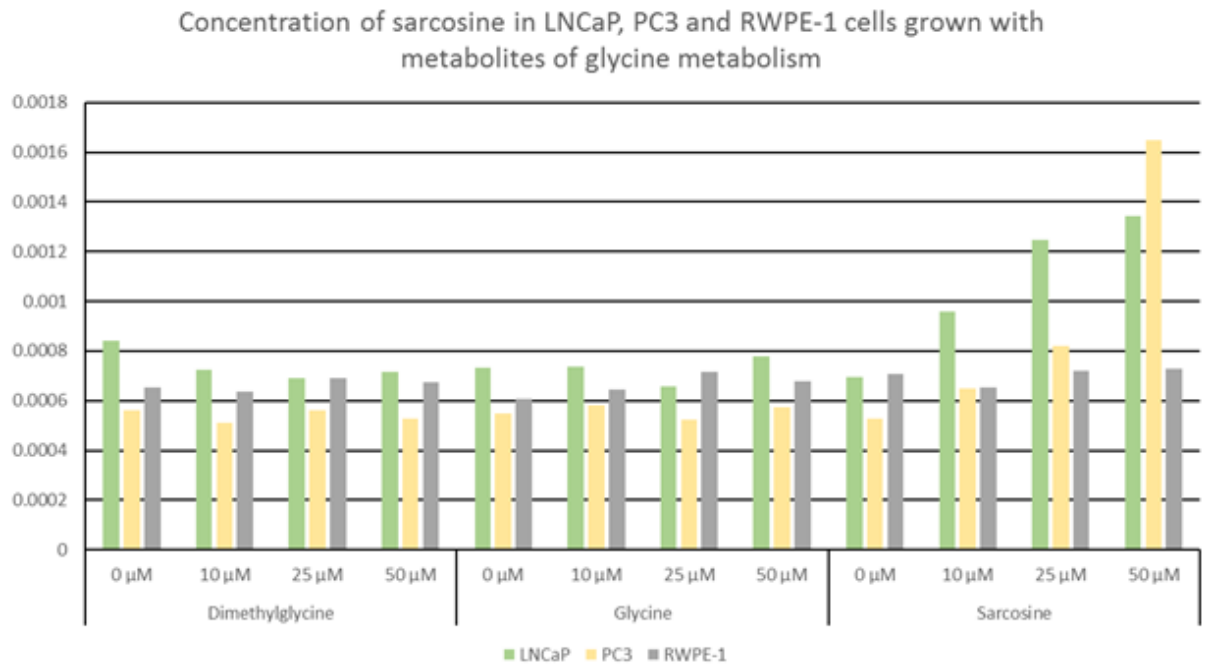

Figure 16: Overview of the relative intensity of the peak corresponding to sarcosine in spectra coming from LNCaP, PC3 and RWPE-1 in incubated with 0 µM, 10 µM, 25 µM and 50 µM of N,N-dimethylglycine, glycine and sarcosine respectively..

A visual analysis of overlaid spectra was done at the area of the spectra corresponding to the singlet of sarcosine (2.74 ppm) (**Fig. 17**). Large singlet signals at the expected location for sarcosine were found in the spectra coming from LNCaP and PC3 grown with high concentrations of sarcosine. No peak was found at the expected location for sarcosine in RWPE-1. No peak corresponding to sarcosine was found in any cell line from samples coming from cells grown with additional glycine, N,N-dimethylglycine or alanine either.

