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Choline and its related metabolites in patients with prostate cancer

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Born in Yabrud (Damas Suburb), Syria 16.03.1980

Dedication

I would like to dedicate this thesis to my family for their unconditional love, support, encouragement, and most of all patience

Abbreviations

ACh	acetylcholine
Acetyl-coA	acetyl-coenzyme A
ADP	adenosine diphosphate
ADT	androgen deprivation therapy
ALAT	alanine aminotransferase
ALP	Alkaline phosphatase
APCI	atmospheric-pressure chemical ionization
ASAT	aspartate aminotransferase
ATP	adenosine triphosphate
BADH	betaine aldehyde dehydrogenase
BHMT	betaine homocysteine methyltransferase
BMI	body mass index
BPH	benign prostatic hyperplasia
CBS	cystathionine-β-synthase
CCT	CTP-phosphocholine cytidylyltransferase
CDP-Cho	cytidine diphosphate choline
Cer	ceramide
CerS	Cer synthase
ChAT	choline acetyltransferase
CHDH	choline dehydrogenase
CHK	choline kinase
CHTs	high-affinity choline transporters
CMP	cytidine monophosphate
CoA	coenzyme A
CPT1	diacylglycerol cholinephosphotransferase 1
CRP	c-reactive protein
CTLs	choline transporter-like proteins
CTP	cytidine triphosphate
CV	coefficient of variation
Cys	cystathionine
DAG	diacylglycerol
DHT	dihydrotestosterone
DMG	dimethylglycine
DMGDH	dimethylglycine dehydrogenase
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
ESI	electrospray ionization
Eth	ethanolamine
FBP	Folate Binding Protein
FMOs	flavin monooxygenases
gamma-GT	gamma glutamyl transferase
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry

GFR	glomerular filtration rate
GNMT	glycine N-methyltransferase
GPC	glycerophosphocholine
GPC-PDE	glycerophosphocholine phosphodiesterase
GPE	glycerophosphoethanolamine
G3P	glycerol-3 phosphate
Hct	hematocrit
Hcy	homocysteine
HDL	high density lipoprotein
HILIC	hydrophilic interaction chromatography
HMG-CoA reductase	3-hydroxy-3-methylglutaryl coenzyme A reductase
HoloTC	holotranscobalamin
HPLC	high-performance liquid chromatography
IDL	intermediate density lipoprotein
IQL	instrumental quantification limit
IUPAC	International Union of Pure and Applied Chemistry
Km	Michaelis constant
Lass	Cer synthase
LC	liquid chromatography
LC-MS	liquid chromatography coupled to mass spectrometry
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LDL	low density lipoprotein
LOD	limit of detection
LOQ	limit of quantification
Lyso-PLA1	lyso-phospholipase A1
Lyso-PtdCho	lysophosphatidylcholine
LPCAT	lysophosphatidylcholine acetyltransferase
MDL	method detection limit
MMA	methylmalonic acid
MQL	method quantification limit
MRI	magnetic resonance imaging
MRM	multiple reaction monitoring
MRSI	magnetic resonance spectroscopic imaging
MS/MS	tandem mass spectrometry
mTHF	5-methyl tetrahydrofolate
MTHFD1	5,10-methylenetetrahydrofolate dehydrogenase
m/z	mass-to-charge ratio
OCTs	organic cation transporters
OCTNs	organic cation/carnitine transporters
PA	phosphatidic acid
PAF	platelet-activating factor
PCa	prostate cancer
PCho	phosphocholine
PCR	polymerase chain reaction
PEth	phosphoethanolamine
PET/CT	positron emission tomography
PEMT	phosphatidylethanolamine methyltransferase

PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
PPi	diphosphate
PSA	prostate-specific antigen
PtdCho	phosphatidylcholine
PtdEth	phosphatidylethanolamine
RBC	red blood cell, erythrocyte
RDA	recommended dietary allowance
RMSE	root mean square error
RNA	ribonucleic acid
R_s	resolution
rs	reference SNP
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SLC	solute carrier
SM	sphingomyelin
SMase	sphingomyelinase
SMS	sphingomyelin synthase
S/N	signal-to-noise ratio
SNP	single nucleotide polymorphism
SPE	solid-phase extraction
TC	transcobalamin
THF	tetrahydrofolate
tHcy	total homocysteine
TIC	total ion chromatogram
TMA	trimethylamine
TMAO	trimethylamine *N*-oxide
TURP	transurethral resection of the prostate
VLDL	very low density lipoprotein
UPLC	ultra performance liquid chromatography
UV	ultra violet
WB	whole blood

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Abstract

Background: Choline is an essential nutrient required for many biological functions such as methyl metabolism, structural integrity of cell membranes, transmembrane signaling, and phospholipid metabolism. A higher intake or status of choline in plasma has been related to higher prostate cancer risk. Choline may affect cancer development through few mechanisms; it can affect DNA methylation and might lead to a disruption of DNA repair; it can modify cell signaling that is mediated by intermediary phospholipid metabolites; and it can support the synthesis of cell membranes and thus support cell proliferation. Choline metabolism in prostate cancer might be dysregulated in order to meet the tumor requirements for phospholipid and methyl groups.

Objective and methods: We hypothesized that the concentrations of choline and choline metabolites in plasma will be higher in men with prostate cancer than those with benign prostatic hyperplasia. The aim of this thesis was to investigate potential differences in choline and its metabolites relate to one-carbon and phospholipid metabolisms between patients with prostate cancer and those with benign prostatic hyperplasia. We studied differences in blood concentrations of choline, betaine, dimethylglycine, S-adenosyl methionine, S-adenosyl homocysteine, homocysteine, and phospholipid species between cases and controls. Quantifications were carried out by using liquid and gas chromatography, coupled with tandem mass spectrometry.

Results: Concentrations of choline and other metabolites did not differ between cases and controls. The concentrations of sphingomyelin species 14:0 and 20:0 were significantly higher in the younger prostate cancer patients (age ≤65 years) compared to the young controls (median SM 14:0 = 10.8 vs. 9.4 µmol/L, p=0.033 and SM 20:0 = 16.2 vs. 13.6 µmol/L, p=0.004). The results according to the pathological grade showed that the same 2 species were significantly increased in the young low-grade prostate cancer patients (Gleason score ≤3+4) compared to the young controls (mean SM 14:0 = 12.1 vs. 10.0 µmol/L, p=0.035, and SM 20:0 = 16.6 vs. 14.1 µmol/L, p=0.027). Sphingomyelin species (16:0, 18:0, 18:1, 20:0, 22:0, 22;1, 23:0, 23:1 24:0, 24:1, and 24:2) were significantly lower in prostate cancer cases than controls in the elderly group (age >65 years). Mean concentrations of sphingomyelin 16:0 and 18:0 were significantly ($p<0.01$) lower in the elderly patients with low-grade (SM 16:0 = 82.9, SM 18:0 = 18.6 µmol/L) or high-grade prostate cancer (Gleason score ≥4+3) (SM 16:0 = 85.6, SM 18:0 = 18.4 µmol/L) compared to the

elderly controls (SM 16:0 = 95.0, SM 18:0 = 21.2 μmol/L). Age was associated with significantly higher choline, S-adenosyl methionine, S-adenosyl homocysteine, total homocysteine, cystathionine levels in the prostate cancer patients. Statin use was associated with lower plasma phospholipid species levels, especially phosphatidylcholine and sphingomyelin species in cases and controls. This lowering effect is synergized by the presence of prostate cancer.

Discussion and conclusions: The current study does not confirm significant differences in plasma concentrations of plasma free choline or metabolites of the methionine cycle between patients with prostate cancer and controls with benign prostate hyperplasia. Because free choline pool may not be a good marker for choline nutrient, the results can not exclude a role for choline in prostate cancer. In line with this, sphingomyelin that originates from choline metabolism was divergent between young patients with prostate cancer and those with benign prostatic hyperplasia (SM 14:0 and 20:0). In addition, sphingomyelin species 16:0 and 18:0 distinguished between prostate cancer and benign prostatic hyperplasia elderly patients. Age and prostate cancer had a significant association with circulating phospholipid profile. These findings support alterations in phospholipid metabolism in prostate cancer and warrant further investigation.

Zusammenfassung

Hintergrund: Cholin ist ein essentieller Mikronährstoff, der an vielen biologischen Funktionen beteiligt ist, wie Methylgruppen-Stoffwechsel, strukturelle Integrität von Zellmembranen, Neurotransmittersynthese und Phospholipidstoffwechsel. Eine erhöhte Zufuhr oder erhöhte Plasmaspiegel von Cholin wurden in Zusammenhang mit einem erhöhten Prostatakrebs-Risiko beobachtet. Unterschiedliche Wirkmechanismen werden dabei diskutiert: Störung der DNA-Methylierung mit daraus resultierenden gehäuft auftretenden DNA-Brüchen; Störung der zellulären Signaltransduktion durch zelluläre Phospholipidmetabolite sowie Störungen des Zellwachstums durch modifizierte Membranensynthese. Beim Prostatakarzinom ist der Cholin-Metabolismus möglicherweise modifiziert durch den gesteigerten Bedarf von Phospholipiden und Methylgruppen der Tumorzellen.

Fragestellung und Methoden: Untersucht wurden 73 Probanden mit benigner Prostatahyperplasie (Kontrollgruppe) und 145 Probanden mit Prostatakarzinom. Die Probanden wurden in Altersgruppen (≤65 und >65 Jahre) eingeteilt. Ausgehend von den Literaturdaten über den Zusammenhang zwischen Cholinstoffwechsel und Karzinommanifestation sind bei Patienten mit Prostatakrebs höhere Konzentration für Cholin und dessen Metaboliten zu erwarten. In der vorliegenden Arbeit wurde ein Panel von Parametern, die den Cholinstoffwechsel, aber auch den C-1- und Phospholipid-Stoffwechsel beschreiben, eingesetzt. Mit der Gas- und Flüssigkeitschromatographie gekoppelten Massenspektrometrie wurden folgende Parameter untersucht: Cholin, Betain, Dimethylglycin, S-Adenosylmethionin, S-Adenosylhomocystein, Homocystein, Cystathionin und Phospholipidformen (Phosphatidylcholin, Lyso-Phosphatidylcholin, Phosphatidylethanolamin und Sphingomyelin).

Ergebnisse: Die Konzentrationen für Cholin und die anderen Metaboliten zeigten keine signifikanten Unterschiede zwischen der Kontrollgruppe und Patienten mit Prostatakarzinom. In der Altersgruppe bis 65 Jahre waren die Konzentrationen für die Sphingomyeline (SM) 14:0 und 20:0 signifikant höher bei Patienten mit Protatakrebs im Vergleich zur Kontrollgruppe (Median SM 14:0 = 10.8 vs. 9.4 µmol/L, p=0.033 und SM 20:0 = 16.2 vs. 13.6 µmol/L, p=0.004). Die Ergebnisse bezogen auf den Gleason-Score zeigten in der gleichen Altersgruppe, dass bei Tumorpatienten mit niedrigem Scorewert (≤3+4) im Vergleich zur Kontrollgruppe die 14:0 und 20:0 Sphingomyeline ebenfalls signifikant erhöht waren (Mittelwert SM 14:0 = 12.1 vs. 10.0 µmol/L, p=0.035, und SM 20:0 = 16.6 vs. 14.1 µmol/L, p=0.027). In der Altersgruppe über 65

Jahre waren die Sphingomyelinformen (16:0, 18:0, 18:1, 20:0, 22:0, 22:1, 23:0, 23:1 24:0, 24:1, und 24:2) signifikant erniedrigt bei Patienten mit Prostatakrebs im Vergleich zu den Kontollprobanden. Die Konzentrationen für Sphingomyeline 16:0 und 18:0 waren signifikant niedriger in der älteren Patientengruppe unabhängig vom Gleason-Score (Gleason-Score ≤3+4: SM 16:0 = 82.9, SM 18:0 = 18.6 µmol/L und Gleason-Score ≥4+3: SM 16:0 = 85.6, SM 18:0 = 18.4 µmol/L) im Vergleich zur Kontrollgruppe (SM 16:0 = 95.0, SM 18:0 = 21.2 µmol/L). In der Gruppe der Patienten mit Prostatakarzinom war das Alter signifikant assoziiert mit höheren Konzentrationen für Cholin, S-Adenosylmethionin, S-Adenosylhomocystein, Homocystein und Cystathionin. Eine Einnahme von Statinen war assoziiert mit erniedrigten Phospholipiden, insbesondere Phosphatidylcholin- und Sphingomyelinformen bei Patienten und Kontrollen. Der Statineffekt wurde in der Gruppe mit Prostatakrebs verstärkt.

Diskussion und Schlussfolgerungen: Die vorliegenden Ergebnisse zeigten keine Unterschiede für Cholin und Metaboliten des Methioninstoffwechsels zwischen den Tumorpatienten und Probanden mit benigner Prostatahyperplasie. Da freies Cholin im Plasma kein Marker mit hoher Aussagekraft für die Cholinversorgung des Tumorgewebes ist, kann eine Relevanz nicht sicher ausgeschlossen werden. Die Sphingomyelinformen 14:0 und 20:0, die im Cholin-Metabolismus entstehen, zeigten in der jüngeren Altersgruppe Unterschiede zwischen der Prostatakarzinom- und der Kontrollgruppe. In der älteren Altersgruppe waren die Sphingomyelinformen 16:0 und 18:0 unterschiedlich. Diese Ergebnisse deuten auf eine mögliche Relevanz von Cholin. Alter und das Vorliegen eines Prostatakarzinoms waren signifikant assoziiert mit dem Phospholipidprofil. Die Ergebnisse lassen interessante Veränderungen des Phospholipidstoffwechsels bei Patienten mit Prostatakarzinom vermuten und geben Anregung für weitere Untersuchungen.

1. Introduction

Dietary factors have been thought to account for about 30% of cancers. Furthermore, many epidemiological evidences and research studies suggesting that diet plays an important role in cancer prevention and/or progression (1;2). Prostate cancer (PCa) is the leading cancer in males of many countries, with nearly a million new cases diagnosed worldwide (3). Both the substantial variation in the incidence of PCa worldwide (more than 25 fold incidence rate of PCa) and the increased risk in the migrant population (moving from low risk to high risk countries) provide a strong evidence that modifiable environmental factors, particularly diet, promote the development of PCa (3;4).

The roles of choline in human health and disease have been rapidly evolving. Choline is an essential nutrient required for many biological functions such as the biosynthesis of acetylcholine, phosphatidylcholine and sphingomyelin or as an important source of methyl groups (5). Choline deficiency appears to play an important role in the pathogenesis of several disorders in humans including neural tube defects (6), fatty liver, muscle damage (7), and cancer (8). Moreover, choline deficiency associated with an increase in inflammation indexes (9) and apoptosis in tissues (10). Therefore, the nutrient choline may be of special interest for prevention and progression of PCa.

Choline is the only nutrient for which dietary deficiency causes liver cancer without any known carcinogen (11;12). Interestingly, choline-deficient rats not only have a higher incidence of spontaneous hepatocarcinoma, but they are markedly sensitized to the effects of administered carcinogens. Choline deficiency is therefore considered to stimulate both cancer-initiating and cancer-promoting activities (12). However, abnormal choline metabolism is a metabolic hallmark that is associated with oncogenesis, as well as one of the characteristic features of PCa (13;14). Multiple roles for choline in cancer development are suggested. Choline can affect DNA methylation and lead to a disruption of DNA repair. It can also modify cell signaling that is mediated by intermediary phospholipid metabolites, and it can support the synthesis of cell membranes and thus support cell proliferation (15). Therefore, this study was undertaken to investigate blood concentrations of choline and related metabolites in men with PCa compared to those with benign prostatic hyperplasia (BPH). We expect differences in related metabolites levels between PCa patients and controls.

1.1 Prostate cancer

The prostate is a tubuloalveolar gland that surrounds the urethra and is located between the bladder neck and the pelvic diaphragm. It encases the ejaculatory ducts and is adjacent to the seminal vesicles **(Figure 1a)**. The gland is composed of a smooth muscle stroma and acini lined by a bilayered epithelium composed of basal cells and mature columnar secretory cells. It is found only in men as a part of the reproductive system. In humans, the gland is divided into zones (transition, central, and peripheral) that attain their greatest distinction in disease states **(Figure 1b)** (16).

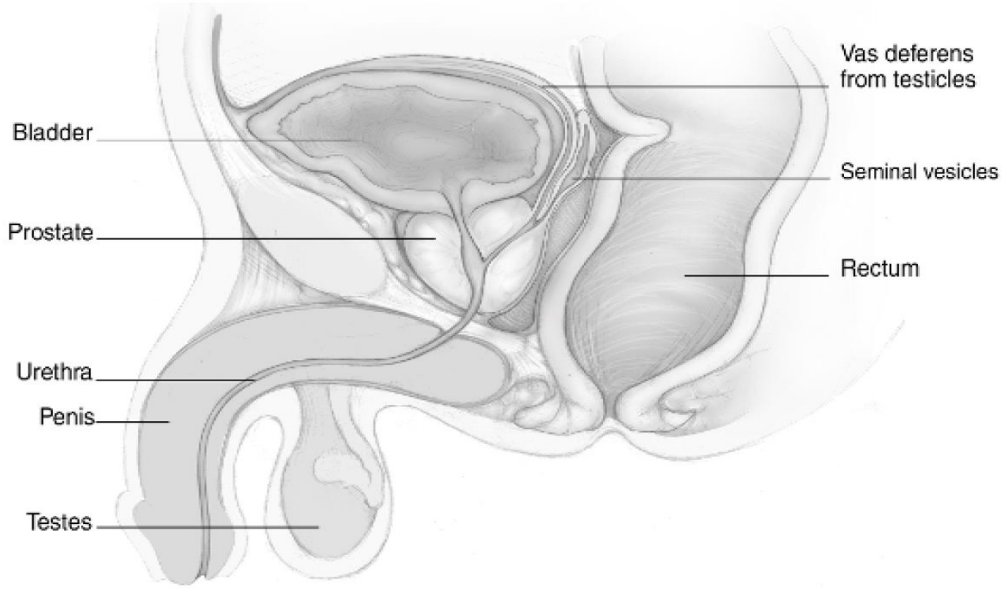

Figure 1a. The prostate diagram

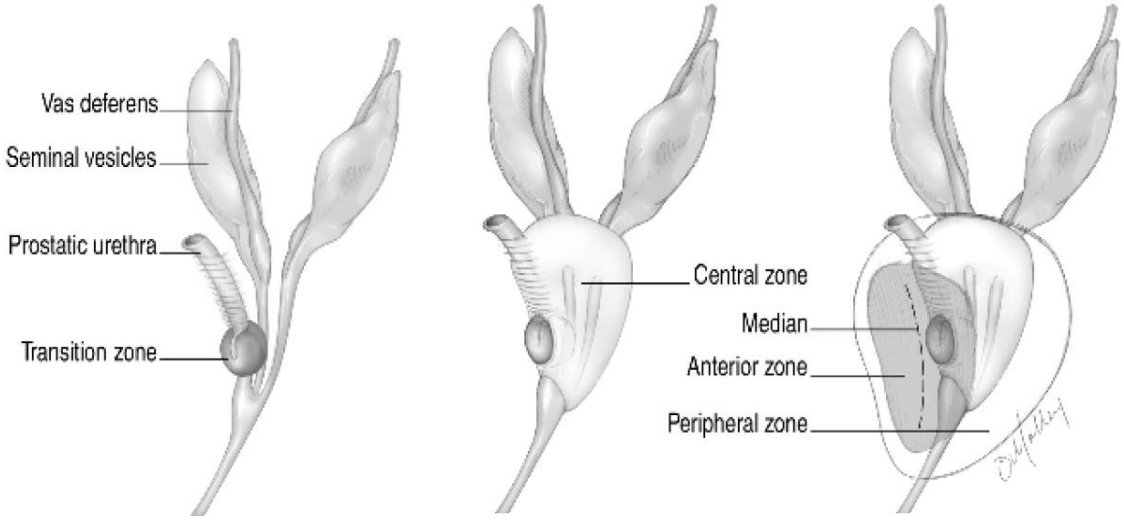

Figure 1b. The prostate zone

The prostate starts to develop before birth and continues to grow until man reaches adulthood. This growth is fueled by androgens in the body. The main androgen, testosterone, is made in the testicles. In the prostate, the enzyme 5alpha-reductase converts testosterone into more active form dihydrotestosterone (DHT). The latter strongly influences prostate function, as well as signals the prostate to grow (16). The weight of the prostate remains relatively constant (approximately 1-2 g) until puberty, at which point it increases to approximately 20-25 g in men between 20 and 30 years old. Increases in gland weight and size after the age of 30 years are largely due to the stromal and epithelial hyperplasia, which leads later to a non-cancerous enlargement of the prostate in older men called BPH (17).

Beyond non-cancerous prostate diseases, the possibility of developing PCa increases as men increase in age, about 97% of all PCa occur in men over 50 years old (18). Different types of cancer are found in the prostate, but almost all of them develop from the secretory glandular cells. This type is known as adenocarcinoma and accounts for more than 95% of all PCa. However, there are a few other rare cancers in the prostate that can also start in the prostate gland, including sarcomas, small cell carcinomas, and transitional cell carcinomas (16).

1.1.1 Epidemiology of prostate cancer

PCa is one of the most common cancers in men. It is the second most frequently diagnosed cancer and the sixth leading cause of cancer death, accounting for 14% (903,500) of the total new cancer cases and 6% (258,400) of the total cancer deaths in males worldwide in 2008. Western Europe shows high incidence rate 94.1 per 100,000 compared with less developed countries 12 per 100,000 (3). Widespread screening for prostate-specific antigen (PSA) and early detection procedures in the developed countries contribute partly to this pattern (19). Since PCa rates increase steeply with age, some part of the variability in the incidence of PCa is attributed to the different age structure between the populations, but large variability remains after rates' correction for age (18). Furthermore, the incidence is also influenced by several risk factors including genetic susceptibility, environmental factors, dietary factors, regional differences in the prevalence and distribution of the major risk factors, and differences in health care and cancer registration (3).

1.1.2 Risk factors for prostate cancer

At present, the major established risk factors for PCa are age, family history and ethnicity. Other important risk factors are hormones, environmental and anthropometric factors, and diet (20). Epidemiological studies of PCa are complex, Since many factors appear to be involved (21).

- **Age:** PCa rates increase with age faster than many other cancers. Autopsy studies show that histological PCa increases with age, presenting 15%-30% in men older than 50 years and 60%-70% in men older than 80 years of age (18).
- **Family history:** Several studies have shown an increased risk (odd's ratios from 2-6) for PCa in men with affected relatives (18). Moreover, in a study of the risk of cancer among 44,788 pairs of twins, 42 percent of PCa cases were attributed to inheritance (19).
- **Ethnicity:** Large variations in PCa incidence were observed in different ethnic groups. For example, African Americans have higher incidence rates than white Americans of similar education and socioeconomic background (18). Furthermore, they are also more likely to develop PCa at an earlier age in addition to higher mortality compared to whites (22).
- **Hormones:** Androgens significantly alter PCa growth rates. Elevated levels of testosterone and DHT may increase PCa risk, but the results are inconsistent. However, androgens' levels are influenced by endogenous factors such as genetic, and exogenous factors (environmental influences) (19;20).
- **Environmental and anthropometric factors:** Epidemiologic evidences support a major contribution of environmental and anthropometric factors to the development of PCa. Many factors are suggested such as cigarette smoking history, physical activity, body mass index (BMI), height, and cadmium exposure (18). For fatal PCa, higher vigorous physical activity level is associated with lower risk. In contrast, recent smoking history, height, and BMI are associated positively with PCa risk (21). Cadmium is linked to PCa risk in some, but not all, epidemiological studies (20).
- **Diet:** A wide variety of dietary factors are implicated in the development of PCa according to descriptive epidemiologic studies of migrants, geographic variations, and temporal studies (20). For instance, a high intake of total energy were associated with a significant increased risk (21) In addition, a high intake of dietary fat showed a strong positive correlation with PCa incidence and mortality (20). Vitamin D deficiency may be a risk factor for PCa, but in contrast, the high intake of total calcium increases PCa risk (22). Retinoids, including vitamin

A had a positive association with PCa risk (20). Phyto-oestrogens, present in a soy-rich diet, were associated with a decreased PCa risk (18). Clinical studies linked the intake of antioxidants, including selenium, α-tocopherol (vitamin E), and lycopene (a carotenoid) to a reduced PCa risk (22). On the other hand, vitamin C intake and alcohol consumption appear to have no significant association with PCa risk (20).

An association between nutrients involved in one-carbon metabolism and cancer risk have been reported (23;24). One-carbon metabolism provides the methyl groups for numerous methyl transferase reactions and plays a critical role in DNA methylation and DNA synthesis. Therefore, it has an impact on both genetic and epigenetic processes in the cell. Furthermore, the perturbation of one-carbon metabolism may lead to chromosome breaks and disruption of DNA repair mechanisms (25). Besides folate, choline, and methionine, other nutrients influence one-carbon metabolism, including vitamin B6, vitamin B12, and vitamin B2 (25). The Northern Sweden Health and Disease Cohort study investigated components of one-carbon metabolism in relation to PCa risk in 561 cases of PCa and 1,034 controls (23). The study showed that elevated plasma concentrations of choline and vitamin B2 were associated with increased risk of PCa, whereas elevated concentrations of methylmalonic acid (marker of vitamin B12 deficiency) were associated with decreased risk (23).

Collectively, a wide variety of dietary factors have been linked to PCa risk with different suggested mechanisms. Furthermore, since several genes may modify the association of specific nutrients with PCa risk, diet-gene interactions may have an important effect (26).

1.1.3 Screening and diagnosis of prostate cancer

The main screening tools for PCa are the digital rectal examination and PSA test. The digital rectal examination is a relatively simple test that allows checking the surface of the prostate with a gloved finger. The physician is able to feel any lumps, hard areas, or anything else that seems unusual on the prostate (27). Despite its poor reliability, sensitivity, and the inability to palpate the entire prostate gland, the rectal examination is still a necessary screening method (28).

PSA is a serine protease produced primarily by epithelial cells lining the acini and ducts of the prostate gland. It appears in the blood via releasing from prostatic stroma. Like other serine proteases, serum PSA exists mostly in a complex form, in addition to a small proportion remains in a free form. PSA is metabolized by the liver with a 2.2- to 3.2-day serum half-life (27). Every prostate disease, such as cancer, inflammation, infection, hyperplasia, and others, can cause PSA

levels to rise. However, BPH is still the most common cause of elevated serum PSA (27). About 30% of men with total PSA values above the currently accepted cut-off point (4.0 ng/mL) will have PCa based on biopsy results (29). The screening results for PCa are significantly better by using PSA test combined with digital rectal examination (27).

Transrectal ultrasound and transrectal ultrasound guided needle biopsies are performed to confirm diagnosis following PSA or digital rectal examination testing. Transrectal ultrasound guided systematic prostate biopsy is the standard test for PCa diagnosis (30). The current protocol using extended biopsy schemes (12 cores) provides a higher sensitivity in detecting PCa compared to previous sextant protocol. The cores are taken from a standard set of locations (30). Recent studies suggested that magnetic resonance imaging (MRI) guided prostate biopsies are superior to transrectal ultrasound guided systematic prostate biopsy and could reduce the false-negative results due to targeting areas considered to be suspicious for PCa (31). However, further studies are needed to confirm the additional benefits of MRI guided prostate biopsies.

Non-invasive clinically imaging techniques that provide data on metabolic activity in vivo such as positron emission tomography (PET/CT) and magnetic resonance spectroscopic imaging (MRSI) were evaluated for detection and staging of PCa (32;33). These techniques detect primarily the abnormalities in choline metabolism that are associated with oncogenesis and tumor progression. PET/CT techniques use positron-emitting isotopes such as ^{11}C-choline, which generally displays a high uptake in tumor tissues (34). However, PET/CT shows a low detection rate for PCa (35). One the other hand, PET/CT plays a promising role for diagnosis of recurrent PCa, as well as for the detection of lymph node metastases (36). MRSI techniques map the distribution of metabolites throughout the entire prostate gland, such as choline and citrate. Elevated choline can be seen in the presence of cancer (34). MRSI shows a better sensitivity than PET/CT for PCa detection (34). Moreover, MRSI is a valid tool for detecting cancer recurrence and monitoring therapy response (37).

Due to the high prevalence of latent PCa, a considerable number of PCa cases are diagnosed incidentally from histopathologic examination of the prostectomy specimens. The common clinical scenario that leads to the diagnosis of incidental PCa is through the performance of prostatectomies for presumed BPH. However, the incidence of such a finding becomes lower as many patients undergo PSA screening test in addition to the increase use of medical therapy for

BPH (30). Further, screening programs have been implemented in the health care system in Europe (38).

1.1.4 Staging and grading of prostate cancer

Following histological diagnosis of PCa, staging is performed to determine the extent of the patient's cancer to predict prognosis and to evaluate and select the appropriate treatment options. Accurate staging is helpful in assessing different treatment options and defining prognostic models (34). Currently, the following systems are used to accomplish this purpose.

1.1.4.1 TNM classification system

The TNM classification is the most widely used means for classifying the extent of cancer spread. It is developed by the Union for International Cancer Control (UICC) and is used as a global method to classify cancers. The classification is based on the extent of the primary tumor (T), the presence and extent of involved lymph nodes (N), and distant metastases (M) (34). The TNM system allows PCa classification according to two distinct systems, the clinical TNM (cTNM) and pathological TNM (pTNM). The first is determined from the information that is available without surgery, whereas pTNM is based on the surgical removal and histological examination of the entire prostate gland, the seminal vesicles and surrounding structures and, if relevant, pelvic lymph nodes. In general, cTNM is used to determine initial treatment strategy, while pTNM is used to determine the requirement for post surgical adjuvant therapy and follow up (39). The 2009 TNM classification for PCa is shown in **Table 1.**

Table 1: The 2009 Tumor Node Metastasis (TNM) classification of PCa (39).

T - Primary tumor		
Stage	**Sub-stage**	**Definition**
TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
T1		Clinically inapparent tumor not palpable or visible by imaging
	T1a	Tumor incidental histological finding in 5% or less of tissue resected
	T1b	Tumor incidental histological finding in more than 5% of tissue resected
	T1c	Tumor identified by needle biopsy (e.g. because of elevated PSA level)
T2		Tumor confined within the prostate [1]
	T2a	Tumor involves one half of one lobe or less
	T2b	Tumor involves more than half of one lobe, but not both lobes
	T2c	Tumor involves both lobes
T3		Tumor extends through the prostatic capsule [2]
	T3a	Extracapsular extension (unilateral or bilateral) including microscopic bladder neck involvement
	T3b	Tumor invades seminal vesicle(s)
T4		Tumor is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator muscles, and/or pelvic wall
N - Regional lymph nodes [3]		
Stage	**Sub-stage**	**Definition**
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1		Regional lymph node metastasis
M - Distant metastasis [4]		
Stage	**Sub-stage**	**Definition**
MX		Distant metastasis cannot be assessed
M0		No distant metastasis
M1		Distant metastasis
	M1a	Non-regional lymph node(s)
	M1b	Bone(s)
	M1c	Other site(s)

[1] Tumor found in one or both lobes by needle biopsy, but not palpable or visible by imaging, is classified as T1c.

[2] Invasion into the prostatic apex, or into (but not beyond) the prostate capsule, is not classified as pT3, but as pT2.

[3] Metastasis no larger than 0.2 cm can be designated pN1 mi.

[4] When more than one site of metastasis is present, the most advanced category should be used.

1.1.4.2 Gleason grading system

The most common method for the histological grading of prostate tissue is the Gleason grading system. In 2005 the Gleason system is altered by International Society of Urological Pathology, and is currently the standard for grading adenocarcinoma of the prostate on core biopsy and operative specimens (39). In this system the tissue is classified into five grades, numbered 1 through 5. The grade increases with increasing malignancy level and therefore cancer aggressiveness **(Figure 2)** (40).

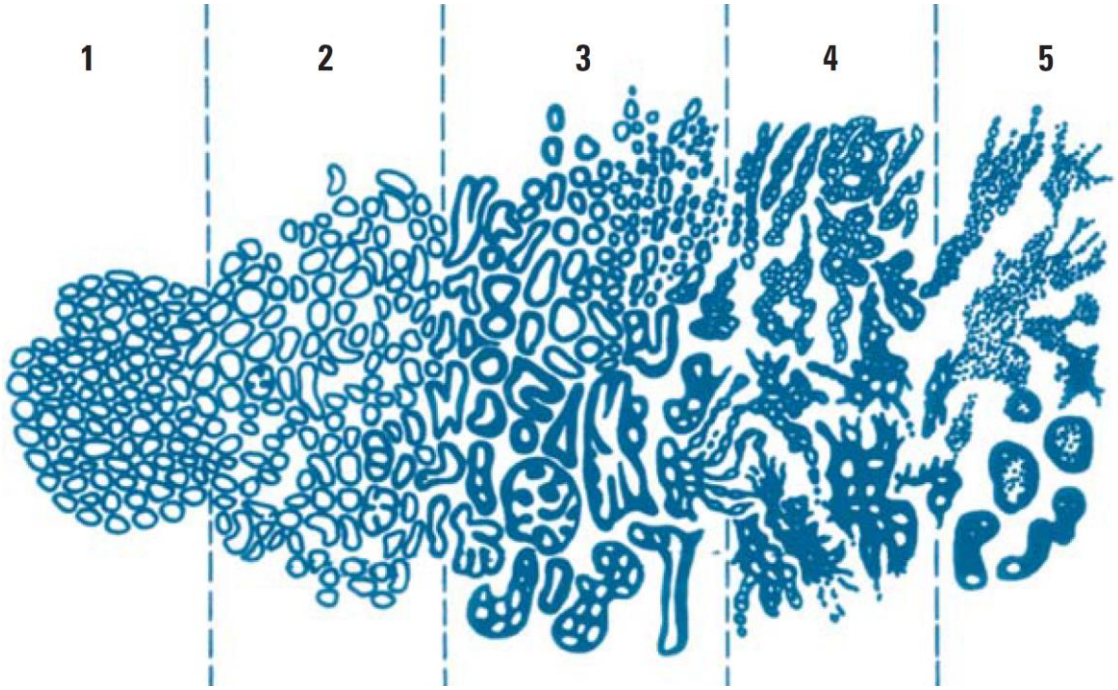

Figure 2. Schematic diagram of Gleason grading system

The Gleason score represents the sum of the most and second most predominant Gleason grades present in the tissue section, and the first number is often more important. All Gleason scores range from 2 (1+1) to 10 (5+5). Although, two scores might be the same, but the underlying grades might be different and therefore, the tumor characteristics might be different. For instance, a Gleason 7 (3+4) cancer would likely prove to be less aggressive than a Gleason 7 (4+3) cancer, even though, both have the same Gleason score (40). In case, a small area of a third pattern (generally more aggressive) is observed, the pathologists should mention details of this "tertiary" pattern. Similarly, a Gleason score 7 (3+4) with a tertiary component of pattern 5 would be considered to be more aggressive than the same score without tertiary pattern 5 (40).

1.1.4.3 Stage grouping system

The American Joint Committee on Cancer (AJCC) in cooperation with the TNM Committee of the Union for International Cancer Control (UICC) develops a stage grouping schema. This stage grouping system is based on TNM classification, Gleason grade, and PSA. If the Gleason score or PSA results are not available, the stage can be based only on the TNM classification. The overall stage is expressed in Roman numerals from I (the least advanced) to IV (the most advanced), as shown in **Table 2**. The classification of stages of cancer is done to help determine the best treatment approach and as a guide for prognosis, as well as provide an outlook for survival (39). However, the prognosis is also affected by other factors such as age, ethnic origin, and socio-economic condition that are completely independent of the tumor's stage. These factors should be also considered in the determination of the individual patient's prognosis (34).

Table 2: Stage grouping (39).

Group	T	N	M	PSA (ng/mL)	Gleason
I	T1a-c	N0	M0	PSA <10	Gleason ≤6
	T2a	N0	M0	PSA <10	Gleason ≤6
	T1-2a	N0	M0	-	-
IIA	T1a-c	N0	M0	PSA <20	Gleason 7
	T1a-c	N0	M0	PSA ≥10, <20	Gleason ≤6
	T2a	N0	M0	PSA ≥10, <20	Gleason ≤6
	T2a	N0	M0	PSA <20	Gleason 7
	T2b	N0	M0	PSA <20	Gleason ≤7
	T2b	N0	M0	-	-
IIB	T2c	N0	M0	Any PSA	Any Gleason
	T1-2	N0	M0	PSA ≥20	Any Gleason
	T1-2	N0	M0	Any PSA	Gleason ≥8
III	T3a-b	N0	M0	Any PSA	Any Gleason
IV	T4	N0	M0	Any PSA	Any Gleason
	Any T	N1	M0	Any PSA	Any Gleason
	Any T	Any N	M1	Any PSA	Any Gleason

1.1.5 Prostate cancer treatment

There are numerous treatment options with regard to the potentially optimal management of patients with organ-confined, locally advanced, and metastatic PCa. However, several factors should take into account before choosing the treatment options including the stage and grade of cancer, comorbidities, and life expectancy (41).

1.1.5.1 Deferred treatment (watchful waiting / active surveillance)

The recent screening and management strategies for PCa lead to the overdiagnosis of clinically insignificant tumors that may never need to be treated. The resultant overtreatment largely occurs because physicians cannot definitively distinguish the indolent from potentially aggressive disease. However, PCa is often a slowly progressive disease, and many men with PCa will not die from their disease, even in the absence of treatment (41). Two conservative management strategies of 'watchful waiting' and 'active surveillance' have been proposed for reducing the risk of overtreatment (38). Watchful waiting referred to the conservative management of PCa patient with life expectancy < 10 years, until the development of local or systemic progression with disease-related complaints. At this point, the patient would then be treated palliatively with transurethral resection of the prostate (TURP) for urinary tract obstruction, and hormonal therapy or radiotherapy for the palliation of metastatic lesions (38). As opposed to watchful waiting, active surveillance aims at the proper timing of curative treatment options (42). The strategy of active surveillance depends on a close monitoring for carefully selected patients with low risk PCa. These patients defined by a Gleason score of 6 or less, a PSA less than 10 ng/mL and cT1c-2a TNM classification (41). Patients under active surveillance must be followed-up carefully with serial PSA measurements, digital rectal examinations, and periodic prostate re-biopsies at 2, 5 and 10 years (41). However, an appropriate therapy for patients whose disease is reclassified as higher risk should be undertaken (39).

1.1.5.2 Radical prostatectomy

The surgical treatment of PCa consists of radical prostatectomy, which involves a removal of the entire prostate gland, along with sufficient surrounding tissues to obtain a negative margin (41). Radical prostatectomy remains the standard treatment for localized PCa (intracapsular disease). The retropubic approach is more commonly performed, as it enables a simultaneous pelvic lymph node assessment to be carried out. In men with localized PCa and a life expectancy of 10 years or more, the goal of the radical prostatectomy must be eradication of the disease and maintaining erectile function and urinary continence (39).

1.1.5.3 Radiation therapy

Radiotherapy for PCa can be divided into two types: external beam radiation therapy and brachytherapy (internal radiation). In external beam radiotherapy, a small amount of radiation is delivered to the prostate over a course of 7 to 8 weeks. On the contrary, radiation is delivered

from within the prostate itself in brachytherapy. To accomplish this, radioactive seeds are implanted directly into the prostate (41). Although, there are no randomized studies that compare radical prostatectomy with radiotherapy, particularly for localized PCa, radiotherapy continues to be an important and valid alternative to surgery as the sole form of curative therapy (39).

1.1.5.4 Hormonal therapy

Hormonal therapy is also called androgen deprivation therapy (ADT). The aim of ADT is to reduce the amount of testosterone and androgens in the bloodstream depriving many PCa cells of the fuel they need to grow. Once androgens are withdrawn, androgen-dependent PCa cells will slow or stop growing, while androgen-independent PCa cells will keep right on growing (43). ADT strategies become the mainstay of management of advanced PCa. More recently, there is a move towards the increasing use of ADT in younger men with earlier or recurrent disease (39).

1.1.5.5 Experimental local treatment of prostate cancer

The fight against PCa goes beyond radical prostatectomy, radiation therapy, and hormonal therapy. Temperature can also kill tumor cells and proves to be highly successful in this war on PCa. Cryosurgery of the prostate and high-intensity focussed ultrasound emerge as alternative therapeutic options (41). High-intensity focussed ultrasound kills the tumor cells by heat with highly focused ultrasonic beams. Nevertheless, it is still considered to be an experimental treatment (39;41). Cryosurgery of the prostate uses freezing techniques to induce cell death. These two techniques have been developed as minimally invasive procedures with the aim of equivalent oncological safety with reduced toxicity (38).

1.1.5.6 Chemotherapy

Until recently, chemotherapy was not a standard treatment for early PCa. However, chemotherapy should be considered as a treatment option when hormonal treatments fail and the cancer cells no longer respond to hormones, as well as in very advanced or metastatic disease (41). Although, the encouraging results of chemotherapy for the management of androgen-independent PCa with metastatic disease, but when a cytotoxic regimen should be initiated is still a matter of controversy (39).

Collectively, there are a variety of treatment options at every stage of PCa, the treatment approaches may involve individual therapy or some combination (39). The goals of the combination between different therapy types are the improvement of operability of the tumor, better local cancer control, and longer survival of the patients. However, the use of

neoadjuvant/adjuvant therapy continues to be studied as a way to maximize cure rates, as well as minimize rates of recurrence (41).

Overdiagnosis of clinically insignificant tumors are attributed to using PSA as screening marker. Determining the most appropriate treatment regimen is a dilemma for many physicians because many aspects of the disease must be taken into consideration before making decisions about treatments. A refinement of prognostic factors for disease progression will be critical in resolving this therapeutic dilemma.

PCa management becomes a major healthcare problem worldwide due to increased detection rate and life expectancy. Research into improving control of this disease is progressing on many fronts including cancer prevention. Since 90-95% of cancer cases are due to lifestyle and environmental factors. Diet may be one of the main environmental factors affecting progression from clinically insignificant to significant PCa, and therefore, diet is a promising candidate for future cancer prevention strategies.

1.2 Choline

1.2.1 Choline structure

In 1849 Adolph Strecker, a German chemist, isolated a compound from pig bile, to which he subsequently applied the name choline (from the Greeke, *chole*, bile) (44). In 1867, Bayer determined the chemical structure of choline (45). Choline (2-hydroxyethyl-trimethyl-ammonium), a simple but unusual compound, consists of a 2-carbon chain in which one carbon is attached to a hydroxyl group and the other to an amine nitrogen. The particularly unusual quality of choline is that this amine nitrogen bonds with a total of four carbon atoms instead of with the usual three, and thus carries a partial positive charge **(Figure 3)** (46).

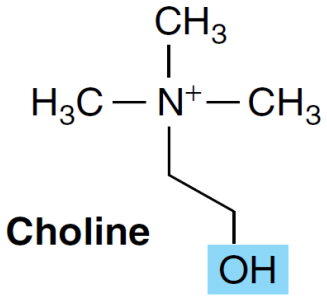

Figure 3. Structure of choline

1.2.2 Dietary choline

Choline is widely distributed throughout the food supply. Excellent sources of dietary choline include liver, eggs, wheat germ, and dried soybeans. In foods, choline is found in free and esterified form [as phosphocholine (PCho), glycerophosphocholine (GPC), phosphatidylcholine (PtdCho), and sphingomyelin (SM)] (47). Dietary choline is absorbed from the lumen of the intestine via transporter proteins (48). The water-soluble forms choline, PCho, and GPC enter the portal circulation and are mostly absorbed by the liver and the rest pass into the systemic circulation. While the lipid-soluble forms PtdCho and SM are hydrolyzed to lysophosphatidylcholine (Lyso-PtdCho) and sphingosine, respectively, then absorbed into lymph as chylomicrons (46;49-51). Due to the differences in the pathways of digestion and absorption, have these forms different bioavailability (52).

In 1998, the U.S. Institute of Medicine's Food and Nutrition Board established an adequate intake (AI) and a tolerable upper limit (UL) for choline (49). The AI is 425 and 550 mg/day for women and men, respectively, with more recommended during pregnancy and lactation. The AI

for infants is estimated from the calculated intake from human breast milk. The main criteria for determining the AI and UL are the amount of choline needed to prevent liver damage, and the choline intake associated with hypotension (the critical adverse effect from high intake of choline), respectively (49). **Table 3** summarizes the AI and UL of dietary choline according to the U.S. Institute of Medicine (49).

Table 3: Dietary reference intake values for choline (49).

Population	Age	Adequate intake (AI)	Tolerable upper limit (UL)
Infants	0 to 6 months	125 mg/day, 18 mg/kg	Not possible to establish *
	6 to 12 months	150 mg/day	Not possible to establish *
Children	1-3 years	200 mg/day	1000 mg/day
	4-8 years	250 mg/day	1000 mg/day
	9-13 years	375 mg/day	2000 mg/day
Males	14-18 years	550 mg/day	3000 mg/day
	19 years and older	550 mg/day	3500 mg/day
Females	14-18 years	400 mg/day	3000 mg/day
	19 years and older	425 mg/day	3500 mg/day
Pregnancy	All ages	450 mg/day	Age-appropriate UL
Lactation	All ages	550 mg/day	Age-appropriate UL

* Not possible to establish; sources of intake should only be mother's milk and infant formulas

1.2.3 Endogenous choline synthesis

Endogenous choline is produced by the sequential addition of three methyl groups to the amine nitrogen of phosphatidylethanolamine (PtdEth); this forms PtdCho, which can then be broken down to liberate the choline via the action of phospholipases (53). The methylation reactions are catalyzed by phosphatidylethanolamine *N* -methyltransferase (PEMT). During this process, S-adenosylmethionine (SAM) is used as a methyl donor for the formation of a new choline moiety. This pathway is primarily active in the liver but also to a small extent within other tissues (54-56).

1.2.4 Sex, gene polymorphism and dietary choline requirement

There are two major factors that affect choline requirements: estrogen status and genetic variation. Whereas 77% of men and 80% of postmenopausal women develop signs of choline deficiency when deprived of dietary choline for up to 42 days, 56% of premenopausal women did not (7). The difference in requirement is explained by inducing the expression of the PEMT by estrogen. Estrogen binds to its receptors which bind to estrogen response elements in the

promoters of the PEMT gene, resulting in an up-regulation in PEMT mRNA expression and increased hepatic enzyme activity (57).

Beyond the estrogen status, additional reasons for this variation in dietary choline requirements must be taken into consideration because some men and women develop organ dysfunction when fed a diet-deprived of choline, while others do not. Choline, methionine, and folate metabolism are interrelated in the reactions of the methyl cycle **(Figure 4)**. Thus, the requirement for dietary choline is modified by availability of these other nutrients in addition to the capacity of endogenous choline synthesis (58). Genetic variation likely underlies these differences in dietary requirements. Several very common single nucleotide polymorphisms (SNPs) in genes in choline and folate metabolism have a profound impact on choline needs (59;60). For example, the PEMT SNP (PEMT; rs12325817) has an obvious effect in women where one allele is sufficient to increase the risk of developing choline deficiency-induced organ dysfunction by 25 fold (60). Another SNP in the PEMT gene (PEMT; rs7946) is present more often in people with fatty liver (61).

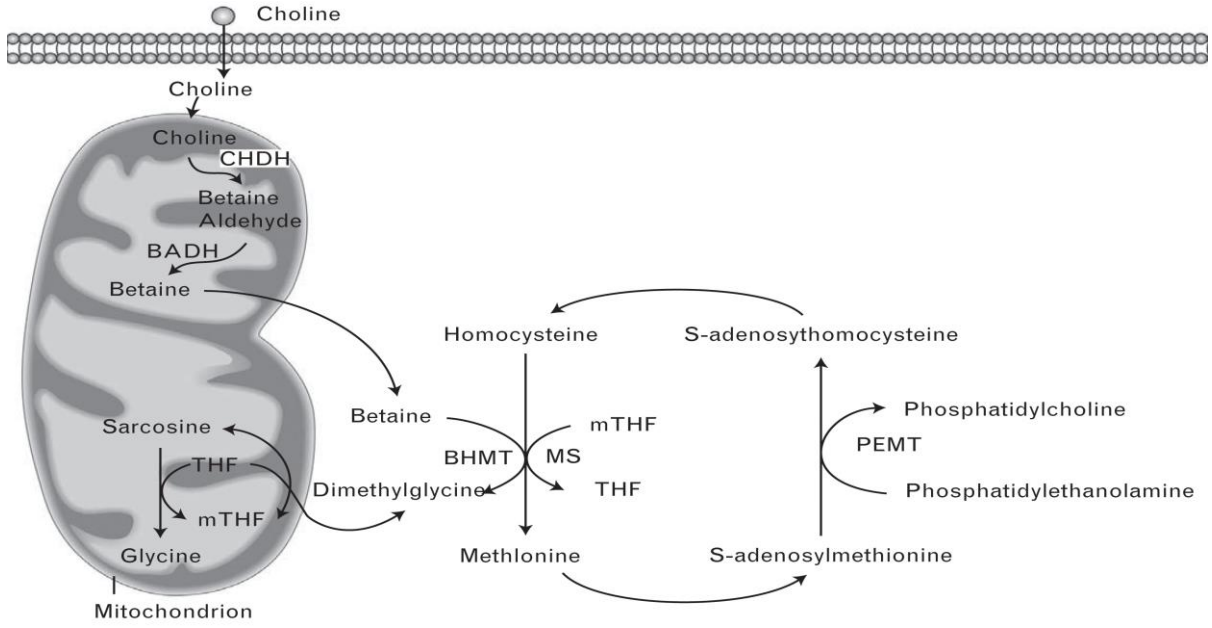

Figure 4. Interrelationships of choline, folate, and methionine in the methyl cycle. BADH, betaine aldehyde dehydrogenase; BHMT, betaine homocysteine methyltransferase; CHDH, choline dehydrogenase; MS, methionine synthase; mTHF, 5-methyl tetrahydrofolate; PEMT, phosphatidylethanolamine-N-methyltransferase; THF, tetrahydrofolate.

Polymorphisms in the folate metabolism can limit the availability of 5-methyl tetrahydrofolate (mTHF) and thereby increase the use of choline as a methyl donor. Premenopausal women who are carriers of the very common 5,10-methylenetetrahydrofolate dehydrogenase SNP (MTHFD1; rs2236225) are more than 15 times as likely as non-carriers to develop choline deficiency-induced organ dysfunction (59). Other genetic polymorphisms that modify choline requirements are choline dehydrogenase (CHDH; rs9001 and rs12676). The first has a protective effect on susceptibility to choline deficiency, while the second CHDH SNP (rs12676) is associated with increased susceptibility (60). Collectively, polymorphisms in the PEMT gene alter endogenous synthesis of choline, and polymorphisms in other genes of choline and folate metabolism influence dietary requirements by changing the utilization of choline moiety as a methyl donor.

1.2.5 Choline biochemistry

Choline is an essential nutrient that is needed for the normal functions of all cells and is crucial for sustaining life (62). Choline, or its metabolites contribute to numerous biological functions, include the structural integrity of cell membranes, methyl metabolism, cholinergic neurotransmission, transmembrane signaling, and lipid and cholesterol transport and metabolism (63). Growing experimental evidences identify important roles for choline in multiple metabolic processes such as gene expression (64), carcinogenesis (65), apoptosis (66), and early brain development (67).

1.2.5.1 Choline transport

After ingestion and subsequent digestion of choline-containing compounds in foods, the free choline that is formed enters the portal circulation of the liver. The rest of the choline in portal venous blood passes into the systemic circulation (46). Choline is a normal constituent of the plasma with a concentration of around 10 μmol/L of free choline (5;49). However, choline is also found in other biological fluids **(Table 4)**. All tissues accumulate choline by diffusion and mediated transport (68). Four choline transporters are described showing different affinity for choline, specificity, and Na^+-dependency (14)

1.2.5.1.1 High-affinity choline transporters (CHTs) are attributed to CHT1 (also known as SLC5A7), which has a Km of 0.5-5 μM for choline. CHT1 is mainly expressed in cholinergic neurons and it is choline-specific and Na^+-dependent (69;70).