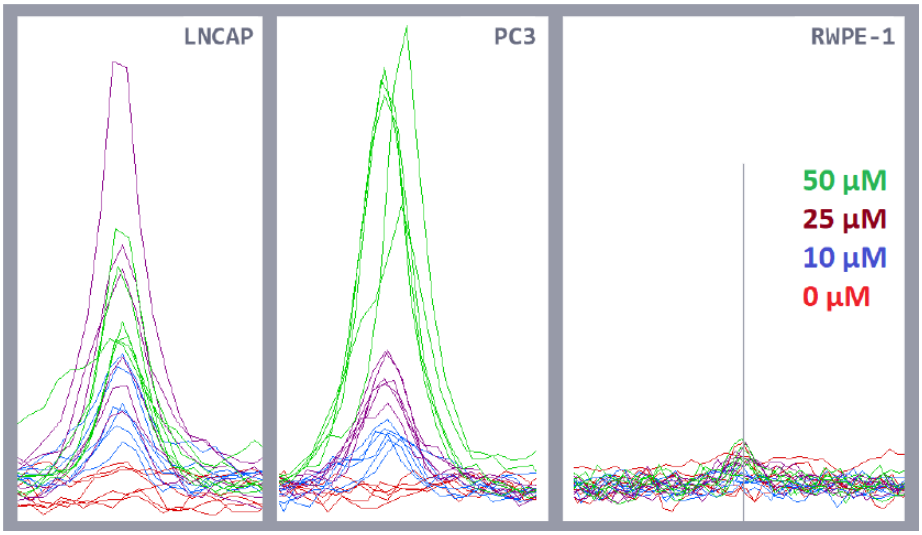

Figure 17: Overlaid spectra from samples of LNCaP-cells, PC3-cells and RWPE-1 cells with 0 μM-, 10 μM-, 25 μM-, and 50 μM of added sarcosine, focused on the region of the spectra where a singlet from sarcosine is expected to be. The grey line in the screen capture from RWPE-1 shows where the centroid of the singlet coming from sarcosine should be.

Discussion

The goal of this project was to determine if treatment with sarcosine had a metabolic effect on malignant prostate epithelial cells and benign prostate epithelial cells which can explain previously observed effects on prostate cancer progression. Using an NMR metabolomics approach we detected a metabolic shift in both cancer cell lines tested when grown with sarcosine. Two cancer cell lines were used, LNCaP, an androgen-sensitive cell line, and PC3, an androgen insensitive cell line. The metabolic shifts of these two cell lines were similar but not identical. In both malignant cell lines, the concentration of glucose was increased when sarcosine was added. The concentrations of alanine and valine were reduced by the sarcosine treatment in both cancer cell lines. On top of the metabolic changes that the cancer cell lines had in common there were unique features of the metabolic effect on each cell type. In PC3 cells, the concentrations of choline and O-phosphocholine were reduced upon addition of sarcosine. In LNCaP cells, the concentrations of creatine and creatine phosphate were higher upon addition of sarcosine, matching the concentration increase of glucose. LNCaP cells also had markedly reduced levels of alanine and valine.

The increase in the concentration of glucose is an intriguing finding. Oxidative stress as a result of glucose deprivation is a common way that cells become apoptotic, especially during metastasis (Piskounova *et al.*, 2015). A tantalizing speculative interpretation of the data generated in this report is that sarcosine prevents apoptosis or growth inhibition signaling by keeping the levels of glucose high. A different interpretation of the increase in glucose concentration is that the increased presence of glucose allows the cells to grow faster due to the glucose being used for biosynthetic- or energetic purposes.

Another interesting finding is the fact that the levels of uridine decrease in PC3-cells grown with sarcosine. Previous research has shown that the most upregulated gene in response to incubation with sarcosine in PC3 cells is the gene encoding thymidylate synthetase (Heger *et al.*, 2016). This protein is responsible for maintaining pyrimidine concentrations, specifically thymidine concentrations, through conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) (**Fig. 18**). It is possible that the levels of uridine in PC3 cells decrease as thymidylate synthetase converts it into thymidine. This finding is also in line with the other findings reported by Heger et al, namely that many of the other genes upregulated by the presence of sarcosine are involved with cell cycle progression. Progression through the cell cycle necessitates utilizing thymidine for DNA replication. This increase in the need for thymidine could potentially account for the increase in consumption of uridine found in our data.