1.2.5.1.2 Choline transporter-like proteins (CTLs) are characterized by intermediate-affinity (Km 20–200 μM) and they are choline specific and Na^+-independent. CTL1 (also known as SLC44A1) is ubiquitously expressed in most human tissues (14;69). It mediates the transport of choline across both mitochondrial and plasma membrane (69;71). The CTL proteins are identified in human, mouse, and rat (69). The CTL family comprises at least six genes where all gene products undergo complex alternative splicing (14).

1.2.5.1.3 Organic cation transporters (OCTs) transfer organic cations in a Na^+-independent and reversible manner. Since 1994, several members of the OCTs family are cloned from different tissues (OCT1, OCT2, and OCT3). They are part of the SLC22 gene family and carry out a low-affinity choline transport in addition to the transport of other organic cations. OCTs facilitate polyspecific cationic transport, whereas their exact role in choline transport is unclear (69).

1.2.5.1.4 Organic cation/carnitine transporters (OCTNs) are part of the SLC22 gene family also and exist as two isoforms. The OCTN1 (also known as SLC22A4) is pH-sensitive and functions in a Na^+-independent manner. The OCTN2 (also known as SLC22A5) can function as a Na^+-dependent co-transporter for certain zwitterions, as well as a Na^+-independent transporter of organic cations. OCTN1 and OCTN2 are structurally related. However, the exact role of OCTNs in choline transport is not known (72;73).

Table 4: Free choline concentrations in human body fluids

Body fluids	Free choline *	References
Plasma	6.7-10.5 μmol/L	(74;75)
Serum	11.9-13.5 μmol/L	(74;75)
Urine	15.5 μmol/L, 1.7-2.6 mmol/mol creatinine	(75;76)
Cerebrospinal fluid	0.7-2.1 μmol/L	(77;78)
Amniotic fluid	22.8-24 μmol/L	(79)
Colostrum	132 μmol/L	(80)
Breast milk	210-228 μmol/L	(80;81)
Semen	16700-24200 μmol/L	(82;83)
Peritoneal dialysate	18.2 μmol/L	(84)

* Values are given as the range of the means or medians from the cited references

1.2.5.2 Acetylcholine biosynthesis

Though only a small fraction of administered choline is acetylated, this pathway is important for synthesis of acetylcholine (ACh) as a neurotransmitter (68). Choline acetyltransferase (ChAT, EC 2.3.1.6) mediates a single reaction, the transfer of an acetyl group from acetyl-coenzyme A (acetyl-coA) to choline, which thereby generates the neurotransmitter acetylcholine in cholinergic neurons (85). This enzyme is highly concentrated in cholinergic nerve terminals. The availability of choline influence ChAT activity (68). In the brain, a specific carrier mechanism transports free choline across the blood brain barrier at a rate proportional to the serum choline concentration, whereas the cholinergic neurons have CHT1 transporter that helps them accumulate choline (70;86). Furthermore, both the PtdCho and the ACh are ultimately broken down to regenerate free choline, thus both of these compounds can also be considered ‘‘reservoirs’’ for free choline in the brain (46).

1.2.5.3 Methyl group metabolism

Methionine, mTHF, and choline important sources of methyl groups in humans (25). The methyl groups of choline can be made available for one-carbon metabolism upon oxidation to betaine. Liver and kidney are the major sites for choline oxidation. This process requires two enzymes: The mitochondrial choline dehydrogenase (CHDH) that converts choline to betaine aldehyde. The latter can then be oxidized in mitochondria or cytoplasm to betaine by betaine aldehyde dehydrogenase (BADH) (68). Betaine homocysteine methyl transferase (BHMT) mediates the transfer of the methyl group from betaine to homocysteine (Hcy) to produce methionine that is in turn converted into S-adenosylmethionine (SAM), the universal methyl donor **(Figure 5)**. As discussed earlier, the metabolism of choline, folate, and methionine are interrelated and therefore disturbances in one of these metabolic pathways are associated with compensatory changes in the others. This means the methyl groups from mTHF, methionine, and choline can be interchangeable (5). For example, methionine can be formed from Hcy using methyl groups donated either by mTHF or by betaine **(Figure 4)** (58).

1.2.5.4 Choline role in trimethylamine N-oxide metabolism

Recently, Wang *et al* reported a new metabolic pathway involving gut flora (87). In this pathway intestinal microbiota metabolism of choline and PtdCho produces trimethylamine (TMA), which is further metabolized to trimethylamine *N*-oxide (TMAO) by means of hepatic flavin monooxygenases (FMOs) **(Figure 6)**. TMAO has been reported to alter cholesterol and

sterol metabolism and contribute to the pathogenesis of atherosclerotic coronary artery disease in animal models (87;88).

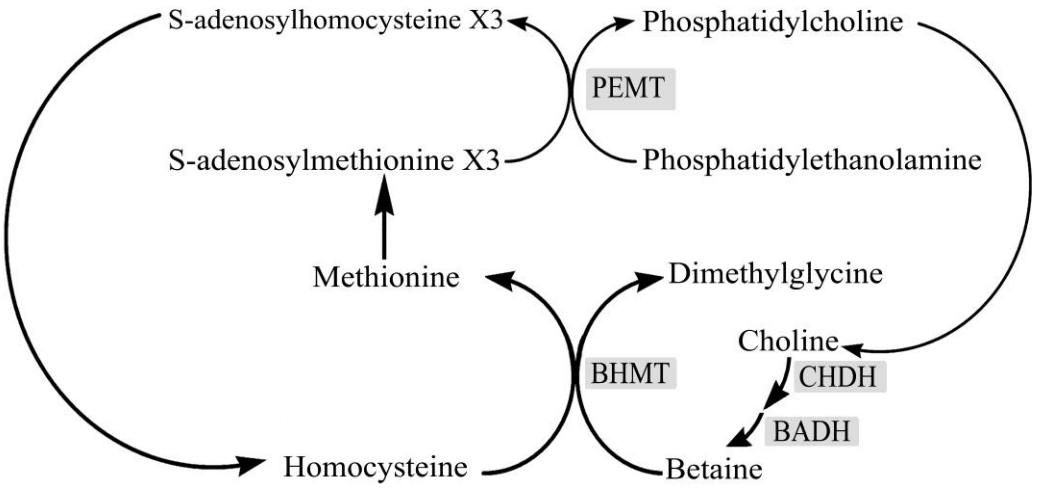

Figure 5. One-carbon metabolism, the choline cycle. BADH, betaine aldehyde dehydrogenase; BHMT, betaine homocysteine methyltransferase; CHDH, choline dehydrogenase; PEMT, phosphatidylethanolamine N-methyltransferase.

Figure 6. Gut flora-dependent metabolic pathway of choline and PtdCho.
FMOs: flavin monooxygenases; PtdCho, Phosphatidylcholine; TMA: trimethylamine; TMAO: trimethylamine-N-oxide.

1.2.5.5 Choline role in phospholipid metabolism

Phospholipid are a class of lipids that consist of two long-chain fatty acids esterified at the *sn*-1 and *sn*-2 positions of glycerol, and contain a head group linked by a phosphate residue at the *sn*-3 position. The head group forms a hydrophilic region and determines the type of phospholipid. Choline forms the head group of PtdCho, Lyso-PtdCho, and SM **(Figure 7)** (89). Phospholipid make up the essential milieu of cellular membranes and play an important role in the lipoprotein metabolism and intracellular signaling (90). The variation in the composition of phospholipid head-group , and the attached fatty acids which vary in the chain length and the degrees of unsaturation resulting in a complexity of phospholipid species (90).

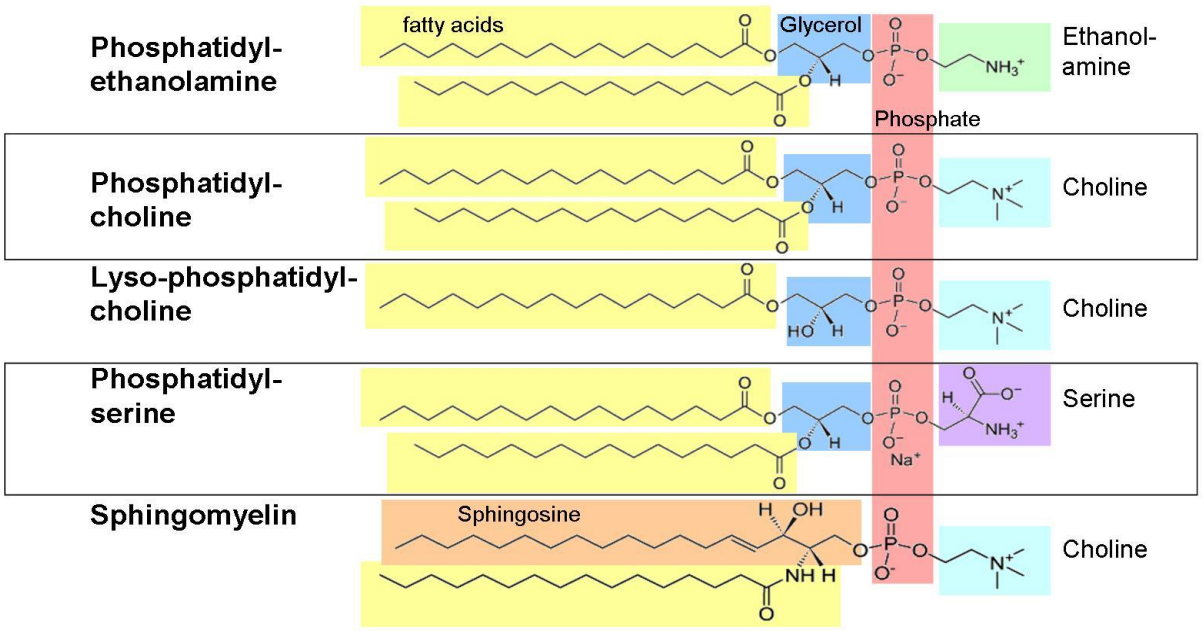

Figure 7. Chemical structure of phospholipid classes.

1.2.5.5.1 Choline role in membrane phospholipid metabolism

Phospholipid are major components of the plasma membrane. PtdCho and its product SM are the predominant phospholipid in most mammalian membranes. In fact, the main metabolic fate of choline is the formulation of PtdCho that accounts for about 95% of the total choline pool in most animal tissues (91). All tissues form PtdCho via the Kennedy pathway (also called CDP-choline pathway) **Figure 8** (92). PtdCho can also be produced via the PEMT pathway that is quantitatively significant only in the liver where it contributes approximately 30% of total hepatic PtdCho synthesis (91).

The Kennedy pathway involves three reactions. In the first step, choline kinase (CHK) catalyzes the phosphorylation of choline into phosphocholine (PCho). This reaction can be a rate-limiting step for PtdCho biosynthesis (93;94). The second reaction in the Kennedy pathway is catalyzed by the CTP-phosphocholine cytidylyltransferase (CCT) that yields cytidine diphosphate choline (CDP-Cho) from PCho and cytidine triphosphate (CTP). CCT mediates the rate-limiting step in the Kennedy pathway and its activity depends on the association with membrane structures, the phosphorylation state, and some transcription factors (92;95). In the final step, the CDP-Cho and diacylglycerol (DAG) are converted into PtdCho by 1,2-diacylglycerol cholinephosphotransferase (CPT1). CPT1 seems not to be a rate-limiting step in PtdCho biosynthesis (91;94).

Choline

On the other hand, three classes of phospholipase mediated the catabolism of PtdCho. Phospholipase C (PLC) converts PtdCho into PCho, while phospholipase A (PLA) and phospholipase D (PLD) can liberate choline. There are three-step reactions that produce choline from PtdCho. First, phospholipase A2 (PLA2) catalyzes the hydrolysis of PtdCho yielding Lyso-PtdCho, which is converted to glycerophosphocholine (GPC) by the enzyme lyso-phospholipase A1 (Lyso-PLA1). The enzyme GPC phosphodiesterase (GPC-PDE) then converts GPC into choline (14). In contrast, choline is produced from PtdCho in one-step reaction via either PLD1 or PLD2 isoform (96).

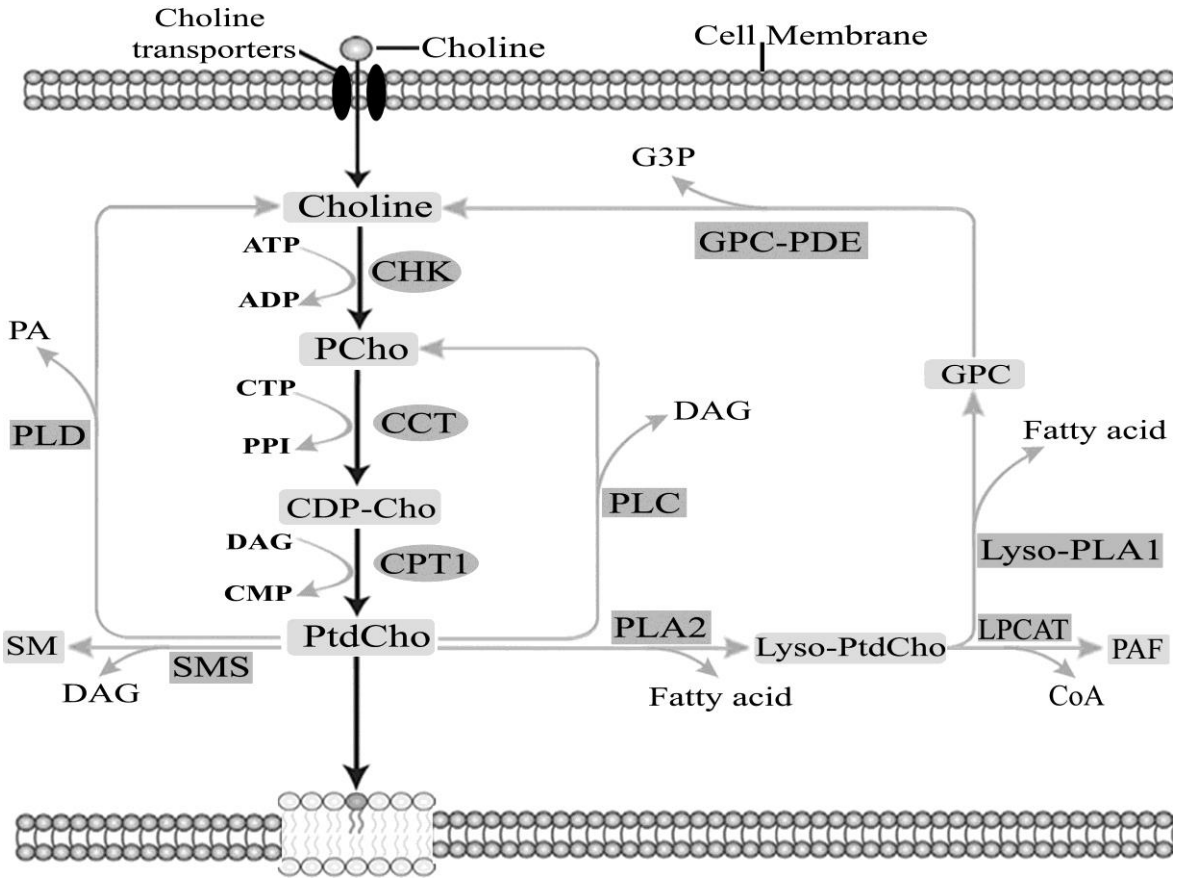

Figure 8. The enzymes involved in choline phospholipid metabolism in the cell. Black arrows represent the biosynthetic pathway. Gray arrows represent the catabolic pathway. ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; CCT, CTP-phosphocholine cytidylyltransferase; CDP-Cho, cytidine diphosphate choline; CHK, choline kinase; CMP, cytidine monophosphate; CoA, Coenzyme A; CPT1, diacylglycerol cholinephosphotransferase 1; CTP, cytidine triphosphate; DAG, diacylglycerol; GPC, glycerophosphocholine; GPC-PDE, glycerophosphocholine phosphodiesterase; G3P, glycerol-3 phosphate; Lyso-PLA1, lyso-phospholipase A1; Lyso-PtdCho, lysophosphatidylcholine; LPCAT, lysophosphatidylcholine acetyltransferase; PA, phosphatidic acid; PAF, Platelet-activating factor; PCho, phosphocholine; PLA2, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; PPi, diphosphate; PtdCho, phosphatidylcholine; SM, sphingomyelin; SMS, sphingomyelin synthase.

1.2.5.5.2 Lipoprotein metabolism

Choline especially via its metabolite, PtdCho, plays an important role in the lipid metabolism. The liver is the major site for both the synthesis of phospholipid and the generation of plasma lipoproteins. Phospholipid, cholesterol and specific proteins, known as apolipoproteins (apos) form a monolayer on the lipoprotein surface surrounding the hydrophobic core of triacylglycerols (TG) and cholesteryl esters **(Figure 9)** (97). Choline-containing phospholipid (PtdCho, Lyso-PtdCho, and SM) are the most abundant phospholipid components in all the lipoprotein classes (more than 90%) **(Table 5)** (98;99). SM is synthesized from PtdCho by transfer of PCho to ceramide (Cer) through SM synthase (SMS) (100), while Lyso-PtdCho is generated from PtdCho by phospholipase A2 (101) **(Figure 8)**.

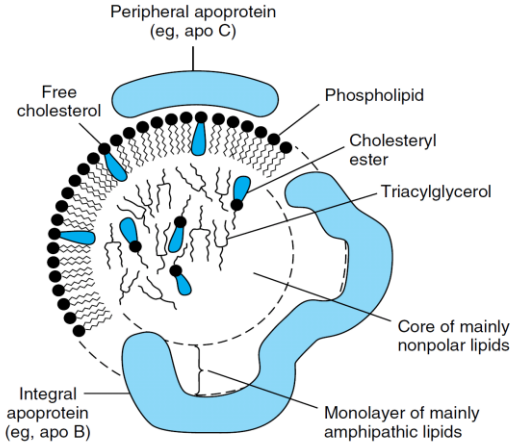

Figure 9. Generalized structure of a plasma lipoprotein.

Table 5: The phospholipid composition of VLDL, LDL and HDL of human serum lipoprotein classes (99).

phospholipid class	VLDL	LDL	HDL
PtdCho	75.9%	74.2%	73.2%
Lyso-PtdCho	4.3%	3.8%	16.6%
SM	14.9%	19.1%	7.4%
PtdEth	2.4%	0.9%	1.2%
Other	2.5%	1.9%	1.5%

HDL, high density lipoprotein; LDL, low density lipoprotein; Lyso-PtdCho, lysophosphatidylcholine; PtdCho, phosphatidylcholine; PtdEth, phosphatidylethanolamine; SM, sphingomyelin ; VLDL, very low density lipoprotein.

TG produced by liver is delivered to other tissues, mainly in the form of very low-density lipoprotein (VLDL) **(Figure 10)** (102). PtdCho is the only phospholipid which is currently

known to be required for lipoprotein assembly and secretion (103). Impaired hepatic PtdCho biosynthesis, either via CDP-choline pathway or PEMT pathway, reduces the levels of circulating VLDL and associate with TG accumulation in the liver (steatosis) (91). Choline-deficient humans also show a decrease in low density lipoprotein (LDL) levels (104). Recent data indicated that impaired PtdCho biosynthesis reduces also plasma high density lipoprotein (HDL) by inhibiting hepatic HDL formation and by increasing HDL uptake from the circulation (103).

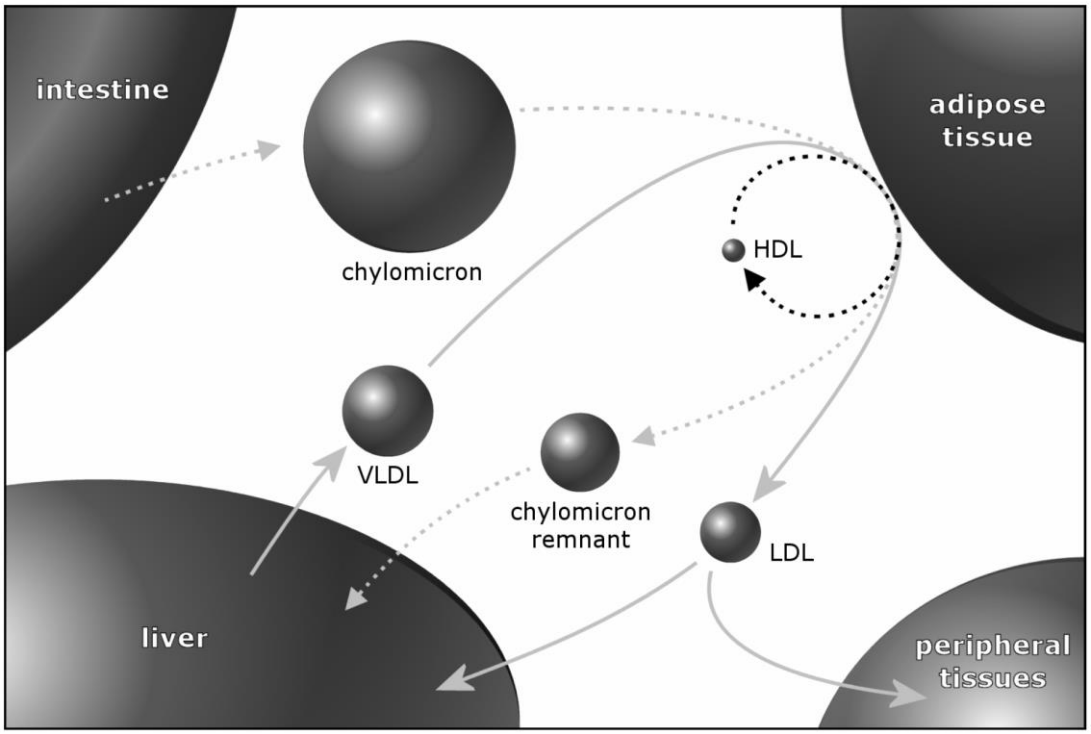

Figure 10. Schematic overview of lipoprotein transport and metabolism. Chylomicrons and VLDL are produced by the intestine and liver, respectively. TG is unloaded from chylomicrons and VLDL through the action of lipoprotein lipase. During this process, HDL functions as the acceptor for the relieved exchangeable apolipoproteins from both chylomicrons and VLDL particles. VLDL become more dense and transformed to an intermediate density lipoprotein (IDL), which is further converted into low-density lipoprotein (LDL). The liver takes up the resulting chylomicron remnants and LDL, the latter is also taken up by peripheral tissues.

1.2.5.5.3 Choline phospholipid-mediated signal transduction

Besides the roles of choline phospholipid metabolites in membranes and lipoproteins metabolism, they function also as cell signaling modulators and mediators (105). The turnover of choline-containing phospholipid by means of specific enzymes, resulting in the formation of breakdown products that are signaling molecules by themselves such as DAG (106), or after conversion to signaling molecules such as phosphatidic acid (PA) (105). Another Important

signaling molecule is platelet-activating factor (PAF, alkyl-acetyl phosphatidylcholine), which has diverse physiological functions in addition to the intercellular signaling effect. PAF is produced mainly from Lyso-PtdCho, where Lyso-PtdCho acetyltransferase (LPCAT) catalyses the transfer of an acetyl residue from acetyl-CoA to Lyso-PtdCho **(Figure 8)** (107).

1.2.6 Choline in health and disease

Humans require choline for sustaining normal life. Studies in animals and humans provide results suggesting the involvement of choline in neurodevelopment and pathogenesis of various chronic diseases, as well as points to a role in risk assessment and disease prevention (108). When healthy humans are fed a choline-deficient diet for 21-42 days they develop biochemical changes such as diminished plasma choline and phosphatidylcholine concentrations, as well as elevated serum aminotransferases and creatine phosphokinase suggesting liver and muscle damage, respectively (7;104).

Choline-deficient humans develop fatty liver owing to a lack of PtdCho, which limit the export of excess TG from liver via VLDL (91). Choline deficiency in humans is also associated with liver and muscle damage (7), and with DNA damage and cell death in peripheral lymphocytes (10). Choline-deficient humans are likely to accumulate Hcy, a risk factor for a wide range of diseases (7). In addition, women who have lower choline intakes during pregnancy have significantly increased risk of giving birth to a child with a neural tube defect (6). Furthermore, women who have lower dietary intakes of choline could have an increased risk and mortality from breast cancer (109;110). However, the implications for choline deficiency on human susceptibility to cancer are not fully elucidated, an important component is likely to be the modifications of DNA, changes in gene expression, and mistakes in DNA repair, in addition to other components that differ from cancer to cancer.

1.3 Prostate cancer and choline

1.3.1 Background

Animal studies demonstrated that choline deficiency can cause cancer, particularly hepatocarcinomas, in the absence of other carcinogens (11;12). Interestingly, choline-deficient rats are markedly sensitized to the effects of administered carcinogens and have higher tumor incidence than rats on the control diet. Choline deficiency is therefore considered to stimulate both cancer-initiating and cancer-promoting activities (12).

Although animal studies imply a causal relationship between choline deficiency and carcinogenesis, the role of this nutrient in human carcinogenesis and tumor progression is not well understood. Few studies observed a positive association between the dietary choline intake or plasma concentration of choline and the risk of some types of cancer (8), including PCa (23), while other studies indicated that a higher intake of choline is associated with reduced risk of cancer (109;110). On the other hand, two studies observed no significant relationship (111;112).

However, abnormal choline metabolism is a metabolic hallmark that is associated with oncogenesis and tumor progression, as well as one of the characteristic features of PCa (13;14). Multiple roles for choline in cancer development were suggested. Choline can affect DNA methylation and lead to a disruption of DNA repair. It can also modify cell signaling that is mediated by intermediary phospholipid metabolites, and it can support the synthesis of cell membranes and thus support cell proliferation. Dietary choline, as a major source of choline, phospholipid, and methyl groups is a potential modifiable risk factor or risk marker for PCa (15).

1.3.2 Choline uptake in prostate cancer cells

As discussed earlier, four different types of choline transporters mediate the cellular uptake of choline (14). An increase in the expression of choline transporters and the transport rate has been documented in PCa compared with normal prostate tissues (113). The transporters involved in increased uptake of choline in human PCa and the mechanism of this enhanced uptake are not completely clear. CTL1 is expressed in PCa cells and the kinetic properties of this transporter are similar to that of human CTL1 (intermediate affinity and Na^+-independent). However, one study showed that choline transporters in the PCa cell line PC-3 have intermediate affinity, but are Na^+-dependent (14;113). Thus, combined transport systems (Na^+-dependent and Na^+-independent) might be involved. Therefore, a potential role for choline transporters in PCa deserves further investigations (15).

1.3.3 The role of choline as a methyl donor

Through its function as a methyl donor, choline has important roles in regulation of gene expression via epigenetic mechanisms. Aberrant epigenetic events such as DNA hypo- and hypermethylation are observed in PCa and play essential roles in prostate carcinogenesis (19;114). Methylation of DNA requires SAM as a methyl donor, which is influenced by choline availability (64). Altered DNA methylation and disruption of DNA repair are reported in a wide variety of human cancers (115), including PCa (19). For example, the glutathione S-transferase 1 can detoxify reactive chemical species through conjugation with reduced glutathione thus preventing or attenuating the development of cancer upon exposure to carcinogens (116). The lower expression of the π-class glutathione S-transferase 1 is related to hypermethylation of CpG island of the promoter region of π-class glutathione S-transferase 1 in more than 90 % of PCa cases (114). Nakayama *et al*. observed hypermethylation of the promoter region of the glutathione S-transferase 1 gene in the majority of areas of carcinoma and high grade prostatic intraepithelial neoplasia lesions, but not in the epithelium and hyperplastic epithelium (117). Therefore, the role of choline in carcinogenesis may be related to the extent of its utilization as a methyl group donor. Since epigenetic mechanisms precede the development of the tumor, follow-up studies can provide information about a potential predictive value for choline levels in cancer. (15).

1.3.4 The role of choline in phospholipid metabolism

A major role for choline is the synthesis of membrane phospholipid. Enhanced choline phospholipid metabolism is reported in cancer cells that need phospholipid in large quantities to enable their rapid proliferation (14). The predominant membrane phospholipid is PtdCho, which with other phospholipid form the characteristic bilayer structure of cells and regulate membrane integrity (89). Biosynthesis and hydrolysis of PtdCho are also essential processes for mitogenic signal transduction events in cells (118). Cancer pathogenesis is discussed in relation to enzymes involved in the synthesis or the degradation of phospholipid.

The enzyme CHK seems to enhance the malignant transformation of cancer cells (119). For example, CHK is overexpressed in PCa thus causing higher PCho and supporting malignant transformation (13). This in turn enhances choline uptake and membrane phospholipid synthesis in malignant cells (13;120). Furthermore, PCho might be involved as a second messenger in the

growth-signaling cascade (94). In line with this evidence, the inhibitors of CHK show antitumor activity and reduces tumor growth (94;120).

Moreover, the endogenous synthesis of choline seems to be upregulated in cancer cells. The higher requirements of choline in cancer cells are supported by the enhanced choline transport activities and increased degradation of PtdCho. The activity of the enzyme PLD that synthesizes choline from PtdCho is increased in tumor cells (96;121). Several lines of evidence indicate that PLD might be implicated in cell proliferation, survival signaling, malignant transformation, tumor progression, and metastatic process (14;96). Furthermore, recent data demonstrate that GPC-PDE constitutes a source of choline from GPC for the Kennedy pathway (122;123). Moreover, GPC-PDE is demonstrated to play a critical regulatory role in cell migration in vitro (123). Recent studies point to a probable interaction between CHK, PLD, and GPC-PDE. A high co-expression of these enzymes in the tumor resulted in higher PCho and lower GPC and led to a characteristically switched PC/GPC ratio (122;123).

PtdCho can be hydrolyzed by PLC to yield PCho as well as DAG, a critical second messenger that activates protein kinase C, an important player in several signal transduction cascades (118). Inhibition of PLC activity in PC3LN3 human prostate carcinoma cells shows a decrease in cell migration, suggesting a possible role for PLC in cell motility and invasion process (124).

Taken together, the higher CHK expression in PCa supports tumor development via PCho formation. A higher uptake of choline by tumor cells might support the additional requirements of the cells (15).

1.3.5 Choline metabolism in relation to oncogenic signaling, hormonal therapy, and hypoxia

Molecules derived by the breakdown of choline-containing phospholipid such as DAG and PCho can act as second messengers in mitogenic signal transduction pathways. Other molecules like lyso-phosphatidic acid can activate enzymes involved in choline metabolism such as PLD (96;118;121). Moreover, PA, the precursor of lyso-phosphatidic acid and DAG, has regulatory properties that are implicated in oncogenic signaling and activation of enzymes involved in choline metabolism (14). The interactions between oncogenic signaling pathways and the choline metabolic pathway are summarized in **Table 6.**

PCa hormonal therapy or ADT might influence choline metabolism or choline uptake, but the evidence is not consistent (35;125;126). The influence of ADT on the uptake of radiolabelled

choline (^{11}C-choline) is tested by using PET/CT. ADT lowers choline uptake in prostate tissues in two studies that tested patients before and after ADT treatment (35;125). A third study reports enhanced choline uptake in patients treated with ADT compared to those not treated with this therapy's type (126). Experimental studies show that androgens increase choline uptake (2.0-fold) and cause accumulation of phospholipid (2.5 fold) in androgen-dependent human prostate LNCaP cell (127;128). In contrast, choline uptake in androgen-independent PC-3 cell is similar both in the presence and the absence of androgens (128). These findings suggest a potential influence of androgens on choline metabolism, but this effect might differ with each cancer type.

Hypoxia might occur because of the decrease in the vascular support when PCa cells grow. The subsequent activation of hypoxia-inducible factor 1 (129) might cause resistance to hormonal, radio- and chemo-therapy (128;129). However, the hypoxia-inducible factor 1 is shown to increase PCho levels in PCa cells (128;130) and to induce the expression of CHK in PC-3 cells, resulting in raised levels of total choline and PCho (130). Furthermore, hypoxia might decrease the uptake of choline in PCa cells, while the choline uptake is enhanced under aerobic conditions (128). Therefore, the relationship between hypoxia and choline metabolism in PCa seems to affect both choline uptake and utilization.

Table 6. The interaction between oncogenic signaling and the choline phospholipid metabolism

Oncogenic signaling	Choline transporter	CHK	CCT	PLD	PLC	PLA2
RAS GTPase		↑ CHK activity (131)		↑ PLD activity (132)	↑ PLC activity (133)	PLA2 mediate downstream of RAS (134)
RAF kinases				↑ PLD activity ǂ (96)		
MAPK/ERK			↓ CCT activity (135)			↑ PLA2 activity (136)
PI3K	PI3K inhibition reduce the choline uptake (137)	↑ CHK activity (131)		↑ PLD activity (138)		
RALGDS		↑ CHK activity (131)		↑ PLD activity ǂ (138)		
JNK			↓ CCT activity (139)			↑ PLA2 activity (136)
hypoxia-inducible factor 1		↑ CHK transcription (130)				
activator protein 1		↑ CHK transcription (140)				
SREBP			↑ CCT activity (141)			
Specificity Protein 1	↑ hCTL1 transcription (142)		↑ CCT transcription (141;143)	↑ PLD transcription (144)		

ǂ negative feedback pathway. CCT, CTP-phosphocholine cytidylyltransferase; CHK, choline kinase; ERK, extracellular signal-regulated kinase; hCTL1, human choline transporter-like protein 1; JNK, JUN N-terminal kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PLA2, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; RAF, rapidly accelerated fibrosarcoma; RALGDS, RAL GTPase guanine nucleotide dissociation stimulator; RAS, rat sarcoma; SREBP, sterol regulatory element binding protein.

1.3.6 Choline phospholipid metabolites in prostate cancer

Accurate clinical staging of PCa is very important for the management of the disease and optimization of the therapy regimen. Total choline levels in prostate tissue have been shown to be positively related to the Gleason score and tumor aggressiveness in patients with PCa (145). Several PET/CT techniques indicate that ^{11}C-choline might be helpful in monitoring the recurrence of PCa, but not for initial staging of the tumor (33;146). MRSI techniques showed that total choline and PCho are correlated with the stage of PCa. Therefore, MRSI might be used for diagnosing and monitoring the progression of PCa (32;33). The low sensitivity and low accuracy of PET/CT in comparison with MRSI might explain the differences in the diagnostic utility of both methods.

The relationship between choline and ethanolamine metabolites levels and PCa stage has been recently investigated in benign and prostate tissues by using MRSI. In addition to Eth, four choline- and ethanolamine (Eth)-containing metabolites (PCho, GPC, and their Eth counterparts phosphoethanolamine (PEth) and glycerophosphoethanolamine (GPE)), were tested (145;147). One study indicated that PCho levels were significantly higher, while Eth levels were lower in cancer versus benign prostate tissues. The other metabolites, PEth, GPE and GPC, showed no significant differences (147). Furthermore, PCho/PEth ratio provided the best discrimination between benign and PCa tissues. Moreover, PCho/PEth, PCho/GPC, PEth/Eth, and GPE/Eth ratios also increased significantly in PCa tissues compared with benign prostate tissues (147). Another study confirmed lower levels of Eth and higher PCho levels in PCa versus benign prostate tissues (145). The levels of PEth, GPE, and GPC were higher in PCa compared with benign prostate tissues in this study. Additionally, the levels of the choline-containing metabolites (PCho and GPC) were significantly elevated in high-grade PCa versus benign prostate tissues whereas the Eth- containing metabolites (Eth, PEth, and GPE) were significantly higher in low-grade PCa versus benign prostate tissues (145). Therefore, it seems that the Eth-containing metabolites (PEth + GPE) can discriminate between individual benign and low-grade PCa tissues. Moreover, the choline- containing metabolites (PCho + GPC) seem to discriminate between high- and low-grade PCa tissues (145).

Taken together, choline may be related to PCa risk via several mechanisms. Choline can affect DNA methylation and lead to a disruption of DNA repair. It can also modify cell signaling that is mediated by intermediary phospholipid metabolites, and it can support the synthesis of cell

membranes and thus support cell proliferation. However, choline metabolism in PCa might be dysregulated in order to meet the tumor requirements for phospholipid and methyl groups. Furthermore, androgens, folate and methionine status, and common polymorphisms in genes involved in choline metabolism can interact with choline metabolism and affect its requirements.

1.4 Study aims

PCa is one of the most common cancers in men. The prevalence has been increasing over the last few decades, largely due to early detection procedures (148). However, risk factors such as age, ethnicity, family history, lifestyle, and androgens were also discussed in relation to the PCa risk (19;22). There are several evidences that dietary factors can contribute to the risk of PCa. A higher intake or status of the nutrient choline in plasma has been related to higher PCa risks (23). Multiple roles for choline in cancer development have been suggested. Choline can affect DNA methylation and lead to a disruption of DNA repair. It can also modify cell signaling that is mediated by intermediary phospholipid metabolites, and it can support the synthesis of cell membranes and thus support cell proliferation (15).

We hypothesized that the concentrations of choline and choline metabolites in plasma will be higher in men with PCa than those with BPH. The main aim of this study was to investigate the metabolites of one-carbon metabolism [choline, betaine, DMG, total folate, SAH, SAM, tHcy, cystathionine (Cys), and methylmalonic acid (MMA) (marker of vitamin B12 status)] and phospholipid classes in cases and controls. Moreover, choline metabolites were studied in relation to tumor grad, age, and statin use. Since choline is a dietary component, the study allows to a better understanding of a potential modifiable factor in PCa.

2. Materials and methods

2.1 Subjects and study design

The study was conducted at the Saarland University Hospital, Department of Clinical Chemistry and Laboratory Medicine - Central Laboratory, Homburg, Germany in collaboration with Department of Urology, Saarland University Hospital, Homburg, Germany. This is a case control study. Participants are men (age > 50 y) with primary PCa (cases, n=152) or with BPH (controls, n=89). Patients were recruited before carrying out the radical prostatectomy surgery, or TURP surgery. Exclusion criteria were: vitamin B supplementation (folic acid> 400 µg / day; B12> 10 µg / day; B6> 10 mg / day), renal failure, advanced liver disease, chronic alcohol consumption, metastases, or methotrexate therapy. The study design is illustrated in **Figure 11**.

The ethics commission of the Saarland approved the study (ref. Nr: 90/12 July 2012). The study was performed according to the Helsinki Declaration and all participants were informed and signed informed consent documents. Patients and controls were recruited between July 2012 and March 2013. Seven patients and sixteen controls were excluded from the study due to exclusion criteria: 9 had renal failure, 2 had advanced liver disease, 2 used vitamin B supplements, one patient used methotrexate therapy, and 9 controls had co-existing cancers **Figure 11**.

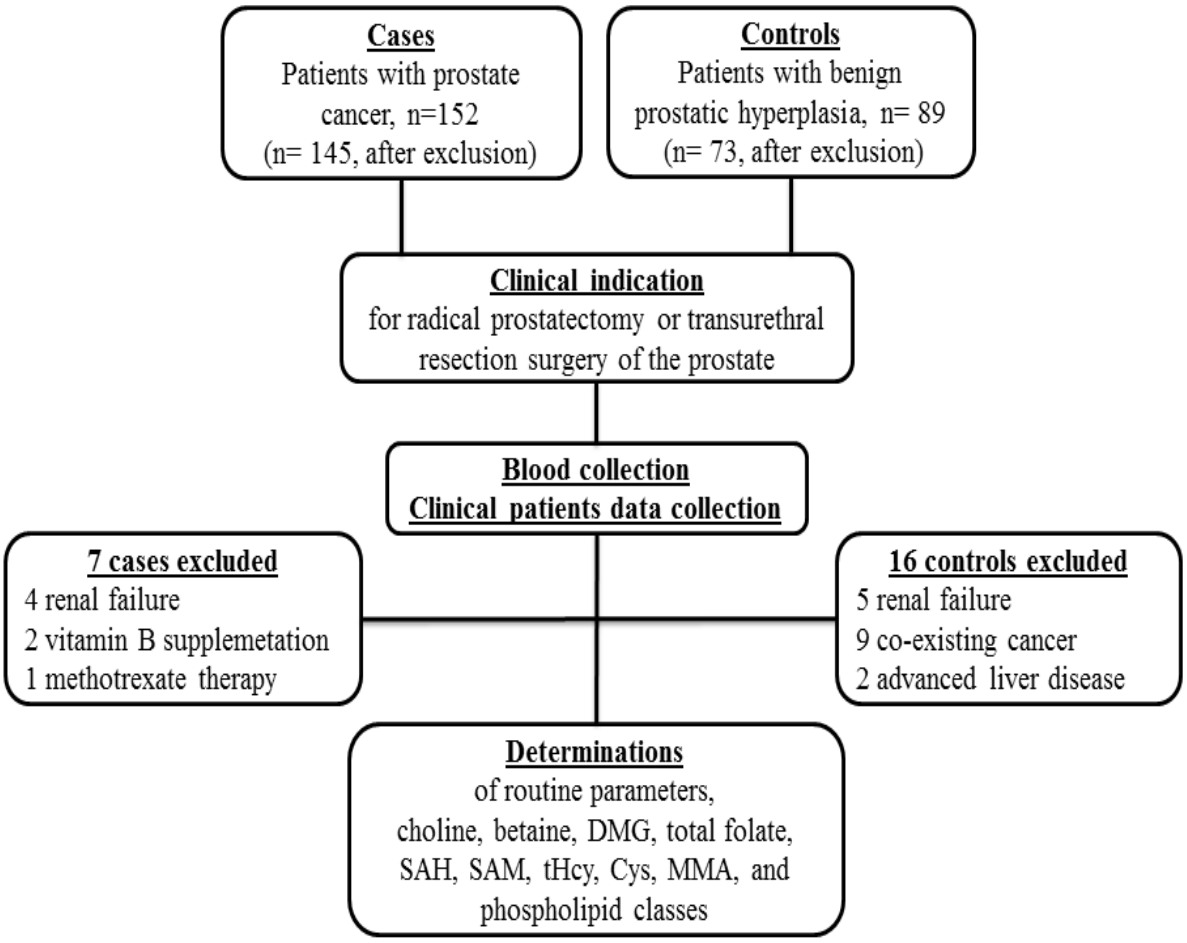

Figure 11. Study design. Cys, cystathionine; DMG, dimethylglycine; MMA, methylmalonic acid; SAH, S-adenosyl homocysteine; SAM, S-adenosyl methionine; tHcy, total homocysteine.

2.2 Blood collection

Blood samples were collected by venous puncture in tubes containing an anticoagulant EDTA and in tubes without anticoagulant. The collected blood in dry tubes was allowed to clot for 30 minutes at room temperature and samples were centrifuged at 4,000 x g and 4°C for 10 minutes. Blood collected in anticoagulated tubes was centrifuged within 30 minutes at 4,000 x g and 4°C for 10 minutes. Serum and plasma were immediately separated, divided into aliquots, and stored at -70°C until analysis. For the SAH and SAM measurement, 500 µL EDTA plasma was acidified using 50 µL 1 N acetic acid and mixed thoroughly, since SAM has to be immediately stabilized by acidifying the sample to avoid the degradation to SAH. For quality control in all UPLC-MS/MS methods, pool samples of serum and EDTA plasma were prepared.

2.3 Materials

2.3.1 Equipments and chemicalss

Lists of the chemicals and equipments used are provided in **Appendix A** and **Appendix B**.

General equipments

- Aurius CE2041 Spectrophotometer (CECIL Instruments Ltd., Cambridge, UK)
- Eppendorf centrifuge 5810 R, A-4-62 Rotor (Eppendorf AG, Hamburg, Germany)
- Eppendorf concentrator 5301 (Eppendorf AG)
- Hettich Mikro 20 centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany)
- RM5-40 Horizontal Mixer (Bennett Scientific Ltd., Newton Abbot, UK)
- Schott Intruments Lab 870 pH meter and N 6000 A electrode (SI Analytics GmbH, Mainz, Germany)
- Thermo Scientific Haake Open-Bath Circulators C10-W19 (Thermo Fischer Scientific, Waltham, USA)
- VARIOMAG Monotherm Heatable Magnetic Stirrer (VARIOMAG-USA, Daytona Beach, USA)
- Waters Acquity UPLC coupled to a MicroMass Quattro Premier XE (Waters Corporation, Milford, USA)

General chemicals

- Acetic acid (glacial; >99.99%; Sigma Aldrich, Munich, Germany)
- Acetonitrile (ULC/MS grade; Biosolve, Valkenswaard, The Netherlands)
- Ammonium acetate (ULC/MS grade; Biosolve)
- Ammonia solution analaR NORMAPUR (25%; VWR International GmbH, Darmstadt, Germany)
- Chloroform (Merck)
- Formic acid (ULC/MS grade; Biosolve)
- Isopropanol (ULC/MS grade; Biosolve)
- Methanol (ULC/MS grade; Biosolve)
- MTBE tert-Butyl methyl ether (for HPLC; >99.8%; Chromasolv)
- Water (18.2 MΩ; Milli-Q water purification system; Millipore, Molsheim, France)

2.4 Methods

2.4.1 Ultra-performance liquid chromatography tandem mass spectrometry

Liquid chromatography technique is frequently used in analytical chemistry and biochemistry. In 2004, UPLC technology was introduced by Waters Corporation. The using of smaller particles that are efficiently packed into columns enhances the column efficiency and enables improved results in resolution, speed, and sensitivity. The coupling of a LC system to a mass spectrometer (MS) **(Figure 12)**, which is known as LC-MS leads to a more sensitive and far more specific detection of the analytes. For mass spectrometric analysis the molecules are ionized and later on sorted and identified according to the *m/z* ratios (149;150).

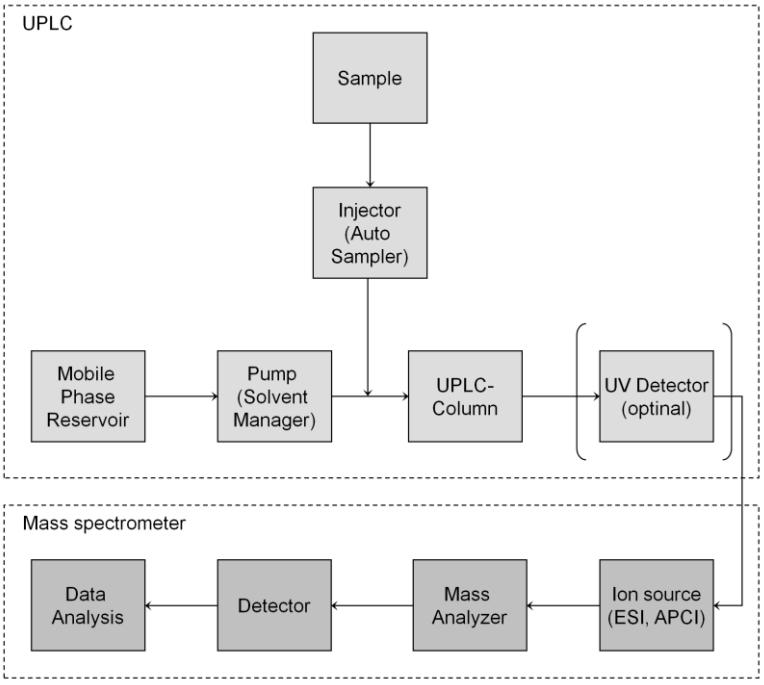

Figure 12. Schematic representation of an UPLC system coupled to a mass spectrometer. The sample is injected via injector device into the UPLC system and separated by the UPLC column. The mobile phase is pumped at high pressure through the column. After separation of the analytes, the sample is ionized in the ion source via ESI or APCI. Identification occurs by means of the mass-to-charge (m/z) ratio. Using a triple quadrupole, a specific analyte can be structurally investigated by fragmentation using a collision gas, creating precursor and product ions. ESI, electrospray; APCI, atmospheric-pressure chemical ionization.

Tandem MS, also known as MS/MS employs two stages of mass analysis in order to examine selectively the fragmentation of particular ions in a mixture of ions. The most widely used MS/MS instrument is the triple quadrupole. It consists of three sets of quadrupoles in series. MS 1 and MS 2 are used as mass analyzers, In between there is a non mass-resolving quadrupole

that used as a collision cell where the fragmentation and focusing of ions is carried out **(Figure 13).** Finally, the streams of sorted ions pass from the analyzer to the detector, which records the relative abundance of each ion type (149;150).

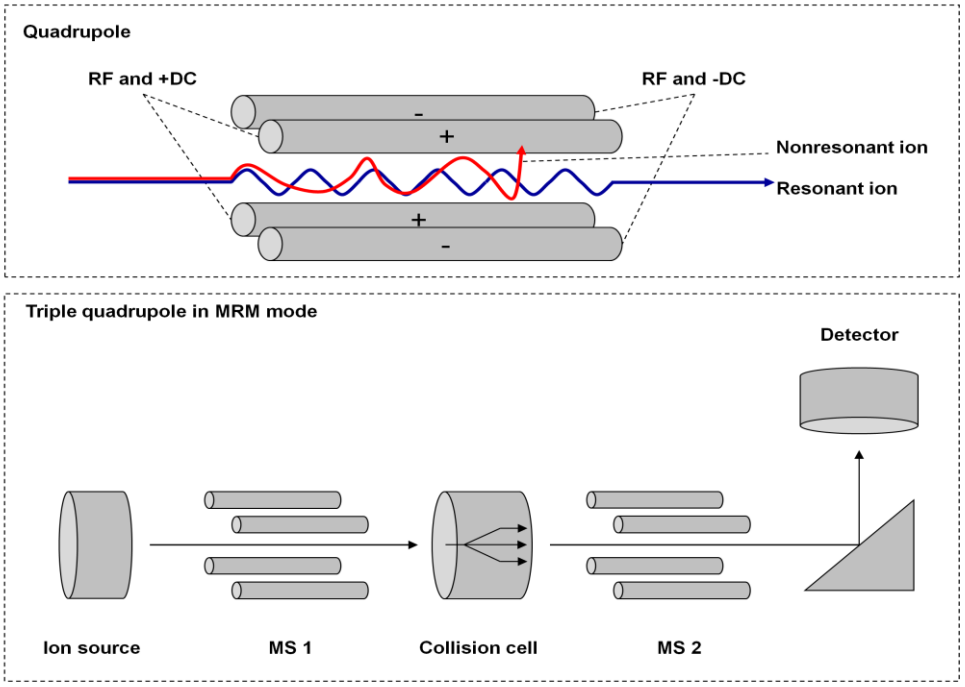

Figure 13. Assembly and functionality of a single quadrupole and a triple quadrupole in multiple reaction monitoring mode of a mass spectrometer. The quadrupole consists of four parallel metallic rods; one pair is at a positive electrical potential, the other one at a negative potential. Constant DC and RF voltages are applied. Ions of a given m/z ratio will resonate and can be detected for a given amplitude of the RF and DC voltages, while nonresonant ions hit the rods. The triple quadrupole consists of three sets of quadrupoles (MS 1, collision cell, and MS 2) in series. In multiple reaction monitoring (MRM) mode, a selected precursor-ion is isolated in MS 1, fragmented in the collision cell, and a selected product-ion is detected in MS 2.

The quantification of analytes using UPLC-MS/MS involves the comparison of the intensity of the analyte signal in a sample with that obtained from standards containing known amounts of the analyte. The internal standard is added as early as possible in the same concentration, correcting the possible loss of analyte during the sample preparation or the sample inlet. Internal standards generally match the analyte of interest as closely as possible but not completely. Stable isotopes labeled analogues of the analytes contain unusual isotopes (e.g. replacing hydrogen with deuterium) in their chemical composition. Since the stable isotopes and the analytes have different masses they can be distinguished from each other without difficulty by MS. For all UPLC-MS/MS methods, the data acquisition was performed by MassLynx V4.1 and the QuanLynx software. Calculations and statistics are presented in **Chapter 6.5**.