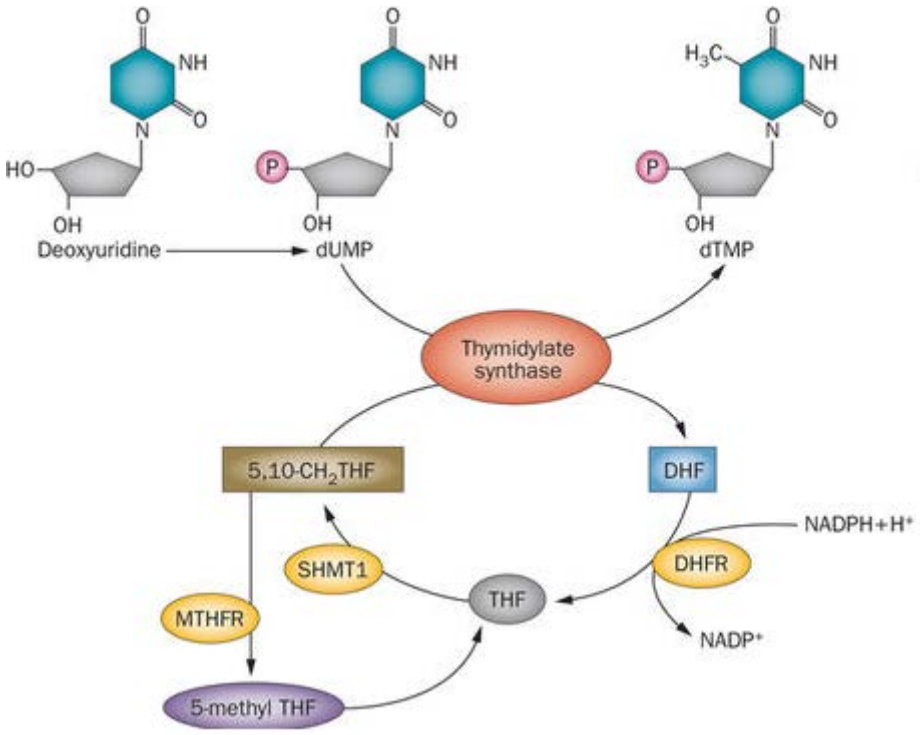

Figure 18: Schematic of the reaction catalyzed by thymidylate synthase. A methyl-group needs to be donated by 5,10-CH_2 THF which is added onto activated deoxyuridine (dUMP).

No metabolic shift was detected in RWPE-1, the benign prostate epithelial cell lines used. We also examined the ability of metabolites similar to sarcosine to induce metabolic shifts. Sarcosine was present in PC3 cells and LNCaP cells grown in sarcosine, but not in RWPE-1 cells. It is not known whether this was the reason that no metabolic effect was detected was because there was no sarcosine present in the cells. An interesting point to consider is that in previous research, where effects on sarcosine accumulation could be shown in benign cells, sarcosine accumulation was achieved by genetic manipulation of the genes responsible for maintaining the levels of sarcosine whereas in our experiment the sarcosine was added to the growth medium.

In the research presented in 2009 by Sreekumar et al it was noted that both sarcosine and glycine could induce an invasive phenotype when glycine could be converted into sarcosine (Sreekumar *et al.*, 2009). We could not detect any metabolic shifts as a result of adding increasing concentrations of glycine. Sreekumar and colleagues also saw a phenotypic difference when benign cells were grown with sarcosine. However, we did not see a metabolic shift in benign cells. One reason we could not see an effect from glycine or alanine could be that we incubated the cells for 48 hours instead of the 24 hour incubation that Sreekumar and colleagues did in their experiments. Both glycine and alanine have high rates of turnover in the cell. The turnover rates of sarcosine and N,N-dimethylglycine are not known and most likely vary from one cell type to another, but since they are not being integrated into protein it is probable that their rates are considerably lower. Pools of both alanine and glycine are high in human cells (glycine = 325.4 ± 126.8 μM, alanine = 427.2 ± 84.4 μM) compared to both sarcosine and N,N-dimethylglycine (Psychogios *et al.*, 2011). Since the amount added was the same in absolute terms for each amino acid, the amount in proportion to the

natural pools were considerably different. It should also be said that the physiological concentration of sarcosine in blood in healthy adults is 0.5-10 μM and that anything above 10 μM is indicative of sarcosinemia. Cancer cells are capable of altering this level to some extent, but 50 μM sarcosine might not be physiologically relevant.