2.4.1.1Quantification of betaine, choline, and dimethylglycine

Concentrations of betaine, choline, and DMG in plasma samples were measured by using an UPLC-MS/MS method according to Kirsch *et al* (75). The method comprises a protein precipitation using acetonitrile. The preparation of stock solutions, calibrators, and quality control samples were described earlier (75).

Standards and internal standards

- Betaine anhydrous (Sigma Aldrich)
- Choline chloride (Sigma Aldrich)
- *N*,*N*,-dimethylglycine (DMG; Sigma Aldrich)
- *N*,*N*,*N*-trimethyl-d_9-glycine hydrochloride (d_9-betaine; Isotec, Sigma Aldrich)
- Choline chloride-trimethyl-d_9 (d_9-choline; Isotec)
- *N*,*N*-dimethyl-d_6-glycine HCl (d_6-DMG; CDN Isotopes)

Sample preparation

EDTA plasma samples were thawed and vortexed, then centrifuged at 10000×g for 5 min at room temperature. EDTA samples, calibrators, and quality controls (100 µL) were added to a 1.5 mL tube containing 300 µL internal standard mix (10 µmol/L d9-betaine, d9-choline, and d6-DMG) in acetonitrile. The addition of acetonitrile caused protein precipitation and the analytes were extracted by vigorous vortexing of the sample for 30 s. The samples were centrifuged for 5 min at 10,000×g at room temperature; the supernatants were transferred to glass vials. Sealed vials were immediately measured.

2.4.1.2 Quantification of S-adenosyl homocysteine and S-adenosyl methionine

Concentrations of SAH and SAM in acidified EDTA plasma samples were measured by using an UPLC-MS/MS method according to Kirsch *et al* (151). The method comprises a phenylboronic acid-containing SPE procedure. The preparation of stock solutions, calibrators, and quality control samples were described in detail earlier (151).

Solid-phase extraction column

- Varian Bond Elut PBA columns (Varian Inc., Palo Alto, USA)

Standards and internal standards

- *S*-(5’-adenosyl)-*L*-homocysteine, crystalline (SAH; Sigma Aldrich)
- *S*-(5′-adenosyl)-*L*-methionine *p*-toluenesulfonate salt, from yeast (SAM; Sigma Aldrich)

- *S*-adenosyl-*L*-methionine-d_3-tetra(*p*-toluenesulfonate) salt ([$^2H_3$]-SAM; CDN Isotopes, Quebec, Canada)
- *S*-(5'-adenosyl)-*L*-homocysteine ([$^{13}C_5$]-SAH; Henkjan Gellekink group, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands)

Sample preparation

Acidified plasma samples were thawed and centrifuged at 2000×g for 5 min at 4 °C. 500 µL of the supernatant was neutralized with 50 µL of 1 mol/L ammonia to a pH of 7.4-7.5. 25 µL internal standard solution mix (concentrations: 3.0µmol/L [$^2H_3$]-SAM and 0.82µmol/L [$^{13}C_5$]-SAH, leading to final concentrations of 195 nmol/L [$^2H_3$]-SAM and 64 nmol/L [$^{13}C_5$]-SAH) was added. Sample cleanup was performed with solid-phase extraction (SPE) columns containing phenylboronic acid (Varian Bond Elut PBA columns, Varian Inc., USA). Before loading the samples, the SPE columns were preconditioned with 1 mL of aqueous acetic acid (pH 2.636), then 5×1 mL of 20 mmol/L aqueous ammonium acetate (pH 7.4). Columns were centrifuged at 500×g for 1 min at 4 °C after each washing step. Each sample was then loaded on top of the cartridges and centrifuged at 250×g (slow start) for 2 min at 4 °C. Water-soluble impurities were removed by washing the column with two 1-mL volumes of 20 mmol/L ammonium acetate, pH 7.4 (500 g, 1 min, 4 °C). Finally, SAM and SAH were eluted from the column using 3×350 µL of aqueous acetic acid (pH 2.636) with a centrifugation step every time at 250×g for 2 min at 4 °C (slow start). The eluates were stored at -70 °C until analysis on UPLC-MS/MS system.

2.4.1.3 Quantification of phospholipid

Concentrations of phospholipid classes (PtdCho, PtdEth, Lyso-PtdCho and SM) in EDTA plasma samples were measured by using an UPLC-MS/MS method according to Rabagny *et al* (152). The method comprises a lipid extraction procedure (153) , followed by sample separation and detection using an UPLC-MS/MS system. The preparation of stock solutions, calibrators, and quality control samples were described in detail earlier (152).

Standards and internal standards

- 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (PtdCho 28:0; Sigma Aldrich)
- 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (PtdCho 36:2; Avanti)
- N-nervonoyl-D-*erythro*-sphingosylphosphorylcholine (SM 24:1; Avanti)
- N-palmitoyl-D-*erythro*-sphingosylphosphorylcholine (SM 16:0; Avanti)

- 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (PtdEth 36:2; Avanti)
- 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (PtdEth 16:0/16:0; Sigma Aldrich)
- 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso-PtdCho 18:0; Avanti)
- 1-palmitoyl-*sn*-glycero-3-phosphocholine (Lyso-PtdCho 16:0; Sigma Aldrich)
- 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine (PtdCho 15:0/15:0; Avanti)
- N-hexanoyl-D-*erythro*-sphingosylphosphorylcholine (SM 6:0; Avanti)
- 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine (PtdEth 15:0/15:0; Avanti)
- 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso-PtdCho 17:0; Avanti)

Sample preparation

EDTA plasma samples were thawed, and plasma lipids were extracted using MTBE according to Matyash *et al* (153). Fifty microliters of an internal standard mix consisting of 3 mmol/L PtdCho 15:0/15:0, 1 mmol/L SM 6:0, 200 µmol/L PtdEth 15:0/15:0, and 2 mmol/L Lyso-PtdCho 17:0 was added into a clean dry glass tube and dried under N2 gas. Plasma, calibrator or control samples (25 µL), and methanol (200 µL) were added to the dried internal standard and mixed thoroughly. MTBE (650 µL) was added and incubated with the samples for 15 min on a horizontal shaker followed by adding water (650 µL), mixing, and incubation at room temperature for 10 min in the dark. This mix was then centrifuged for 10 min at 5,000×g and 20°C. The upper organic phase was transferred to a 1.5-mL glass vial. The extraction step with MTBE was repeated on the remaining aqueous phase after the first extraction, and the final extract was vacuum dried at 45°C. The dried lipids were resuspended in 500 µL methanol and immediately measured.

2.4.2 Gas chromatography tandem mass spectrometry

2.4.2.1 Quantification of homocysteine, cystathionine, and methylmalonic acid

The quantification of tHcy, Cys, and MMA was performed by a modified GC-MS method according to Allen *et al* and Stabler *et al* (154;155). Cys and tHcy were simultaneously determined in serum. The GC-MS system consisted of a HP GC System, 6890 Series, G 1530 with a HS 5973 Mass Selective Detector, G 1099AX and a HP-5ms GC column (cross-linked 5% PH ME siloxane; 30 m x 0.25 mm x 0.25 µm; Agilent Technologies, Waldbronn, Germany).

Solid-phase extraction

- Poly-prep chromatography columns, 0.8 x 4 cm
- Anionic resin: AG MP-1, 100 – 200 mesh, chloride form (Bio-RAD)

Internal standards

- Methyl-d3-malonic acid (d3-MMA; CDN Isotopes)
- DL-homocystine-3,3,3',3',4,4,4',4'-d8 (d8-Hcy; Cambridge Isotope Laboratories Inc., Andover, USA)
- DL-(2-amino-2-carboxyethyl)-homocysteine-3,3,4,4-d4 (d4-Cys; CDN Isotopes)

Additional chemicals and equipment

- Acetic acid, glacial EMPROVE (Merck Chemicals)
- Dithiothreitol (AppliChem GmbH, Darmstadt, Germany)
- Hydrochloric acid (Merck Chemicals)
- N-methyl-N-tert-butyldimethylsilyltrifluoro-acetamid (MBDSTFA; Machery and Nagel, Düren, Germany)
- Sodium hydroxide (Merck Chemicals)
- 5 mL glass tubes (5-SV – EPA Screw Top Vials; Chromacol)
- Panasonic NN-5256 microwave (Panasonic Deutschland GmbH, Hamburg, Germany)

Column preparation

Anionic resin was washed with 1 N HCl, then methanol. The resin was left to dry for 1-2 days at 50°C. Poly-prep columns were packed with ~100 mg anionic resin and 1 mL methanol was added.

Methylmalonic acid assay

In 5 mL glass tubes, 1 mL water, 200 µL sample, and 50 µL d_3-MMA (1.635 nmol/L) were added. Methanol was removed from the poly-prep columns and columns were conditioned with 3.3 mL water. Preparated samples were loaded using glass Pasteur pipettes. The columns were washed with 3 mL water and 3 x 3 mL of 0.01 N acetic acid/methanol solution. The samples were eluted using 800 µL elution solution (4 N acetic acid/1 N HCl (9:1, v/v)).

Total homocysteine and cystathionine assay

In 5 mL glass tubes, 1 mL water, 200 µL sample, 15 µL d_8-Hcy (392 µmol/L), 20 µL d_4-Cys (413.36 pmol/50 µL), and 30 µL DTT (10 mg/mL in 1 N NaOH; freshly prepared) were added. DTT is necessary for releasing the protein bound Hcy. After vortexing, the samples were incubated at 45°C for 35 minutes for the reduction of disulfides of homocystine to Hcy. The methanol was removed from the poly-prep columns and the columns were conditioned with 1 mL methanol and 800 µL water. Preparated samples were loaded using glass Pasteur pipettes. The

columns were washed with 3 x 3 mL water and 3 mL methanol. The samples were eluted in a glass vial using 1.1 mL elution solution (0.4 N acetic acid in methanol).

Derivatization of the analytes

Eluted samples from both assays were taken to dryness at 60°C in an Eppendorf concentrator. Dried samples were derivatized with 20 µL acetonitrile and 10 µL MBDSTFA. After vortexing, the samples were treated in the microwave at 440 Watts for 5 minutes. The samples were vortexed and analyzed by GC-MS. For both assays, serum pool samples were included at each batch of samples. The reference ranges for the analytes determined by this method were: tHcy < 12 µmol/L, Cys < 301 nmol/L, and MMA ≤ 271 nmol/L.

2.4.3 Quantification of total folate

The quantification of total folate was performed using a chemiluminescent microparticle immunoassay performed on the ARCHITECT *i* System (Abbott). After the release of folate from endogenous folate binding protein by traetment with dithiothreitol. Folate Binding Protein (FBP) coated paramagnetic microparticles is added. Folate present in the sample binds to the FBP coated microparticles. After washing, pteroic acid-acridinium labeled conjugate is added and binds to unoccupied sites on the FBP-coated microparticles. Following hydrogen peroxide and sodium hydroxide (pre-trigger and trigger solutions) are then added to the reaction mixture; the resulting chemiluminescent reaction is measured as relative light units. An inverse relationship exists between the amount of folate in the sample and the relative light units detected by the ARCHITECT *i* optical system. The assay has a linearity from 1.6 to 20 ng/mL, and a measuring interval from 1.5 to 20.0 ng/mL. Specimens with a folate serum value exceeding 20.0 ng/mL are diluted using the automated dilution procedure. Limit of detection (LOD) was 0.5 ng/mL (1.1 nmol/L), and the limit of quantitation (LOQ) was 1.5 ng/mL (3.4 nmol/L). The interassay CV was ≤ 12% for serum samples from 3.5 to 20 ng/mL, and the Standard Deviation (SD) ≤ 0.42.

2.4.4 Determination of routine parameters

The blood count was determined from EDTA WB either by the SYSMEX SF 3000 or the SYSMEX XE 5000 platform (SYSMEX Germany GmbH, Norderstedt, Germany). From lithium-heparin plasma following parameters were determined using the automated Roche/Hitachi Cobas® 8000 modular analyzer (Roche Diagnostics GmbH): creatinine, uric acid, urea, glucose, glomerular filtration rate (GFR), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), gamma glutamyl transferase (gamma-GT), Alkaline phosphatase (ALP), C-reactive

protein (CRP). Total PSA in serum was determined using the automated Roche/Hitachi Cobas® 8000 modular analyzer (Roche Diagnostics GmbH). Reference ranges for the routine parameters are summarized in **Appendix C**.

2.5 Calculations and statistical analyses

For all UPLC-MS/MS assays, calibrators were used for the construction of a standard curve by plotting the response (y = area analyte/area internal standard) against the corresponding concentrations (c) of the calibrators. The slope (m) and the intercept (i) of the standard curve are used for calculating the concentration of the unknown sample:

$$Concentration\ of\ the\ unknown\ analyte = \frac{y - i}{m} \qquad (1)$$

Recovery [%] was calculated as:

$$Recovery\ [\%] = \frac{Measured\ concentration}{Expected\ \ concentration + concentration\ added} \times 100 \qquad (2)$$

Quantification of tHcy, Cys, and MMA was carried out using following equations:

$$Concentration\ tHcy = \frac{Area\ sample \times concentration\ internal\ \ standard}{Area\ internal\ \ standard} \times 39.2 \qquad (3)$$

$$Concentration\ Cys = \frac{Area\ sample \times concentration\ internal\ \ standard}{Area\ internal\ \ standard} \times 1{,}000 \qquad (4)$$

$$Concentration\ MMA = \frac{Area\ sample \times concentration\ internal\ \ standard}{Area\ internal\ \ standard} \times 4{,}087.5 \qquad (5)$$

Statistics

Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences, version 19.0). ANOVA and the Tamhane-T test were used for testing possible differences in the means of continuous variables between several groups. The differences in continuous variables between two independent groups were tested by the Mann-Whitney-U test and those in categorical variables by the Chi square test. The correlation analyses were performed by using the Spearman-Rho test. All tests used were 2-sided and p values ≤ 0.05 were considered to be statistically significant.

3. Results

The study included 152 patients and 89 controls. According to the exclusion criteria, 23 participants (7 patients, 16 controls) were excluded from the study: 9 had renal failure, 2 had advanced liver disease, 2 used vitamin B supplements, one patient used methotrexate, and 9 controls had co-existing cancers. Since data indicated that the metabolic phenotype showed a strong age-dependent variation, as well as an altered hormone status with aging (156-158). For instance, elderly men showed higher choline and betaine levels than younger men (159). Furthermore, early-onset PCa exhibits important biological differences compared to late-onset PCa (160). Therefore, we divided the study population in two groups according to age for statistical comparison of variables (young, ≤65years; elderly, >66 years).

3.1 Young group

65 patients [median (10^{th} – 90^{th} percentiles) age = 60.2 (53.0-64.1) years] and 22 controls 60.5 (53.0-64.6) years were compared. The main characteristics and routine markers for younger participants are presented in **Table 7**. There were no significant differences in routine markers according to age between patients and controls. The concentrations of PSA were significantly higher in patients with PCa compared to those with BPH (median 8.1 vs. 3.6 ng/mL, p= 0.001) in the younger participants.

Table 7: Characteristic and routine markers of the prostate cancer patients and controls (age ≤65 years)

Variable	Benign prostatic hyperplasia	Prostate cancer	p
Number	22	65	-
Age, years	60.5 (53.0-64.6)	60.2 (52.6-64.1)	0.359
GFR, ml/min	76.3 (57.9-106.7)	83.7 (68.9-100.7)	0.061
Glucose, mg/dl	92 (81-142)	96 (77-116)	0.792
GOT, U/L	25 (20-38)	25 (18-38)	0.822
GPT, U/L	26 (19-43)	28 (19-51)	0.497
gamma GT, U/L	31 (17-69)	32 (17-80)	0.594
Alk phosphatase, U/L	66 (44-84)	64 (45-77)	0.353
Hb, g/dl	14.7 (13.7-16.8)	15.1 (13.9-15.9)	0.484
Hct, %	43 (40-49)	43 (40-46)	0.646
MCV, fl	89 (81-95)	87 (84-94)	0.301
CRP, mg/L	1.1 (0.4-8.1)	1.5 (0.4-4.7)	0.615
PSA, ng/mL	3.6 (0.7-12.0)	8.1 (3.8-34.3)	**0.001**

The data are medians (10^{th} – 90^{th} percentiles).
p values are according to the Mann-Whitney-U test.

Results

Concentrations of plasma and serum choline biomarkers of the young group are presented in **Table 8**. Choline, betaine, and DMG concentrations were lower in PCa patients, but without significant differences. Only SAM showed significant difference and was lower in PCa patients compared to controls (median 112 vs. 127 nmol/L, p=0.046).

Table 8: Metabolite concentrations of the prostate cancer patients and controls (age ≤65 years)

Analyte	Benign prostatic hyperplasia	Prostate cancer	p
Number	22	65	-
Betaine, µmol/L	40 (29-62)	36 (25-53)	0.074
Choline, µmol/L	9.6 (6.6-12.7)	8.4 (6.0-11.5)	0.080
DMG, µmol/L	3.9 (2.4-6.3)	3.1 (2.4-5.4)	0.084
Choline/betaine ratio	0.24 (0.15-0.34)	0.24 (0.15-0.35)	0.632
Betaine/DMG ratio	10.2 (7.2-16.3)	10.8 (6.3-16.8)	0.755
Total folate, nmol/L	16.1 (8.8-27.9)	18.4 (9.3-34.2)	0.099
MMA, nmol/L	192 (143-566)	194 (124-276)	0.470
tHcy, µmol/L	15.4 (10.1-19.7)	13.7 (10.8-19.3)	0.476
Cys, nmol/L	280 (149-1048)	222 (143-481)	0.062
SAH, nmol/L	16.1 (7.0-27.9)	14.7 (10.4-21.3)	0.770
SAM, nmol/L	127 (100-168)	112 (91-152)	**0.046**
SAM/SAH ratio	7.8 (4.8-15.5)	7.6 (6.2-10.9)	0.482

The data are medians (10^{th} – 90^{th} percentiles).
p values are according to the Mann-Whitney-U test.

Plasma concentrations of phospholipid classes without consideration of statin use are summarized in **Table 9**. Median concentrations of SM 14:0 and 20:0 were significantly higher in the young patients compared to controls (10.8 vs. 9.4 µmol/L, p=0.033 and 16.2 vs. 13.6 µmol/L, p= 0.004), respectively.

Table 9: Phospholipid classes and species of the prostate cancer patients and controls (age ≤65 years, without consideration of statin use)

Analyte	Benign prostatic hyperplasia	Prostate cancer	p
Number	22	65	-
PtdCho, µmol/L			
PtdCho 32:1	12.8 (3.1-29.9)	14.4 (3.5-29.5)	0.615
PtdCho 34:1	267.3 (211.6-444.1)	282.3 (205.4-381.9)	0.961
PtdCho 34:2	522.0 (409.3-703.6)	526.2 (415.2-671.5)	0.946
PtdCho 34:3	11.6 (7.2-22.8)	13.3 (6.7-22.2)	0.703
PtdCho 36:2	294.3 (235.4-360.6)	289.1 (207.9-361.5)	0.482
PtdCho 36:3	126.7 (97.7-171.2)	127.0 (97.7-173.7)	0.755

Table 9: Phospholipid classes and species of the prostate cancer patients and controls (age ≤65 years, without consideration of statin use)

Analyte	Benign prostatic hyperplasia	Prostate cancer	p
PtdCho 36:4	175.3 (137.0-244.2)	182.7 (129.2-244.7)	0.755
PtdCho 36:5	24.5 (13.5-45.4)	24.3 (11.5-43.8)	0.938
PtdCho 38:3	38.3 (24.7-60.3)	39.5 (23.5-61.1)	0.488
PtdCho 38:4	126.2 (106.9-197.9)	125.5 (89.0-177.7)	0.682
PtdCho 38:5	63.3 (42.2-83.0)	60.7 (45.7-86.2)	0.977
PtdCho 38:6	62.4 (37.6-101.6)	64.2 (42.9-103.8)	0.899
Sum of PtdCho	1716 (1417-2255)	1789 (1413-2291)	0.899
Lyso-PtdCho, µmol/L			
Lyso-PtdCho 16:0	88.9 (57.4-125.9)	88.5 (61.4-113.5)	0.876
Lyso-PtdCho 18:0	24.1 (17.1-34.2)	23.4 (14.7-35.0)	0.807
Lyso-PtdCho 18:1	20.5 (13.1-28.3)	18.9 (11.4-26.9)	0.565
Lyso-PtdCho 18:2	20.3 (14.2-36.2)	19.7 (11.1-32.3)	0.407
Lyso-PtdCho 20:4	5.2 (3.6-7.0)	4.9 (1.5-8.2)	0.612
Sum of Lyso-PtdCho	160 (111-207)	156 (112-206)	0.625
PtdEth, µmol/L			
PtdEth 34:1	2.2 (1.0-4.4)	2.1 (0.8-3.8)	0.558
PtdEth 34:2	1.6 (0.8-2.9)	1.4 (0.6-3.1)	0.283
PtdEth 36:2	5.5 (2.3-10.2)	4.9 (2.3-8.9)	0.401
PtdEth 36:3	2.2 (1.0-4.5)	2.4 (0.9-4.6)	0.660
PtdEth 36:4	2.0 (1.0-4.0)	2.0 (1.1-4.2)	0.977
PtdEth 38:4	2.8 (1.2-4.7)	2.8 (1.1-5.2)	0.838
PtdEth 38:5	3.3 (2.0-6.9)	3.5 (1.6-5.8)	0.675
PtdEth 38:6	2.7 (1.6-5.1)	2.7 (1.3-5.8)	0.876
PtdEth 40:5	0.7 (0.3-1.8)	1.0 (0.5-2.2)	0.069
Sum of PtdEth	25.0 (12.7-41.1)	23.6 (11.8-40.8)	0.605
SM, µmol/L			
SM 14:0	9.4 (5.5-14.1)	10.8 (8.3-16.0)	**0.033**
SM 16:0	88.1 (68.8-123.1)	94.5 (69.4-115.4)	0.458
SM 16:1	12.9 (10.2-17.7)	14.0 (10.9-18.2)	0.056
SM 18:0	18.5 (13.0-26.2)	20.4 (14.6-28.6)	0.105
SM 18:1	7.8 (4.6-10.7)	8.1 (5.5-12.0)	0.109
SM 20:0	13.6 (9.6-21.5)	16.2 (12.1-21.0)	**0.004**
SM 22:0	23.8 (16.3-34.1)	26.2 (19.6-34.8)	0.103
SM 22:1	17.5 (13.0-27.6)	20.7 (15.0-25.7)	0.051
SM 23:0	10.8 (6.7-14.9)	11.4 (8.3-15.2)	0.105
SM 23:1	8.6 (6.4-14.6)	10.6 (6.8-13.7)	0.059
SM 23:2	5.2 (3.4-8.1)	5.0 (3.2-8.0)	0.807
SM 24:0	18.2 (11.1-24.7)	20.8 (14.0-27.2)	0.133
SM 24:1	49.6 (35.3-73.0)	52.4 (39.1-73.6)	0.211
SM 24:2	20.1 (16.2-29.0)	22.3 (16.0-31.1)	0.423
Sum of SM	302 (239-437)	333 (254-414)	0.072

The data are medians (10^{th} – 90^{th} percentiles).
p values are according to the Mann-Whitney-U test.

3.2 Elderly group

The elderly group consisted of 80 patients [median (10^{th} – 90^{th} percentiles) age = 70.7 (66.6-77.8)] years and 51 controls [median (10^{th} – 90^{th} percentiles) age = 73.9 (66.6-82.1) years]. The main characteristics and routine markers are presented in **Table 10**. Age showed a significant difference between patients and controls (p=0.011). The concentrations of PSA showed significant differences between patients with PCa and patients with BPH (median 6.4 vs. 3.4 ng/mL, p<0.001). Concentrations of Hct and MCV were significantly lower in patients compared to controls (42 vs. 43% and 88 vs. 90 fl, p= 0.038 and 0.034), respectively.

Table 10: Characteristic and routine markers of the prostate cancer patients and controls (age >65 years)

Variable	Benign prostatic hyperplasia	Prostate cancer	p
Number	51	80	-
Age, years	73.9 (66.6-82.1)	70.7 (66.6-77.8)	**0.011**
GFR, ml/min	72.4 (55.2-97.0)	74.2 (55.1-92.9)	0.770
Glucose, mg/dl	96 (80-156)	100 (80-139)	0.662
GOT, U/L	24 (17-33)	25 (17-34)	0.522
GPT, U/L	25 (16-41)	26 (17-39)	0.715
gamma GT, U/L	34 (18-69)	30 (18-68)	0.545
Alk phosphatase, U/L	61 (46-93)	62 (42-87)	0.458
Hb, g/dl	15.0 (13.3-16.0)	14.6 (12.8-16.1)	0.350
Hct, %	43 (39-47)	42 (37-46)	**0.038**
MCV, fl	90 (82-95)	88 (82-92)	**0.034**
CRP, mg/L	1.7 (0.4-9.2)	1.2 (0.4-6.8)	0.119
PSA, ng/ml	3.4 (0.9-8.1)	6.4 (1.3-15.8)	**<0.001**

The data are medians (10^{th} – 90^{th} percentiles).
p values are according to the Mann-Whitney-U test.

Concentrations of plasma and serum biomarkers in the elderly group are presented in **Table 11**. Choline, betaine, and DMG concentrations in PCa patients were comparable with those of the controls, and did not show significant differences. MMA concentrations were significantly lower in PCa patients compared to controls (median 203 vs. 228 nmol/L, p= 0.025). After adjusting for age, the difference in MMA between patients and controls became non-significant (p=0.939).

Table 11: Metabolite concentrations of the prostate cancer patients and controls (age >65 years)

Analyte	Benign prostatic hyperplasia	Prostate cancer	p [a]	p [b]
Number	51	80	-	-
Betaine, µmol/L	41 (25-60)	40 (26-59)	0.661	
Choline, µmol/L	9.7 (6.9-13.8)	10.0 (6.6-14.8)	0.944	
DMG, µmol/L	3.6 (2.6-6.1)	3.7 (2.5-5.7)	0.895	
Choline/betaine ratio	0.25 (0.16-0.35)	0.25 (0.17-0.36)	0.887	
Betaine/DMG ratio	10.6 (6.8-15.6)	10.7 (7.6-16.7)	0.861	
Total folate, nmol/L	17.4 (10.0-39.2)	19.9 (9.3-35.7)	0.819	0.925
MMA, nmol/L	228 (171-390)	203 (140-401)	**0.025**	0.939
tHcy, µmol/L	16.0 (11.3-25.8)	16.2 (11.4-22.0)	0.730	0.649
Cys, nmol/L	312 (185-754)	314 (173-927)	0.781	0.634
SAH, nmol/L	18.8 (10.0-32.4)	18.2 (11.7-29.9)	0.494	0.805
SAM, nmol/L	137 (105-179)	128 (101-188)	0.174	0.982
SAM/SAH ratio	7.4 (4.4-12.6)	6.9 (5.1-9.8)	0.681	0.309

The data are medians (10^{th} – 90^{th} percentiles).
a p values are according to the Mann-Whitney-U test.
b p values are according to Multivariable analysis, adjusted for age.

Phospholipid concentrations in elderly group without consideration of statin use are summarized in **Table 12**. Contrary to the young group, SM 14:0 did not exhibit a significant difference between patients and controls (p=0.182). On the other hand, SM 16:0, 18:0, 18:1, 20:0, 22:0, 23:0, 23:1, and 24:1 were significantly lower in patients compared to controls. After adjusting for age, the differences remained significant for these SM species. In addition, the differences in SM 22:1 (p: 0.116→0.034), 24:0 (p: 0.052→0.007), and 24:2 (p: 0.058→0.019) between patients and controls became significant after adjusting for age.

Table 12: Phospholipid classes and species of the prostate cancer patients and controls (age >65 years, without consideration of statin use)

Analyte	Benign prostatic hyperplasia	Prostate cancer	p [a]	p [b]
Number	51	80	-	-
PtdCho, µmol/L				
PtdCho 32:1	11.9 (5.4-22.6)	11.7 (2.5-30.6)	0.962	
PtdCho 34:1	248.4 (194.5-334.7)	246.7 (181.2-371.7)	0.674	
PtdCho 34:2	489.3 (377.1-655.2)	489.6 (351.7-636.6)	0.388	
PtdCho 34:3	11.2 (7.2-17.6)	10.6 (5.2-21.0)	0.582	
PtdCho 36:2	264.0 (192.5-340.0)	257.0 (170.6-329.3)	0.902	
PtdCho 36:3	110.4 (84.6-147.6)	115.7 (85.7-152.0)	0.552	
PtdCho 36:4	161.9 (117.1-220.5)	167.9 (131.9-224.4)	0.124	

Table 12: Phospholipid classes and species of the prostate cancer patients and controls (age >65 years, without consideration of statin use)

Analyte	Benign prostatic hyperplasia	Prostate cancer	p [a]	p [b]
PtdCho 36:5	20.3 (8.7-48.2)	22.7 (8.7-57.0)	0.661	
PtdCho 38:3	34.3 (22.8-54.1)	35.7 (22.0-50.5)	0.947	
PtdCho 38:4	117.4 (77.0-155.4)	118.7 (86.1-160.5)	0.293	
PtdCho 38:5	59.3 (42.5-84.6)	61.3 (42.4-95.9)	0.336	
PtdCho 38:6	68.4 (38.7-103.0)	68.0 (48.1-109.0)	0.872	
Sum of PtdCho	1634 (1294-1981)	1610 (1236-2094)	0.865	
Lyso-PtdCho, µmol/L				
Lyso-PtdCho 16:0	83.7 (52.7-123.0)	75.9 (52.7-107.8)	0.143	
Lyso-PtdCho 18:0	22.0 (14.6-30.7)	19.7 (13.9-28.0)	0.155	
Lyso-PtdCho 18:1	16.8 (10.6-25.7)	17.5 (11.1-25.6)	0.759	
Lyso-PtdCho 18:2	18.0 (10.2-28.6)	17.5 (10.6-29.6)	0.966	
Lyso-PtdCho 20:4	4.7 (2.1-7.4)	4.1 (2.1-6.9)	0.395	
Sum of Lyso-PtdCho	156 (96-205)	138 (93-188)	0.333	
PtdEth, µmol/L				
PtdEth 34:1	1.8 (0.8-4.4)	1.6 (0.6-3.9)	0.247	
PtdEth 34:2	1.5 (0.3-2.5)	1.3 (0.4-2.4)	0.249	
PtdEth 36:2	4.3 (1.7-9.0)	4.0 (2.0-8.3)	0.865	
PtdEth 36:3	2.1 (0.9-3.9)	1.9 (0.7-4.8)	0.614	
PtdEth 36:4	2.0 (0.5-4.2)	1.7 (0.7-3.2)	0.574	
PtdEth 38:4	2.6 (1.2-6.5)	2.3 (1.3-4.6)	0.555	
PtdEth 38:5	3.4 (1.2-7.1)	3.0 (1.3-6.8)	0.234	
PtdEth 38:6	3.4 (1.0-6.7)	2.5 (1.2-6.6)	0.167	
PtdEth 40:5	0.9 (0.2-2.2)	0.9 (0.3-2.3)	0.955	
Sum of PtdEth	21.5 (10.0-47.9)	19.4 (10.2-40.0)	0.350	
SM, µmol/L				
SM 14:0	10.4 (6.8-14.4)	9.4 (6.6-14.8)	0.182	0.676
SM 16:0	93.5 (71.2-121.0)	83.2 (61.0-103.7)	**0.001**	**<0.001**
SM 16:1	13.8 (9.8-16.5)	12.7 (6.6-16.7)	0.246	0.297
SM 18:0	21.0 (15.5-26.9)	18.6 (14.1-23.3)	**0.002**	**<0.001**
SM 18:1	8.1 (5.7-11.5)	7.5 (5.0-10.2)	**0.040**	**0.018**
SM 20:0	15.5 (10.4-20.9)	14.2 (9.7-18.5)	**0.034**	**0.008**
SM 22:0	23.6 (17.0-31.4)	21.2 (15.1-28.1)	**0.017**	**0.002**
SM 22:1	19.0 (13.7-24.0)	17.9 (13.0-22.9)	0.116	**0.034**
SM 23:0	11.1 (7.0-14.7)	9.3 (6.0-12.7)	**0.005**	**0.001**
SM 23:1	10.5 (7.7-13.0)	8.9 (6.4-11.8)	**0.002**	**0.001**
SM 23:2	4.9 (3.2-7.0)	4.7 (3.1-6.7)	0.970	0.931
SM 24:0	16.8 (11.2-23.2)	15.2 (10.8-20.1)	0.052	**0.007**
SM 24:1	50.9 (37.5-67.2)	46.5 (33.9-61.4)	**0.018**	**0.006**
SM 24:2	21.9 (15.6-29.0)	19.6 (13.5-27.8)	0.058	**0.019**
Sum of SM	324 (241-399)	297 (218-361)	**0.005**	**0.001**

The data are medians (10^{th} – 90^{th} percentiles). [a] p values are according to the Mann-Whitney-U test. [b] p values are according to ANOVA test, adjusted for age.

3.3 Comparison of phospholipid and metabolite concentrations between controls, low-grade, and high-grad prostate cancer

To assess the relationship between the pathologic grade and the metabolic biomarker, we divided PCa patients for statistical comparisons according to Gleason score into two groups [low-grade (≤3+4) and high-grade (≥4+3)]. We tested the differences in the metabolite concentrations in the young and elderly groups.

3.3.1 In the young group

The young group consisted of 62 patients (40 low-grade and 22 high-grade PCa), and 22 controls. The metabolite concentrations are presented in **Tables 13, 14**. PSA showed a significant difference either between controls and low-grade (mean 5.2 vs. 10.3 ng/mL, p=0.001) or between controls and high-grade PCa (mean 5.2 vs. 21.1 ng/mL, p=0.001). There were no significant differences associated to statin users distribution between the three groups according to Chi-square test (p=0.421). Mean concentrations of SM 14:0 and 20:0 were significantly higher in low-grade PCa compared to controls (12.1 vs. 10.0 µmol/L, p=0.035 and 16.6 vs. 14.1 µmol/L, p= 0.027), respectively. A similar observation has been shown between patients and controls in young group (age ≤ 65 years, without consideration of statin use) **(Table 9).**

Table 13: Comparison of metabolite concentrations between controls, low-grade, and high-grad prostate cancer (age ≤65 years)

Analyte	Controls	Prostate cancer		p [a]
		low-grade (≤3+4)	high-grad (≥4+3)	
Number	22	40	22	-
Betaine, µmol/L	43 (13.3)	37 (11.0)	41 (13.6)	ns
Choline, µmol/L	9.4 (2.1)	8.3 (2.0)	8.8 (2.4)	ns
Choline/betaine ratio	0.23 (0.07)	0.24 (0.06)	0.23 (0.07)	ns
Total folate, nmol/L	7.4 (3.5)	9.5 (3.9)	9.9 (6.7)	ns
MMA, nmol/L	252 (183)	205 (137)	207 (56)	ns
tHcy, µmol/L	16.2 (7.9)	14.2 (3.4)	15.5 (6.0)	ns
Cys, nmol/L	434 (413)	318 (490)	309 (177)	ns
SAH, nmol/L	16.6 (8.4)	14.4 (4.1)	15.8 (4.0)	ns
SAM, nmol/L	128 (25.3)	110 (17.3) [c (p=0.012)]	126 (29.6)	ns
SAM/SAH ratio	9.9 (6.6)	8.2 (2.2)	8.2 (1.9)	ns
PSA, ng/ml	5.2 (4.5)	10.3 (8.2) [c]	21.1 (25.0) [c]	**0.001**

The data are mean (SD). [a] p values are according to ANOVA and Tamhane-T tests unless otherwise specified. [b] p value is according to chi-square test. [c] significant differences with controls. ns, non-significant.

Table 14: Comparison of phospholipid classes and species between controls, low-grade, and high-grad prostate cancer (age ≤65 years)

Analyte	Controls	Prostate cancer		p [a]
		low-grade (≤3+4)	high-grad (≥4+3)	
Number	22	40	22	-
Statins user n (%)	3 (13.6%)	6 (15%)	5 (22.7%)	0.421 [b]
PtdCho, µmol/L				
PtdCho 32:1	15.0 (10.6)	15.0 (11.3)	15.9 (6.8)	ns
PtdCho 34:1	285.8 (81.3)	279.5 (73.2)	272.0 (42.6)	ns
PtdCho 34:2	534.7 (94.4)	535.5 (86.9)	506.5 (104.2)	ns
PtdCho 34:3	13.5 (6.3)	13.4 (5.9)	13.1 (4.5)	ns
PtdCho 36:2	298.0 (50.8)	291.2 (48.6)	268.8 (66.0)	ns
PtdCho 36:3	130.0 (27.4)	133.5 (26.2)	122.0 (22.1)	ns
PtdCho 36:4	182.2 (35.4)	185.7 (46.3)	181.8 (41.1)	ns
PtdCho 36:5	26.3 (13.1)	29.0 (18.2)	22.2 (9.2)	ns
PtdCho 38:3	40.1 (12.7)	42.6 (14.2)	38.9 (11.3)	ns
PtdCho 38:4	134.2 (32.1)	131.7 (37.3)	127.0 (32.5)	ns
PtdCho 38:5	64.0 (15.5)	67.3 (19.6)	57.3 (10.8)	ns
PtdCho 38:6	65.8 (20.4)	72.7 (29.9)	61.1 (15.8)	ns
Sum of PtdCho	1790 (297)	1797 (294)	1687 (265)	ns
Lyso-PtdCho, µmol/L				
Lyso-PtdCho 16:0	87.6 (22.1)	89.8 (22.5)	82.5 (16.8)	ns
Lyso-PtdCho 18:0	24.6 (6.7)	24.9 (7.4)	22.4 (6.0)	ns
Lyso-PtdCho 18:1	20.3 (5.9)	20.0 (7.5)	18.4 (6.4)	ns
Lyso-PtdCho 18:2	22.3 (8.1)	21.0 (8.6)	20.8 (10.8)	ns
Lyso-PtdCho 20:4	5.2 (1.3)	4.9 (2.9)	5.5 (2.1)	ns
Sum of Lyso-PtdCho	160 (35.1)	161 (43.1)	150 (36.8)	ns
PtdEth, µmol/L				
PtdEth 34:1	2.5 (1.3)	2.2 (1.3)	2.2 (1.0)	ns
PtdEth 34:2	1.7 (0.8)	1.5 (1.0)	1.4 (0.8)	ns
PtdEth 36:2	5.8 (2.8)	5.4 (2.9)	5.0 (3.7)	ns
PtdEth 36:3	2.5 (1.2)	2.4 (1.5)	2.3 (1.2)	ns
PtdEth 36:4	2.2 (1.0)	2.3 (1.5)	2.2 (1.0)	ns
PtdEth 38:4	2.9 (1.4)	2.9 (1.3)	2.9 (1.5)	ns
PtdEth 38:5	3.8 (1.7)	3.8 (2.2)	3.3 (1.4)	ns
PtdEth 38:6	3.0 (1.4)	3.2 (1.9)	3.0 (1.6)	ns
PtdEth 40:5	1.0 (0.7)	1.2 (0.8)	1.0 (0.5)	ns
Sum of PtdEth	25.5 (9.4)	24.9 (12.4)	23.4 (10.4)	ns
SM, µmol/L				
SM 14:0	10.0 (3.6)	12.1 (3.4) [c (p=0.09)]	10.4 (2.6)	**0.035**
SM 16:0	92.6 (17.0)	95.7 (17.0)	90.5 (19.5)	0.522
SM 16:1	13.3 (2.5)	14.8 (2.9)	13.4 (2.6)	0.070
SM 18:0	18.9 (4.7)	20.7 (4.9)	21.0 (5.0)	0.304
SM 18:1	7.3 (2.1)	8.2 (2.3)	8.3 (2.5)	0.303
SM 20:0	14.1 (4.2)	16.6 (3.0) [c (p=0.06)]	15.7 (3.1)	**0.027**
SM 22:0	24.5 (6.7)	27.3 (5.4)	25.3 (5.3)	0.155

Table 14: Comparison of phospholipid classes and species between controls, low-grade, and high-grad prostate cancer (age ≤65 years)

Analyte	Controls	Prostate cancer		p [a]
		low-grade (≤3+4)	high-grad (≥4+3)	
SM 22:1	18.8 (4.8)	21.0 (3.5)	19.3 (4.3)	0.086
SM 23:0	10.5 (2.9)	11.8 (2.5)	10.8 (2.4)	0.103
SM 23:1	9.5 (3.1)	10.8 (2.3)	9.7 (3.0)	0.115
SM 23:2	5.3 (1.5)	5.3 (1.8)	5.4 (1.7)	0.954
SM 24:0	18.6 (5.5)	20.5 (4.8)	19.9 (5.1)	0.387
SM 24:1	51.0 (12.1)	55.1 (10.5)	51.6 (13.7)	0.354
SM 24:2	21.6 (4.2)	23.5 (5.2)	20.7 (5.7)	0.101
Sum of SM	316 (65.3)	343 (55.4)	322 (65.9)	0.186

The data are mean (SD). [a] p values are according to ANOVA and Tamhane-T tests unless otherwise specified. [b] p value is according to chi-square test. [c] significant differences with controls. ns, non-significant.

3.3.2 In the elderly group

The elderly group consisted of 72 patients (50 low-grade and 22 high-grade PCa) and 51 controls. PSA was higher in low-grade PCa compared to controls (mean 7.8 vs. 4.4 ng/mL, p=0.001). There were no significant differences in the prevalence of statin users between the three groups according to Chi-square test (p=0.863). Mean concentrations of SM 16:0 and 18:0 were significantly lower either between controls and low-grade or between controls and high-grade PCa. Whereas, SM 18:1, 22:0, 23:0, and 23:1 were significantly lower in low-grade patients compared to the controls **(Tables 15, 16).**

Table 15: Comparison of metabolite concentrations between controls, low-grade, and high-grad prostate cancer (age >65 years)

Analyte	Controls	Prostate cancer		p [a]
		low-grade (≤3+4)	high-grad (≥4+3)	
Number	51	50	22	-
Betaine, µmol/L	42 (14.4)	43 (11.9) [d (p=0.03)]	36 (10.0)	ns
Choline, µmol/L	10.2 (2.6)	10.3 (3.1)	9.8 (2.5)	ns
Choline/betaine ratio	0.26 (0.08)	0.25 (0.07)	0.28 (0.07)	ns
Total folate, nmol/L	9.4 (5.4)	10.5 (5.2) [d (p=0.034)]	7.6 (3.6)	ns
MMA, nmol/L	258 (93)	247 (154)	232 (114)	ns
tHcy, µmol/L	17.4 (5.5)	16.7 (5.0)	17.3 (5.7)	ns
Cys, nmol/L	418 (302)	396 (330)	412 (265)	ns
SAH, nmol/L	20.9 (9.7)	20.0 (7.3)	18.7 (5.9)	ns
SAM, nmol/L	142 (32.8)	136 (35.3)	135 (36.7)	ns
SAM/SAH ratio	7.7 (2.9)	7.3 (2.0)	7.6 (2.2)	ns
PSA, ng/ml	4.4 (3.9)	7.8 (5.3) [c]	9.8 (9.8)	**0.001**

The data are mean (SD). [a] p values are according to ANOVA and Tamhane-T tests unless otherwise specified. [b] p value is according to chi-square test. [c] significant differences with controls. [d] significant differences between low- and high-grad. ns, non-significant.

Table 16: Comparison of phospholipid classes and species between controls, low-grade, and high-grad prostate cancer (age >65 years)

Analyte	Controls	Prostate cancer		p [a]
		low-grade (≤3+4)	high-grad (≥4+3)	
Number	51	50	22	-
Statins user n (%)	15 (29.4%)	20 (40%)	6 (27.3%)	0.863 [b]
PtdCho, µmol/L				
PtdCho 32:1	12.8 (6.4)	12.1 (9.4)	14.6 (8.8)	ns
PtdCho 34:1	258.2 (54.9)	247.9 (70.4)	262.3 (54.5)	ns
PtdCho 34:2	503.0 (102.9)	475.6 (99.8)	512.4 (80.3)	ns
PtdCho 34:3	11.7 (4.2)	11.0 (5.6)	11.9 (5.4)	ns
PtdCho 36:2	259.9 (56.1)	252.9 (57.8)	266.9 (35.8)	ns
PtdCho 36:3	115.5 (22.5)	113.3 (25.5)	126.8 (21.4)	ns
PtdCho 36:4	162.4 (39.2)	167.9 (37.5)	184.0 (50.1)	ns
PtdCho 36:5	25.5 (18.2)	26.0 (19.4)	28.3 (23.0)	ns
PtdCho 38:3	36.8 (11.3)	33.3 (10.5)	40.7 (10.3)	ns
PtdCho 38:4	116.5 (30.5)	118.5 (30.6)	127.3 (35.4)	ns
PtdCho 38:5	60.5 (16.5)	62.0 (17.1)	64.8 (19.5)	ns
PtdCho 38:6	73.3 (25.7)	69.3 (20.6)	74.7 (26.6)	ns
Sum of PtdCho	1636 (264)	1590 (295)	1715 (264)	ns
Lyso-PtdCho, µmol/L				
Lyso-PtdCho 16:0	85.0 (24.2)	80.6 (21.4)	77.0 (19.8)	ns
Lyso-PtdCho 18:0	22.2 (6.2)	21.2 (5.7)	19.4 (4.3)	ns
Lyso-PtdCho 18:1	17.5 (5.5)	18.6 (5.9)	16.6 (5.1)	ns
Lyso-PtdCho 18:2	18.8 (6.9)	20.3 (6.7)	17.4 (5.9)	ns
Lyso-PtdCho 20:4	4.6 (1.8)	4.6 (1.8)	3.9 (1.9)	ns
Sum of Lyso-PtdCho	148 (40.5)	145 (35.4)	134 (33.7)	ns
PtdEth, µmol/L				
PtdEth 34:1	2.2 (1.4)	1.9 (1.3)	1.7 (1.4)	ns
PtdEth 34:2	1.5 (0.9)	1.5 (0.9)	1.1 (0.8)	ns
PtdEth 36:2	5.0 (3.1)	4.6 (2.3)	4.7 (2.9)	ns
PtdEth 36:3	2.4 (1.4)	2.4 (1.5)	2.0 (1.6)	ns
PtdEth 36:4	2.1 (1.4)	1.9 (0.8)	1.8 (1.5)	ns
PtdEth 38:4	3.0 (1.9)	2.7 (1.1)	2.5 (1.5)	ns
PtdEth 38:5	4.2 (2.8)	3.5 (2.0)	3.3 (2.5)	ns
PtdEth 38:6	3.7 (2.4)	3.1 (1.8)	3.3 (3.3)	ns
PtdEth 40:5	1.1 (0.9)	1.0 (0.6)	1.1 (1.0)	ns
Sum of PtdEth	25.2 (13.5)	22.6 (10.4)	21.5 (14.7)	ns
SM, µmol/L				
SM 14:0	10.5 (2.6)	10.1 (3.6)	10.7 (3.3)	0.681
SM 16:0	95.0 (17.8)	82.9 (16.4) [c]	85.6 (13.3) [c]	**0.001**
SM 16:1	13.5 (3.1)	12.5 (2.8)	13.9 (2.8)	0.102
SM 18:0	21.2 (4.5)	18.6 (3.5) [c]	18.4 (3.1) [c]	**0.002**
SM 18:1	8.4 (2.3)	7.4 (1.9) [c]	7.5 (1.9)	**0.035**
SM 20:0	15.5 (3.5)	14.2 (3.1)	14.4 (2.7)	0.088
SM 22:0	23.8 (5.2)	21.4 (4.7) [c]	22.0 (4.4)	**0.045**

Table 16: Comparison of phospholipid classes and species between controls, low-grade, and high-grad prostate cancer (age >65 years)

Analyte	Controls	Prostate cancer		p [a]
		low-grade (≤3+4)	high-grad (≥4+3)	
SM 22:1	19.1 (3.8)	17.6 (3.6)	18.4 (3.8)	0.162
SM 23:0	10.9 (3.0)	9.4 (2.3) [c]	9.5 (2.6)	**0.012**
SM 23:1	10.5 (2.2)	9.3 (2.2) [c]	9.3 (2.1)	**0.012**
SM 23:2	4.9 (1.6)	4.9 (1.7)	5.0 (1.5)	0.985
SM 24:0	16.9 (4.3)	15.3 (3.6)	16.1 (4.3)	0.117
SM 24:1	51.0 (10.3)	46.3 (10.2)	46.6 (9.6)	0.052
SM 24:2	22.1 (4.9)	19.8 (5.3)	21.0 (4.9)	0.092
Sum of SM	322 (57.5)	290 (52.4) [c]	298 (50.4)	**0.008**

The data are mean (SD). [a] p values are according to ANOVA and Tamhane-T tests unless otherwise specified. [b] p value is according to chi-square test. [c] significant differences with controls.

3.4 Choline metabolites and SM species according to age

SM concentrations increased with age in controls. These age changes were reversed in patients group, therefore, we tested the differences of metabolite concentrations in controls and patients groups according to age.

3.4.1 In the controls

This group consisted of 22 young and 51 elderly participants (median age 60.5 vs. 73.9, p<0.001). The main characteristics and metabolites values are presented in **Table 17**. No significant difference of PSA levels between young and elderly were observed. MMA was significantly lower in elderly compared to young group (median 171 vs. 192 nmol/L, p=0.025).

Table 17: Metabolite concentrations in the controls according to age

Analyte	age ≤65 years	age >65 years	p
Number	22	51	-
Age, years	60.5 (53.0-64.6)	73.9 (66.6-82.1)	**<0.001**
Betaine, µmol/L	40 (29-61)	41 (25-60)	0.857
Choline, µmol/L	9.6 (6.6-12.8)	9.7 (6.9-13.8)	0.342
DMG, µmol/L	3.9 (2.4-6.3)	3.6 (2.8-6.1)	0.639
Choline/betaine ratio	0.24 (0.15-0.34)	0.25 (0.16-0.35)	0.279
Betaine/DMG ratio	10.2 (7.2-16.3)	10.6 (6.8-15.6)	0.971
Total folate, nmol/L	17.2 (10.0-33.3)	16.3 (7.9-62.1)	0.107
MMA, nmol/L	192 (143-566)	171 (228-390)	**0.025**
tHcy, µmol/L	15.4 (10.1-19.7)	16.0 (11.3-25.8)	0.186
Cys, nmol/L	280 (149-1048)	312 (185-754)	0.501
SAH, nmol/L	16.1 (7.0-27.9)	18.8 (10.0-32.4)	0.075
SAM, nmol/L	127 (100-168)	137 (105-179)	0.092
SAM/SAH ratio	7.8 (4.8-15.5)	7.4 (4.4-12.6)	0.186
PSA, ng/ml	3.6 (0.7-12.0)	3.4 (0.9-8.1)	0.696

The data are medians (10^{th} – 90^{th} percentiles). p values are according to the Mann-Whitney-U test.