NMR spectroscopy was used for the analyses conducted in this study. While reliable, NMR is not as sensitive as for instance mass spectrometry and a limitation of this study is the number of metabolites that it captures. It is possible that many metabolites with lower concentrations were dramatically affected but that they were not captured in the analysis. Analyzing the samples with mass spectrometry will give information on the presence and concentrations of more metabolites, as well as aid in the identification of the metabolites whose identity was not certain in this analysis. It would also be prudent to incubate the cells with their respective treatments for 24 hours instead of 48 hours in order to capture changes caused by metabolites that have higher turnover rates, if such changes occur.

Conclusion

Sarcosine has previously been shown to be able to drive prostate cancer progression. The goal of this experiment was to explore potential mechanistic explanations for how sarcosine can induce the phenotypic alterations associated with prostate cancer progression. Specifically, we wanted to know if sarcosine could cause a metabolic shift in the metabolism of prostate cancer cells. We found that this is indeed the case. The metabolites found to be differential are not connected by a single pathway and the relationship seems to be systemic, rather than specific. Broad, important pathways such as energy metabolism, amino acid metabolism, nucleotide metabolism and mitochondrial metabolism were affected. While this does not represent a complete mechanistic explanation for how sarcosine drives prostate cancer progression, it offers important clues to be pursued in the future.

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Supplementary data

*Supplementary table 1: Model metrics for the PLS-DA models used to identify differential metabolites. *Not significant.*

Model	Component	R2X	R2X (cum)	R2Y	R2Y (cum)	Q2	Q2 (cum)	Significance
PC3 Sarcosine	1st component	0.273	0.273	0.233	0.233	0.0948	0.0948	R1
	2nd component	0.231	0.504	0.0824	0.315	-0.169	0.00425	R2
LNCaP Sarcosine	1st component	0.222	0.222	0.276	0.276	0.159	0.159	R1
	2nd component	0.241	0.463	0.179	0.456	0.159	0.292	R1
LNCaP N,N-Dimethylglycine	1st component	0.288	0.288	0.221	0.221	0.13	0.13	R1
LNCaP Alanine	1st component	0.189	0.189	0.23	0.23	0.0693	0.0693	NS*

Supplementary table 2: Buckets used for univariate statistical analysis, the signal which that bucket corresponds, the VIP- and VIP-cv values from the multivariate model and the P-values from the ANOVA. Not significant.*

Metabolite	Bucket	Signal (ppm)	VIP	VIP – cv	P-value in ANOVA
Valine	1.055	1.0485 - 1.0530	2.18 (LNCaP1st comp.)	1.15	0.013
			2.55 (PC3 1st comp.)	1.36	0.031
Glucose	3.495	3.4910 - 3.505	2.53 (LNCaP 1st comp.)	1.08	0.005
			2.86 (PC3 1st comp.)	1.44	< 0.001
Alanine	1.475	1.4695 - 1.4805	5.00 (LNCaP 1st comp.)	1.09	< 0.001
			1.5296 (PC3 1st comp.)	1.06	< 0.001
Creatine	3.945	3.945 - 3.951	2.95 (LNCaP 1st comp.)	1.19	< 0.001
Creatine phosphate	3.955	3.952 - 3.9595	2.12 (LNCaP 1st comp.)	0.89	< 0.001
Acetate	1.925	1.9145 - 1.9305	9.02 (LNCaP 1st comp.)	1.19	0.052*
Lactate	1.325	1.3295 - 1.3215	6.01 (LNCaP 2nd comp.)	2.38	0.006
Proline	4.135	4.1310 - 4.1355	2.75 (LNCaP 2nd comp.)	0.91	0.041
SCFA	1.285	1.259 - 1.313	6.20 (LNCaP DMG 1st comp.)	2.61	< 0.001
Uridine	7.875	7.8680 - 7.8765	2.01 (PC3 1st comp.)	0.87	< 0.001
Malonate	3.115	3.106 - 3.1225	3.09 (PC3 1st comp.)	1.07	< 0.001
Choline	3.205	3.1975 - 3.2095	7.98 (PC3 1st comp.)	4.44	< 0.001
O-phosphocholine	3.225	3.2265 - 3.234	4.43 (PC3 1st comp.)	3.06	< 0.001