Results

There were no significant differences associated to statin users distribution between the controls according to Chi-square test (p=0.237). Although most SM species were higher in elderly group, only SM 23:1 showed a significant difference compared to the young group (median 10.5 vs. 8.6 µmol/L, p=0.046) **(Table 18).**

Table 18: SM species in the controls according to age

Analyte	age ≤65 years	age >65 years	p [a]
Statins user n (%)	3 (13.6%)	15 (29.4%)	0.237 [b]
SM, µmol/L			
SM 14:0	9.4 (5.5-14.1)	10.4 (6.8-14.4)	0.414
SM 16:0	88.1 (68.8-123.1)	93.5 (71.2-130.0)	0.486
SM 16:1	12.9 (10.2-17.7)	13.8 (9.8-16.5)	0.631
SM 18:0	18.5 (13.0-26.2)	21.0 (15.5-26.9)	0.068
SM 18:1	7.8 (4.6-10.7)	8.1 (5.7-11.5)	0.086
SM 20:0	13.8 (9.6-21.5)	15.5 (10.4-20.9)	0.068
SM 22:0	23.8 (16.3-34.1)	23.6 (17.0-31.4)	0.773
SM 22:1	17.5 (13.0-27.6)	19.0 (13.7-24.0)	0.580
SM 23:0	10.8 (6.7-14.9)	11.1 (7.0-14.7)	0.456
SM 23:1	8.6 (6.4-14.6)	10.5 (7.7-13.0)	**0.046**
SM 23:2	5.2 (3.4-8.1)	4.9 (3.2-7.0)	0.414
SM 24:0	18.2 (11.1-24.7)	16.8 (11.2-23.2)	0.248
SM 24:1	49.6 (35.3-73.0)	50.9 (37.5-67.2)	0.847
SM 24:2	20.1 (16.2-29.0)	21.9 (15.6-29.0)	0.674
Sum of SM	302 (239-437)	324 (241-399)	0.449

The data are medians (10^{th} – 90^{th} percentiles). [a] p values are according to the Mann-Whitney-U test unless otherwise specified. [b] p value is according to chi-square test.

3.4.2 In the patients

The PCa group contained 65 young and 80 elderly patients (median age 60.2 vs. 70.7, p<0.001). The main characteristics and metabolites concentrations are presented in **Table 19**. Choline was significantly higher in elderly group compared to young group (median 10.0 vs. 8.4 µmol/L, p=0.001). In addition, tHcy, Cys, SAH, and SAM showed significant differences and were higher in elderly group compared to young group **(Table 19)**.

There were no significant differences in the prevalence of statin users between the groups according to Chi-square test (p=0.010). In contrary to the observation in the controls, all SM species, except SM 23:2 were significantly lower in elderly group compared to young group **(Table 20)**. After adjusting for statin use the differences in SM concentrations between the young and elderly patients became non-significant.

Table 19: Metabolite concentrations in patients according to age

Analyte	age ≤65 years	age >65 years	p
Number	65	80	-
Age, years	60.2 (52.6-64.1)	70.7 (66.6-77.8)	**<0.001**
Betaine, µmol/L	36 (25-53)	40 (26-59)	0.129
Choline, µmol/L	8.4 (6.0-11.5)	10.0 (6.6-14.8)	**0.001**
DMG, µmol/L	3.1 (2.4-5.4)	3.7 (2.5-5.7)	0.052
Choline/betaine ratio	0.24 (0.15-0.35)	0.25 (0.17-0.36)	0.191
Betaine/DMG ratio	10.8 (6.3-16.8)	10.7 (7.6-16.7)	0.735
Total folate, nmol/L	18.3 (9.3-34.1)	19.8 (9.3-35.7)	0.982
MMA, nmol/L	194 (124-276)	203 (140-401)	0.070
tHcy, µmol/L	13.7 (10.8-19.3)	16.2 (11.4-22.0)	**0.004**
Cys, nmol/L	221 (143-481)	314 (173-926)	**<0.001**
SAH, nmol/L	14.7 (10.4-21.3)	18.2 (11.7-29.9)	**<0.001**
SAM, nmol/L	112 (91-152)	128 (101-188)	**<0.001**
SAM/SAH ratio	7.6 (6.2-10.9)	6.9 (5.1-9.8)	**0.014**
PSA, ng/ml	8.1 (3.8-34.3)	6.4 (1.3-15.8)	**0.029**

The data are medians (10^{th} – 90^{th} percentiles).
p values are according to the Mann-Whitney-U test.

Table 20: SM species in the patients according to age

Analyte	age ≤65 years	age >65 years	p [a]	p [c]
Statins user n (%)	11 (16.9%)	29 (36.3%)	**0.010** [b]	
SM, µmol/L				
SM 14:0	10.8 (8.3-16.0)	9.4 (6.6-14.8)	**0.001**	0.324
SM 16:0	94.5 (69.4-115.4)	83.2 (61.0-103.7)	**<0.001**	0.713
SM 16:1	14.0 (10.9-18.2)	12.7 (9.6-16.7)	**0.002**	0.891
SM 18:0	20.4 (14.6-28.6)	18.6 (14.1-23.3)	**0.007**	0.344
SM 18:1	8.1 (5.5-12.0)	7.5 (5.0-10.2)	**0.043**	0.636
SM 20:0	16.2 (12.1-21.0)	14.2 (9.7-18.5)	**<0.001**	0.563
SM 22:0	26.2 (19.6-34.8)	21.2 (15.1-28.1)	**<0.001**	0.591
SM 22:1	20.7 (15.0-25.7)	17.9 (13.0-22.9)	**<0.001**	0.937
SM 23:0	11.4 (8.3-15.2)	9.3 (6.0-12.7)	**<0.001**	0.750
SM 23:1	10.6 (6.8-13.7)	8.9 (6.4-11.8)	**0.002**	0.622
SM 23:2	5.0 (3.2-8.0)	4.7 (3.1-6.7)	0.083	0.756
SM 24:0	20.8 (14.0-27.2)	15.2 (10.8-20.1)	**<0.001**	0.306
SM 24:1	52.4 (39.1-73.6)	46.5 (33.9-61.4)	**<0.001**	0.514
SM 24:2	22.3 (16.0-31.1)	19.6 (13.5-27.8)	**0.013**	0.366
Sum of SM	333 (254-414)	297 (218-361)	**<0.001**	0.604

The data are medians (10^{th} – 90^{th} percentiles). [a] p values are according to the Mann-Whitney-U test unless otherwise specified. [b] p value is according to chi-square test. [c] p values adjusted for statin use.

3.4.3 In the patients with low- and high-grad PCa

Since several SM species showed significant differences between controls and patients according to Gleason score, especially in low-grad PCa group, we tested the differences of metabolite concentrations in high- and low-grad PCa groups according to age.

3.4.3.1 In the patients with low-grad PCa

The low-grad PCa group consisted of 38 young and 49 elderly participants (median age 59.5 vs. 69.9, p<0.001). The main characteristics and metabolites concentrations are presented in **Table 21**. Choline, betaine, and DMG were significantly higher in elderly compared to young participants. In addition, MMA, tHcy, Cys, SAH, and SAM were also significantly higher in older participants.

Table 21: Metabolite concentrations in low-grad PCa according to age

Analyte	age ≤65 years	age >65 years	p
Number	38	49	-
Age, years	59.5 (49.8-64.0)	69.9 (66.3-75.4)	**<0.001**
Betaine, µmol/L	33 (23-50)	41 (29-61)	**0.022**
Choline, µmol/L	8.3 (5.7-10.6)	10.1 (6.5-15.1)	**0.004**
DMG, µmol/L	3.0 (2.2-4.3)	3.9 (2.7-5.7)	**0.001**
Choline/betaine ratio	0.24 (0.15-0.32)	0.23 (0.17-0.36)	0.620
Betaine/DMG ratio	11.5 (7.7-17.0)	10.7 (7.6-18.0)	0.350
Total folate, nmol/L	21.3 (9.9-33.6)	22.0 (11.1-38.7)	0.506
MMA, nmol/L	177 (119-267)	209 (133-390)	**0.019**
tHcy, µmol/L	13.3 (11.0-18.0)	16.1 (11.4-23.6)	**0.006**
Cys, nmol/L	201 (134-352)	309 (173-723)	**<0.001**
SAH, nmol/L	14.1 (9.2-16.9)	17.7 (11.7-30.7)	**<0.001**
SAM, nmol/L	108 (87-132)	129 (101-191)	**<0.001**
SAM/SAH ratio	7.6 (6.4-11.4)	6.9 (4.8-9.8)	**0.047**
PSA, ng/ml	8.1 (4.3-21.1)	7.0 (1.5-15.5)	0.208

The data are medians (10^{th} – 90^{th} percentiles).
p values are according to the Mann-Whitney-U test.

The prevalence of statin users was higher in the elderly group compared with the young group. Similar to the observation of SM concentrations in the patients according to age, all SM species, except SM 18:0, 18:1; and 23:2 were significantly lower in the elderly group compared to the young group **(Table 22)**. After adjusting for statin use the differences of SM concentrations between the two groups became non-significant, except SM 24:0 (p=0.049). In addition, the difference related to SM 18:0 became significant (p=0.031).

Table 22: SM species in low-grad PCa according to age

Analyte	age ≤65 years	age >65 years	p [a]	p [c]
Statins user n (%)	6 (15.8%)	20 (40.8%)	**0.011** [b]	
SM, µmol/L				
SM 14:0	11.8 (8.3-16.2)	9.4 (6.5-14.3)	**0.002**	0.404
SM 16:0	94.5 (70.4-113.8)	82.2 (58.1-109.1)	**0.001**	0.182
SM 16:1	14.1 (11.0-17.9)	12.6 (9.2-15.9)	**<0.001**	0.616
SM 18:0	19.8 (15.1-28.4)	18.5 (13.9-22.9)	0.075	**0.031**
SM 18:1	8.1 (5.6-11.7)	7.4 (4.8-10.0)	0.142	0.084
SM 20:0	16.7 (12.6-21.4)	14.3 (9.7-18.5)	**0.001**	0.179
SM 22:0	26.5 (19.3-34.8)	21.2 (15.0-27.6)	**<0.001**	0.189
SM 22:1	20.6 (16.6-25.2)	18.1 (12.4-22.0)	**<0.001**	0.276
SM 23:0	11.5 (8.6-15.5)	9.6 (6.0-12.7)	**<0.001**	0.264
SM 23:1	10.8 (8.1-13.4)	9.2 (6.1-11.8)	**0.003**	0.396
SM 23:2	5.0 (3.1-7.4)	4.6 (3.1-7.0)	0.239	0.370
SM 24:0	20.9 (13.4-26.1)	15.1 (11.0-20.0)	**<0.001**	**0.049**
SM 24:1	52.4 (43.1-69.6)	46.5 (33.3-61.6)	**<0.001**	0.077
SM 24:2	22.7 (16.9-31.1)	19.5 (13.5-27.2)	**0.002**	0.067
Sum of SM	337 (259-418)	297 (210-359)	**<0.001**	0.091

The data are medians (10^{th} – 90^{th} percentiles). [a] p values are according to the Mann-Whitney-U test unless otherwise specified. [b] p value is according to chi-square test. [c] p values adjusted for statin use.

3.4.3.2 In the patients with high-grad PCa

The high-grad PCa group consisted of 21 young 19 elderly participants (median age 60.3 vs. 72.3, p<0.001). The main characteristics and metabolites values are presented in **Table 23**. No significant differences in metabolite concentrations between young and elderly were observed.

Table 23: Metabolite concentrations in high-grad PCa according to age

Analyte	age ≤65 years	age >65 years	p
Number	21	19	-
Age, years	60.3 (54.3-64.2)	72.3 (66.6-77.8)	**<0.001**
Betaine, µmol/L	38 (26-65)	32 (23-52)	0.173
Choline, µmol/L	8.1 (6.9-13.6)	9.9 (7.2-10.9)	0.074
DMG, µmol/L	3.6 (2.7-6.9)	3.2 (2.3-6.4)	0.222
Choline/betaine ratio	0.24 (0.12-0.31)	0.27 (0.23-0.41)	**0.037**
Betaine/DMG ratio	10.6 (5.6-17.1)	10.4 (4.9-13.7)	0.814
Total folate, nmol/L	18.3 (9.3-37.7)	14.7 (9.3-30.1)	0.238
MMA, nmol/L	229 (134-290)	186 (141-431)	0.944
tHcy, µmol/L	14.6 (9.3-20.9)	16.5 (11.4-21.5)	0.133
Cys, nmol/L	223 (140-670)	316 (149-937)	0.139
SAH, nmol/L	14.7 (10.8-21.0)	17.5 (11.2-24.5)	0.060
SAM, nmol/L	116 (91-173)	121 (106-164)	0.336
SAM/SAH ratio	8.8 (5.8-10.9)	7.2 (5.0-11.3)	0.213
PSA, ng/ml	11.6 (4.6-41.4)	6.8 (1.2-26.0)	0.062

The data are medians (10^{th} – 90^{th} percentiles). p values are according to the Mann-Whitney-U test.

The prevalence of statin users was not significantly different according to age in the group of high-grad PCa patients. Only SM 22:0 and 24:0 were significantly lower in elderly group compared to young group **(Table 24).**

Table 24: SM species in high-grad PCa according to age

Analyte	**age ≤65 years**	**age >65 years**	**p** [a]
Statins user n (%)	5 (23.8%)	6 (31.6%)	0.731 [b]
SM, µmol/L			
SM 14:0	10.0 (6.0-13.8)	9.6 (7.5-15.5)	0.707
SM 16:0	91.4 (64.2-117.6)	88.0 (61.7-102.0)	0.398
SM 16:1	13.5 (9.7-17.9)	12.9 (10.2-18.3)	0.814
SM 18:0	20.8 (13.1-27.2)	19.0 (13.8-22.1)	0.064
SM 18:1	8.2 (4.6-11.9)	7.6 (5.0-10.3)	0.302
SM 20:0	15.1 (10.8-20.6)	14.6 (11.4-18.5)	0.189
SM 22:0	25.5 (16.7-32.5)	21.8 (14.9-29.1)	**0.033**
SM 22:1	18.7 (13.8-26.8)	19.1 (13.6-23.1)	0.639
SM 23:0	10.6 (7.3-14.3)	9.8 (6.1-12.6)	0.133
SM 23:1	9.5 (6.1-15.1)	10.0 (6.4-11.7)	0.869
SM 23:2	4.8 (4.1-8.5)	5.3 (2.5-6.8)	0.725
SM 24:0	18.7 (13.9-27.2)	16.9 (10.6-24.0)	**0.039**
SM 24:1	50.7 (34.3-75.4)	49.2 (31.7-59.5)	0.302
SM 24:2	20.6 (13.4-31.0)	22.1 (12.7-28.2)	0.725
Sum of SM	320 (235-409)	304 (214-357)	0.302

The data are medians (10^{th} – 90^{th} percentiles). [a] p values are according to the Mann-Whitney-U test unless otherwise specified. [b] p value is according to chi-square test.

3.5 Choline metabolites and phospholipid classes according to statin use

Since we found significant difference for SM species, especially in the elderly group, we examined the association of statin use in controls and patients groups.

3.5.1 In the controls

This group consisted of 18 statin users and 55 non-statin users (median age 71.8 vs. 69.5, p=0.219). The main characteristics and metabolites concentrations are presented in **Table 25**. No significant differences between statin users and non-statin users were observed.

Table 25: Metabolite concentrations in the controls according to statin use

Analyte	non-statin users	statin users	p
Number	55	18	-
Age, years	69.5 (56.0-80.5)	71.8 (63.2-82.8)	0.219
Betaine, µmol/L	40 (27-57)	41 (25-65)	0.556
Choline, µmol/L	9.7 (6.8-12.9)	9.9 (6.5-14.1)	0.556
DMG, µmol/L	3.6 (2.6-5.7)	4.4 (2.6-7.7)	0.481
Choline/betaine ratio	0.25 (0.16-0.35)	0.23 (0.15-0.38)	0.980
Betaine/DMG ratio	10.6 (7.0-16.7)	9.9 (6.2-14.8)	0.711
Total folate, nmol/L	17.2 (10.0-33.3)	16.3 (7.9-62.1)	0.673
MMA, nmol/L	215 (164-355)	218 (140-509)	0.908
tHcy, µmol/L	15.6 (10.9-24.4)	16.1 (11.1-37.4)	0.609
Cys, nmol/L	286 (164-908)	323 (180-749)	0.682
SAH, nmol/L	17.7 (8.6-27.4)	20.9 (9.7-49.1)	0.058
SAM, nmol/L	132 (103-177)	136 (103-201)	0.450
SAM/SAH ratio	7.8 (4.8-13.4)	6.7 (3.6-12.0)	0.073

The data are medians (10^{th} – 90^{th} percentiles).
p values are according to the Mann-Whitney-U test.

On the other hand, statin users had lower concentrations of all SM species compared to non-statin users. These decreases were significant except for SM 14:0, 18:0, 18:1, and 20:0 **(Table 26).**

Table 26: Phospholipid classes in the controls according to statin use

Analyte	non-statin users	statin users	p
Number	55	18	-
PtdCho, µmol/L			
PtdCho 32:1	12.3 (5.7-22.9)	11.8 (2.9-24.5)	0.514
PtdCho 34:1	259.4 (213.1-357.0)	241.0 (149.4-365.0)	0.318
PtdCho 34:2	530.4 (407.7-662.1)	461.8 (322.8-563.6)	**0.002**
PtdCho 34:3	11.5 (8.3-20.0)	9.9 (2.4-18.2)	0.131
PtdCho 36:2	291.3 (206.4-353.4)	225.5 (167.1-288.2)	**<0.001**
PtdCho 36:3	118.5 (98.4-158.2)	103.6 (77.0-137.6)	**0.001**

Table 26: Phospholipid classes in the controls according to statin use

Analyte	non-statin users	statin users	p
PtdCho 36:4	169.1 (124.2-238.1)	163.0 (94.2-238.5)	0.155
PtdCho 36:5	20.2 (9.7-48.6)	23.7 (6.4-45.4)	0.898
PtdCho 38:3	38.5 (24.2-59.2)	31.8 (21.8-41.1)	**0.008**
PtdCho 38:4	121.1 (88.9-185.6)	117.5 (68.0-140.5)	0.060
PtdCho 38:5	61.4 (43.8-84.4)	57.8 (35.0-81.4)	0.163
PtdCho 38:6	68.4 (41.6-104.2)	63.1 (33.0-97.2)	0.420
Sum of PtdCho	1704 (1430-2146)	1513 (1043-1792)	**0.002**
Lyso-PtdCho, µmol/L			
Lyso-PtdCho 16:0	92.6 (58.6-113.9)	77.0 (38.3-129.5)	0.163
Lyso-PtdCho 18:0	23.4 (16.8-32.6)	18.2 (11.0-29.8)	**0.007**
Lyso-PtdCho 18:1	17.9 (11.6-25.2)	15.6 (8.3-27.8)	0.344
Lyso-PtdCho 18:2	19.0 (13.8-30.9)	16.5 (7.8-28.6)	0.094
Lyso-PtdCho 20:4	4.8 (3.0-6.9)	4.0 (1.1-7.6)	0.219
Sum of Lyso-PtdCho	160 (106-206)	133 (72-223)	0.082
PtdEth, µmol/L			
PtdEth 34:1	2.0 (0.9-4.1)	2.1 (0.6-6.3)	0.427
PtdEth 34:2	1.5 (0.5-2.6)	1.7 (0.4-2.6)	0.654
PtdEth 36:2	4.4 (2.0-9.2)	4.8 (1.9-10.8)	0.959
PtdEth 36:3	2.1 (0.9-3.8)	2.4 (1.2-6.5)	0.300
PtdEth 36:4	2.0 (0.7-3.8)	2.1 (0.6-4.2)	0.489
PtdEth 38:4	2.6 (1.1-5.1)	2.7 (1.5-7.2)	0.898
PtdEth 38:5	3.3 (1.3-6.4)	4.3 (1.5-12.0)	0.331
PtdEth 38:6	3.1 (1.1-6.2)	3.4 (1.5-9.7)	0.489
PtdEth 40:5	0.7 (0.2-2.1)	1.3 (0.2-2.9)	0.063
Sum of PtdEth	23.9 (10.5-40.6)	23.0 (10.3-57.5)	0.591
SM, µmol/L			
SM 14:0	10.4 (7.0-14.4)	9.3 (6.4-13.6)	0.073
SM 16:0	94.5 (83.4-123.3)	79.6 (62.6-115.4)	**<0.001**
SM 16:1	13.5 (11.1-17.5)	11.2 (6.8-16.2)	**0.006**
SM 18:0	21.0 (15.0-26.0)	19.2 (15.2-29.7)	0.565
SM 18:1	8.1 (5.2-10.9)	7.3 (5.5-12.5)	0.692
SM 20:0	15.0 (10.5-21.1)	13.2 (9.7-19.6)	0.138
SM 22:0	24.9 (16.9-32.8)	19.8 (13.8-24.9)	**0.001**
SM 22:1	19.4 (14.9-25.5)	16.8 (12.8-22.6)	**0.010**
SM 23:0	11.6 (7.6-15.4)	8.5 (6.4-13.4)	**0.014**
SM 23:1	10.5 (7.3-14.4)	8.5 (6.5-12.7)	**0.043**
SM 23:2	5.2 (3.4-7.1)	3.8 (2.2-7.1)	**0.030**
SM 24:0	18.5 (13.1-24.0)	12.7 (9.8-20.6)	**<0.001**
SM 24:1	52.3 (38.3-71.3)	43.6 (34.6-55.8)	**0.002**
SM 24:2	22.9 (17.8-29.4)	19.0 (13.4-23.5)	**0.002**
Sum of SM	334 (255-411)	271 (230-364)	**0.001**

The data are medians (10^{th} – 90^{th} percentiles).
p values are according to the Mann-Whitney-U test.

3.5.2 In the patients

The PCa group contained 40 statin users and 105 non-statin users (median age 68.6 vs. 64.3, p=0.047). The main characteristics and metabolites concentrations are presented in **Table 27**. In addition to age, MMA, Cys, SAH, and SAM showed significant differences and were higher in the statin users group compared to non-statin users.

Table 27: Metabolite concentrations in the prostate cancer patients according to statin use

Analyte	non-statin users	statin users	p
Number	105	40	-
Age, years	64.3 (54.9-76.2)	68.6 (58.3-75.3)	**0.047**
Betaine, µmol/L	35 (25-57)	41 (30-61)	**0.029**
Choline, µmol/L	8.6 (6.1-13.0)	9.8 (6.9-14.3)	0.105
DMG, µmol/L	3.2 (2.4-5.3)	3.8 (2.6-6.1)	**0.026**
Choline/betaine ratio	0.24 (0.17-0.36)	0.24 (0.17-0.33)	0.757
Betaine/DMG ratio	10.7 (7.5-16.7)	10.9 (6.7-17.5)	0.958
Total folate, nmol/L	18.4 (9.3-32.9)	23.6 (10.4-40.8)	0.110
MMA, nmol/L	195 (124-296)	211 (142-427)	**0.043**
tHcy, µmol/L	14.9 (11.3-20.6)	15.9 (11.1-22.0)	0.335
Cys, nmol/L	242 (150-673)	339 (182-916)	**0.035**
SAH, nmol/L	15.6 (10.8-24.5)	18.2 (13.8-28.8)	**0.002**
SAM, nmol/L	116 (97-160)	133 (104-177)	**0.005**
SAM/SAH ratio	7.3 (5.3-10.7)	6.8 (5.5-9.8)	0.158

The data are medians (10^{th} – 90^{th} percentiles).
p values are according to the Mann-Whitney-U test.

In comparison with SM species in controls groups, all SM species were significantly lower in statin users group, with one exception for SM 18:0 **(Table 28).**

Table 28: Phospholipid classes in the prostate cancer patients according to statin use

Analyte	non-statin users	statin users	p
Number	105	40	-
PtdCho, µmol/L			
PtdCho 32:1	13.5 (3.2-29.5)	11.8 (3.4-33.1)	0.782
PtdCho 34:1	262.0 (199.8-373.9)	246.8 (143.1-372.0)	0.110
PtdCho 34:2	526.3 (421.1-655.3)	423.0 (304.7-601.2)	**<0.001**
PtdCho 34:3	12.6 (5.8-21.3)	10.7 (6.1-17.1)	**0.039**
PtdCho 36:2	281.6 (220.3-356.1)	235.7 (149.2-316.9)	**<0.001**
PtdCho 36:3	125.5 (96.2-168.8)	113.6 (77.5-159.6)	**0.010**
PtdCho 36:4	175.4 (129.2-225.1)	184.1 (141.2-276.4)	**0.033**
PtdCho 36:5	24.3 (9.3-51.8)	22.9 (9.7-47.8)	0.611

Table 28: Phospholipid classes in the prostate cancer patients according to statin use

Analyte	non-statin users	statin users	p
PtdCho 38:3	37.6 (24.0-58.4)	37.5 (20.6-53.6)	0.071
PtdCho 38:4	116.0 (85.9-168.3)	132.9 (92.1-203.4)	**0.024**
PtdCho 38:5	60.7 (44.4-94.9)	64.3 (42.0-93.9)	0.898
PtdCho 38:6	67.4 (43.1+113.2)	62.5 (49.1-87.9)	0.222
Sum of PtdCho	1735 (1378-2186)	1582 (1160-2090)	**0.012**
Lyso-PtdCho, µmol/L			
Lyso-PtdCho 16:0	84.8 (60.2-109.3)	72.4 (52.7-121.7)	**0.004**
Lyso-PtdCho 18:0	22.6 (14.6-30.0)	19.6 (13.0-30.0)	**0.028**
Lyso-PtdCho 18:1	18.4 (11.2-25.8)	17.1 (10.8-26.5)	0.374
Lyso-PtdCho 18:2	19.0 (11.5-30.3)	17.8 (10.1-29.6)	0.476
Lyso-PtdCho 20:4	4.2 (1.6-6.9)	5.4 (2.5-8.2)	**0.018**
Sum of Lyso-PtdCho	149 (106-200)	129 (91-201)	**0.032**
PtdEth, µmol/L			
PtdEth 34:1	1.8 (0.7-3.5)	2.0 (0.8-5.1)	0.386
PtdEth 34:2	1.4 (0.5-3.1)	1.3 (0.6-2.7)	0.901
PtdEth 36:2	4.8 (2.1-9.2)	4.2 (2.2-7.7)	0.465
PtdEth 36:3	2.0 (0.8-4.6)	2.4 (1.1-4.8)	0.408
PtdEth 36:4	1.8 (0.8-3.7)	1.9 (1.0-3.4)	0.195
PtdEth 38:4	2.4 (1.1-4.9)	3.2 (2.0-4.3)	**0.004**
PtdEth 38:5	3.0 (1.5-6.1)	3.7 (1.8-7.1)	**0.040**
PtdEth 38:6	2.6 (1.2-5.7)	2.9 (1.5-6.7)	0.157
PtdEth 40:5	1.0 (0.3-2.2)	1.0 (0.4-2.2)	0.204
Sum of PtdEth	21.2 (10.1-40.3)	23.2 (14.0-41.6)	0.263
SM, µmol/L			
SM 14:0	10.6 (8.0-16.3)	8.6 (5.1-13.4)	**<0.001**
SM 16:0	93.5 (74.7-112.1)	72.5 (57.9-95.8)	**<0.001**
SM 16:1	13.9 (11.1-18.3)	11.5 (8.3-15.6)	**<0.001**
SM 18:0	19.9 (15.0-26.7)	17.8 (13.4-24.9)	**0.004**
SM 18:1	7.9 (5.5-11.1)	7.4 (4.6-10.6)	0.180
SM 20:0	15.7 (11.7-19.7)	13.8 (9.6-18.6)	**0.002**
SM 22:0	24.9 (18.9-32.9)	19.6 (14.0-27.0)	**<0.001**
SM 22:1	20.1 (14.8-25.0)	16.1 (12.4-22.9)	**<0.001**
SM 23:0	10.9 (8.2-14.3)	8.1 (5.7-12.4)	**<0.001**
SM 23:1	10.4 (7.5-13.1)	7.7 (5.4-12.0)	**<0.001**
SM 23:2	5.1 (3.4-7.6)	4.5 (2.6-6.5)	**0.013**
SM 24:0	17.8 (13.2-25.9)	13.8 (10.3-20.8)	**<0.001**
SM 24:1	52.3 (40.7-68.7)	40.2 (29.2-54.0)	**<0.001**
SM 24:2	22.3 (16.4-29.5)	16.8 (12.6-25.4)	**<0.001**
Sum of SM	332 (266-403)	254 (198-333)	**<0.001**

The data are medians (10^{th} – 90^{th} percentiles).
p values are according to the Mann-Whitney-U test.

3.5.3 The interactions of disease and statin use on SM species in the elderly group

Our results showed that statin use has similar lowering effect on SM species concentrations either between controls or patients. In addition, a similar observation was obtained in PCa patients in elderly group. Thereby, we tested for interactions between statin use and PCa disease in elderly group.

The non- user's elderly group consisted of 51 patients and 36 controls. SM species 16:0, 18:0, 23:0, and 23:1were significantly lower in patients compared to controls. However, there is tendency that PCa patients have lowers concentrations of the other SM species compared to controls **(Table 29).**

Table 29: Sphingomyelin species in the non-statin users in the prostate cancer patients and controls (age >65 years)

Analyte	Benign prostatic hyperplasia	Prostate cancer	p
Number	36	51	-
SM, µmol/L			
SM 14:0	10.4 (7.2-14.7)	9.7 (7.2-16.8)	0.343
SM 16:0	96.8 (76.6-123.0)	91.4 (67.4-109.1)	**0.014**
SM 16:1	14.7 (10.5-17.5)	13.2 (9.6-18.2)	0.569
SM 18:0	21.5 (14.4-25.5)	19.7 (14.2-24.1)	**0.040**
SM 18:1	8.2 (5.3-11.0)	7.6 (4.6-10.2)	0.205
SM 20:0	15.6 (10.1-21.1)	14.4 (10.4-18.5)	0.168
SM 22:0	24.9 (16.6-32.1)	22.7 (16.2-28.9)	0.069
SM 22:1	20.1 (13.7-24.4)	19.1 (12.6-23.1)	0.326
SM 23:0	11.8 (6.7-15.7)	9.9 (6.3-12.8)	**0.039**
SM 23:1	10.9 (7.4-13.6)	10.1 (6.8-11.7)	**0.017**
SM 23:2	5.2 (2.0-7.0)	5.2 (2.3-7.0)	0.952
SM 24:0	18.2 (11.4-23.9)	17.0 (10.4-22.8)	0.138
SM 24:1	53.6 (36.9-71.2)	49.3 (35.6-62.3)	0.155
SM 24:2	23.2 (15.8-29.4)	22.0 (13.5-28.4)	0.322
Sum of SM	337 (253-410)	311 (227-374)	0.059

The data are medians (10^{th} – 90^{th} percentiles).
p values are according to the Mann-Whitney-U test.

The stain user's elderly group consisted of 29 patients and 15 controls. Similar to non-stain user's elderly group SM species 18:0, 23:0, and 23:1were significantly lower in patients compared to controls. Whereas, SM 16:0 showed a difference in the same direction and tended to be significant (p= 0.052). Additionally, SM 24:1 showed a significant difference in the same direction **(Table 30).**

Table 30: Sphingomyelin species in the statin users in the prostate cancer patients and controls (age >65 years)

Analyte	Benign prostatic hyperplasia	Prostate cancer	p
Number	15	29	-
SM, µmol/L			
SM 14:0	9.4 (6.3-13.9)	8.6 (5.1-13.5)	0.407
SM 16:0	78.3 (61.9-115.5)	72.8 (57.9-90.3)	**0.050**
SM 16:1	11.3 (6.7-16.3)	11.3 (7.8-14.7)	0.720
SM 18:0	20.1 (14.8-30.7)	16.8 (13.7-21.6)	**0.030**
SM 18:1	7.5 (6.0-13.3)	7.4 (4.6-10.2)	0.141
SM 20:0	15.1 (9.8-20.5)	12.3 (9.4-18.5)	0.116
SM 22:0	21.3 (14.0-26.3)	18.2 (13.3-25.2)	0.141
SM 22:1	16.9 (12.8-23.1)	15.3 (10.6-21.9)	0.281
SM 23:0	8.7 (6.4-13.8)	7.4 (5.5-11.4)	**0.046**
SM 23:1	8.6 (7.4-13.4)	7.5 (5.3-12.0)	**0.019**
SM 23:2	3.7 (2.0-7.3)	4.4 (2.5-6.5)	0.450
SM 24:0	13.4 (9.4-20.7)	12.6 (10.1-16.9)	0.480
SM 24:1	44.3 (35.3-56.8)	40.1 (28.5-50.0)	**0.028**
SM 24:2	20.2 (13.0-23.9)	16.1 (12.5-22.7)	0.128
Sum of SM	278 (223-376)	250 (189-327)	0.069

The data are medians (10^{th} – 90^{th} percentiles).
p values are according to the Mann-Whitney-U test.

4. Discussion

Choline can influence cancer development with several suggested mechanisms; choline, via betaine, is important for choline-mediated one-carbon metabolism and can affect DNA methylation. It also plays a central role in phospholipid metabolism and can modify cell signaling and support structural integrity of cell membranes. One-carbon metabolism consists of a set of interrelated pathways: remethylation (betaine and folate cycle), transmethylation (methionine cycle), and transsulfuration of Hcy via Cys by the cystathionine-β-synthase enzyme. On the other hand, Kennedy and PEMT pathways are involved in choline phospholipid metabolism. Abnormal choline metabolism is a metabolic hallmark associated with tumor onset and progression. We hypothesized that the concentrations of choline and choline metabolites in plasma will be higher in cancer patients than noncancer matched controls. In this case-control study, we investigated for differences in blood concentrations of one-carbon metabolism related metabolites (choline, betaine, DMG, total folate, SAH, SAM, tHcy, Cys, and MMA) and phospholipid metabolites (phospholipid: PtdCho, PtdEth, Lyso-PtdCho and SM) in males with PCa and those with BPH. Furthermore, we studied these metabolites in relations to tumor grade, age, and statin use.

4.1 One-carbon metabolism

4.1.1 One-carbon metabolites in PCa and BPH

Our study found no significant differences in the concentrations of choline, betaine, DMG, total folate, tHcy, Cys, and MMA between cases and controls, in the young or elderly groups **(Table 8 & 11)**. However, all one-carbon metabolism metabolites except of total folate were slightly lower in the PCa patients compared to controls in the young group. On the other hand, choline, DMG, total folate, tHcy, and Cys were slightly higher in the PCa patients compared to controls in the elderly group. MMA was significantly lower in the elderly PCa patients, but this difference became non-significant after adjustment for age **(Table 11)**. Our results do not support differences in plasma concentrations of choline or other one-carbon metabolites between the patients and controls. However, all studies showed that folate and tHcy blood levels were not associated with PCa risk (161-165), whereas circulating MMA concentration showed an inverse association with the risk of PCa (23). Our findings on total folate and tHcy are in accordance with those of some other studies (161-165).

Two studies examined the relation between choline and the risk of PCa. In one case-control study from the Northern Sweden Health and Disease Cohort, there was a positive association

between elevated plasma choline concentrations and an increased risk of PCa (23), while other epidemiological prospective study found that dietary choline intake was associated with an increased risk of lethal PCa (166). Nevertheless, betaine concentration was not associated with PCa risk (23). No studies regarding the association between DMG and cancer risk exist in the literature. Although the Northern Sweden Health and Disease Cohort study showed a positive association between plasma choline levels and PCa risk (23), the differences of choline and betaine concentrations between PCa patients and controls (healthy subjects) were not statistically significant (10.4 vs. 10.0 µmol/L, p=0.07 for choline, and 32.7 vs. 31.4 µmol/L, p=0.18 for betaine), respectively (23). The young group in our study had a similar median age compared with that of the Northern Sweden Health and Disease Cohort study (60.2 vs. 59.8 years), respectively. In comparison, our results for choline and betaine were also non-significant, but on the contrary, we found slightly lower concentrations of betaine and choline in the PCa patients compared to BPH (8.4 vs. 9.6 µmol/L, p=0.08 for choline, and 36.0 vs. 40.0 µmol/L, p=0.07 for betaine), respectively. The control group by the above mentioned study consisted of healthy subjects, therefore differ markedly from the representative group in our study (BPH patients), and this is likely to be one cause of difference. One other possible explanation might be that the blood collection occurred after diagnosis in our study, thus disease effects on metabolites concentrations cannot be ruled out. In the Northern Sweden Health and Disease Cohort study, the median time between blood collection and diagnosis was about 6.9 years, suggesting that most participants were apparent healthy and free of cancer.

Since betaine demethylation is the only pathway known to produce DMG, this metabolite may serve as an index of the methyl group transfer from betaine to Hcy (167). Although the DMG concentrations were slightly lower in the young PCa and slightly higher in the elderly PCa patients compared to controls in each group, betaine/DMG as well as choline/betaine ratios were closed or equal to the corresponding ratios in controls in the two groups, supporting no differences in methylation process of Hcy via betaine cycle between the PCa and BPH patients.

4.1.2 One-carbon metabolites in low-grade, high-grade PCa, and BPH

Our data showed that choline and betaine concentrations were slightly lower in the young high-grade and low-grade PCa patients compared to controls, but these differences were not significant (8.8 and 8.3 vs. 9.4 µmol/L, p=0.13 for choline, and 41.0 and 37.0 vs. 43.0 µmol/L, p=0.19 for betaine), respectively. We found also no significant differences in the concentrations

of choline between high-grade, low-grade PCa, and controls in the elderly group (9.8, 10.3, and 10.2 µmol/L, p=0.81), respectively. Changes in the concentrations of choline have been observed in PCa tissues using MRSI techniques (32). These concentrations vary between benign, low-grade, and high-grade PCa tissues (145;147). Moreover, an enhanced choline uptake has been documented in PCa compared with normal prostate tissues (113). However, the relationship between blood and intracellular choline has not been sufficiently studied (168;169). It is not clear if plasma levels reflect the tissues variation. Our results cannot demonstrate differences in circulating plasma choline concentrations between groups due to the tumor grade.

Interestingly, the elderly high-grade PCa patients showed significantly lower concentrations of betaine and total folate compared to low-grade PCa (36.0 vs. 43.0 µmol/L, p=0.03 for betaine, and 6.7 vs. 10.5 nmol/L, p=0.03 for total folate), respectively, probably because of higher requirements of the advanced prostate tumor to methyl groups via betaine and folate. However, further studies will have to test whether the circulating concentrations of choline, betaine, or folate are representative of concentrations in prostatic tissues and whether either concentration is correlated with prostate cancer cell proliferation.

4.2 SAM and SAM/SAH ratio

In our study, no significant differences were found in SAM, SAH or their ratio between PCa and controls in the young and in the elderly group. SAM/SAH ratios were slightly lower in the PCa groups compared to controls (7.6 vs. 7.8, p=0.48 in the young group, and 6.9 vs. 7.4, p=0.31 in the elderly group), respectively. SAM does not readily cross the plasma membrane, hence each mammalian cell is responsible for synthesizing its own SAM from circulating methionine, Hcy, or SAH (170). SAH, in contrast, leaks from the cell once its accumulation exceeds the buffering capacity of specific SAH-binding proteins in the cytoplasm. Moreover, SAM/SAH ratio could be altered due to changes in activity of SAM-dependent methyltransferases, particularly glycine N-methyltransferase (GNMT) and PEMT (171), and accordingly GNMT- or PEMT- knockout mice showed increased SAM availability accompanied with aberrant DNA methylation (172-174). For all these reasons, plasma SAM/SAH ratio may not provide a meaningful index of intracellular methylation status.

Only the young PCa patients showed a marginally significant decrease in SAM concentrations compared to controls (median 112 vs. 127 nmol/L, p=0.046). In addition, we observed that the young PCa patients displayed slightly higher PtdCho sum concentrations

accompanied with slightly lower sum concentrations of PtdEth compared to controls. The indicators of such inverse relation suggest an increased synthesis of PtdCho from PtdEth by PEMT, and this may be the reason of decreased SAM concentrations in the young PCa patients. Interestingly, Tchantchou *et al.* indicated that SAM can exert a direct effect on glutathione S-transferase activity, where the reduced levels of SAM were associated with an impaired activity of glutathione S-transferase (175). In accordance with previos study, low levels of glutathione S-transferase activity was associated with an elevated risk for developing PCa at a young age (176).

4.3 Choline phospholipid metabolism

4.3.1 Phospholipid classes in PCa and BPH

To the best of our knowledge, this is the first study investigating plasma phospholipid in patients with PCa compared to BPH. Our results showed that the sum concentrations of PtdCho and SM were slightly higher and that of Lyso-PtdCho and PtdEth slightly lower in the young PCa patients compared to controls, but these differences were not significant. The further analysis of the results according to the pathological grade showed also no significant differences in the sum concentrations of phospholipid classes between the low-grade, high-grade young PCa and control. However, we observed that the increase in concentrations of PtdCho and its product SM were associated with a decrease in PtdEth and choline concentrations. Therefore, we can argue that such correlations may be reflecting the enhanced synthesis of PtdCho from choline via Kennedy pathway, and from PtdEth via PEMT pathway by cancer cells. Since choline /betaine ratios were almost equal without significant differences in the young PCa and controls, it is plausible that choline in the young patients is used to build up more PtdCho instead of using to generate the methyl donor betaine. These data, in conjunction with the previous suggestion that the young PCa patients had a raised synthesis of PtdCho from PtdEth due to the elevated PEMT activity, support the hypothesis of enhanced synthesis of PtdCho from choline and PtdEth. In accordance, men whose diagnosed with PCa at a young age have a faster growing and more aggressive cancer than men diagnosed at an older age (160), thus they have to synthesize more PtdCho and SM to meet the increased demand of cell membrane synthesis.

In the elderly group, the results of PtdCho and SM were in the other direction, where the sums of PtdCho and SM concentrations decreased slightly in the elderly patients compared to controls, but the differences were significant only for the sum of SM (P=0.001) **(Table 12)**. Here, on the contrary, choline concentrations were unexpectedly almost equal without significant

differences in the elderly PCa and controls (10.0 vs. 9.7 µmol/L, p=0.944), respectively. Thus, unlike the young group, the inverse association between PtdCho and choline levels was not observed. However, the decrease in the sum concentrations of phospholipid might be explained by higher statin usage in elderly PCa compared to young PCa (36.3 % vs. 16.9 %), respectively. An additional possible explanation is that the majority of older men have slow-growing or indolent disease (41;160), which might have a close demand of PtdCho to that of BPH.

Lipids, including fatty acids, triglycerides, cholesterol, and phospholipid, were found to be associated with malignant tumors (177). There are increasing evidences showed that blood and tissues fatty acids levels are associated with PCa risk (178). New recent studies highlighted the potential of plasma phospholipid fatty acids (179-182), as well as phospholipid in biological fluids to serve as biomarkers of PCa (183-186). However, these studies showed inconsistent results **(Table 31)**. The variations in assessment methods may partially explain the inconsistent results (179;187). Moreover, the studies differed in the blood materials used to measure phospholipid and phospholipid fatty acids (181;183;188). Another reason may well be the differences in study population between countries. The methodological considerations played also a role in these conflicting findings.

However, three studies have investigated plasma phospholipid in PCa patients (184-186). The first study by Cvetkovic *et al* investigated four classes of plasma phospholipid in PCa patients and healthy subjects. In this study, PCa patients displayed significantly lower levels of PtdCho, Lyso-PtdCho, PtdEth, and SM compared to controls. Moreover, these four classes decreased in high-grade PCa patients (Gleason score ≥7) compared to low-grade PCa patients (Gleason score <7) (184). In the second, Zhou *et al* showed a different pattern, where plasma levels of phospholipid classes PtdCho, Lyso-PtdCho, PtdEth, and SM have significantly increased in PCa patients compared to controls (1.9-2.7 fold). Furthermore, many phospholipid species were suggested as plasma phospholipid biomarkers for diagnosis of PCa including; three PtdCho species (38:4, 38:5, and 40:7), four Lyso-PtdCho species (18:0, 18:1, 18:2, and 20:4), and three SM species (16:0, 16:1, and 18:0). Several other species such as PtdCho (34:1, and 36:1), Lyso-PtdCho (16:0), PtdEth (36:2), and SM (18:1, 22:0, and 24:0) showed significant differences between PCa patients and control, but they had a lower diagnostic sensitivity (185). In the third study, Patel *et al* analyzed serum phospholipid in newly diagnosed PCa patients compared to healthy subject, he indicated that PtdCho species (38:5, 40:3, and 42:4) may serve as early

predictive serum markers for the presence of PCa (186). Our findings regarding PtdCho and SM in the young group are in line with that of Zhou *et al's* study that had participants with a similar mean age (63.6±8.5 year) to the young group in our study (median age 60.2 years) (185). On the other hand, Cvetkovic *et al's* study that analyzed plasma phospholipid in newly diagnosed elderly patients (median age 74 years) had a similar observation to the elderly group in our study (median age 70.7 years) (184).

Lyso-PtdCho levels were slightly lower in the PCa patients in the young and the elderly group. This reduction can be attributed to the increased enzymatic activity of lysophosphatidylcholine acyltransferase (LPCAT) in PCa (189;190), the enzyme involved in PAF biosynthesis from Lyso-PtdCho **(Figure 8)** and in the Lyso-PtdCho remodelling (191). In accordance, new data referred that LPCAT overexpression might greatly affect biological activities of cancer and correlates to the progression of PCa (189;192). Our results regarding Lyso-PtdCho are in line with previous reports that showed a decrease in Lyso-PtdCho concentrations in cancer patients (184;193;194).

Table 31: Selected lipids metabolomics studies in prostate cancer.

Study design	Experimental model	Key results	Reference
phospholipid fatty acids studies			
Case-control	PCa vs. BPH (plasma and tissue)	-Decrease in arachidonic (20:4) and docosapentaenoic (22:5) acids in phospholipid of malignant tissues. -Higher levels of oleic acid (18:1) in plasma and tissue phospholipid in PCa patients.	(182)
Nested case-control	Incident PCa vs. controls (plasma)	-Positive association between palmitic acid (16:0) and risk of low-grade PCa. -Inverse association between stearic (18:0) acid and the risk of low-grade PCa. -Positive associations between myristic (14:0), α-Linolenic (18:3, ω3), and eicosapentaenoic (20:5, ω3) acids and risk of high-grade PCa.	(179)
Nested case-control	Incident PCa vs. controls (plasma)	-Higher levels of long chain ω3-polyunsaturated fatty acids (20:5, 22:5, and 22:6) are associated with increased PCa risk.	(180)
Nested case-control	Incident PCa vs. controls (plasma)	-Higher levels of long chain ω3-polyunsaturated fatty acids (20:5, 22:5, and 22:6) are associated with increased PCa risk. -Higher linoleic acid (18:2, ω6) level is associated with reduced PCa risk.	(181)

Table 31: Selected lipids metabolomics studies in prostate cancer.

Study design	Experimental model	Key results	Reference
Nested case-control	Incident PCa vs. controls (WB)	-Higher long chain ω3-polyunsaturated fatty acids (20:5, 22:5, and 22:6) levels were inversely related to PCa risk.	(188)
phospholipid studies			
Case-control	PCa vs. control subjects (plasma)	-Decrease in PtdCho, Lyso-PtdCho, PtdEth, and SM levels in PCa patients compared to controls. -Decrease in PtdCho, Lyso-PtdCho, PtdEth, and SM levels in high grade-PCa patients (Gleason score ≥7) compared to low grade-PCa patients (Gleason score <7).	(184)
Case-control	PCa vs. control subjects (plasma)	-Increase in PtdCho, Lyso-PtdCho, PtdEth, and SM levels in PCa patients compared to controls.	(185)
Case-control	PCa vs. control subjects (serum)	-PtdCho 40:3, PtdCho 42:4, and egg PtdCho 38:5 identified as unique candidate for PCa diagnosis	(186)

4.3.2 Phospholipid species in PCa and BPH

Our results showed that, in general, there is a slightly increase in SM species in the young PCa patients compared to BPH, but the differences were significant only for SM 14:0 and 20:0 (10.8 vs. 9.4 µmol/L, p=0.033 and 16.2 vs. 13.6 µmol/L, p= 0.004), respectively **(Table 9)**. The results according to Gleason score showed also similar observations regarding SM 14:0 and 20:0, but the significant differences were only between the low-grade young PCa patients and BPH (12.1 vs. 10.0 µmol/L, p=0.035 for SM 14:0, and 16.6 vs. 14.1 µmol/L, p= 0.027 for SM 20:0) **(Table 14)**. Zhou *et al's* study reported higher SM species (16:0, 16:1, 18:0, 18:1, 22:0, 22;1, 24:0, and 24:1) concentrations in the PCa patients compared to controls (185). This is in line with our finding of higher SM species in the young PCa group. This overall increase in concentrations of SM species in the young PCa may be related to increased activity of SMS that synthesize SM by transfer PCho moiety from PtdCho to Cer, and resulting in release of DAG **(Figure 8)**. Indeed, the biological roles of SMSs go beyond formation of SM. Several lines of evidence indicated that SMSs have considerable biological potential as a regulator of the proapoptotic factor Cer and mitogenic factor DAG, hence they play a critical role in cell growth and survival (195;196). However, we can argue that the increase in SM 14:0 and 20:0 concentrations may reflect an increased production of their precursors Cer 14:0 and 20:0 than other Cer subspecies. In other word, increased proportion of myristic (C14:0) and arachidic (C20:0) acids in SM. Accordingly,

a positive association between serum- or plasma phospholipid myristic acid and PCa risk has been reported (179;197;198). In addition, one study reported a higher serum phospholipid arachidic acid in the PCa cases compared to controls (197).

In the elderly group, SM species (16:0, 18:0, 18:1, 20:0, 22:0, 22;1, 23:0, 23:1 24:0, 24:1, and 24:2) were significantly lower in the PCa cases than controls **(Table 12)**. The further data analysis according to the pathological grade showed that SM 16:0 and 18:0 concentrations were significantly lower among the low-grade, high-grade PCa compared to the controls. Whereas, SM 18:1, 22:0, 23:0, and 23:1 concentrations were significantly lower in the low-grade PCa compared to controls **(Table 16)**. The higher usage of statin in the elderly PCa compared to controls (36.3 % vs. 29.4 %) might contribute to this decrease, but it does not fully explain these differences. However, this decrease may be partly resulting from the decrease of SM precursors PtdCho or Cer. Apparent Cer production via Cer synthases (CerS) has been reported in cancer cells (199;200). The different substrate selectivity and genetic variation of CerS family have also a significant effect on the biosynthesis of some SM species. For example, the CerS4 SNPs showed an association with circulating SM 18:0, 18:1, 20:0, and 20:1 (100;201) . However, SM biosynthesis is modified by many additional factors such as aging, obesity, and the delivery of Cer to SMS that involves the Cer transport protein (100;202). Alteration in SM metabolism affects many aspects of cell behavior, such as the sensitivity of cells to proapoptotic agents (100). Unfortunately, we did not find any information on SM species among the elderly PCa patients. However, the findings from lipid studies have documented a positive association between palmitic acid (C16:0) and PCa risk (179;197;198;203), and an inverse association for stearic acid (C18:0) (179). Plasma phospholipid oleic acid (C18:1) levels were significantly lower in the PCa cases in one study (203), and higher in another study (182).

Taken together, our findings showed variations in phospholipid metabolism between PCa and controls. We even observed a different tendency of variations with respect to the age at diagnosis, supporting the hypothesis that there are important metabolic differences between young and elderly PCa. Our results highlight that SM 14:0 and 20:0 may differentiate between young patients (≤ 65 years) with PCa and those with BPH, and SM 16:0 and 18:0 can distinguish between elderly PCa patients (>65 years) and BPH patients.

4.4 Metabolites concentrations according to age

The present study showed also age-related differences in metabolites concentrations in the controls and the PCa group. These changes were stronger in the PCa group. Although some of the differences can be attributed in part to age-associated changes in the metabolism process, but they more strongly suggest an effect of cancer on the levels of these metabolites.

4.4.1 In the control group

The comparisons of metabolite concentrations between the older and younger controls showed slightly differences which were not statistically significant, unlike that of MMA and SM 23:1 **(Table 17 & 18)**. Concentrations of choline, betaine, and DMG were markedly unchanged with age. This is in line with previous results showing that betaine levels remain stable for years in normal subjects (204). The concentration of MMA decreased significantly in the elderly compared to young controls (median 171 vs. 192 nmol/L, p=0.025). Elevated serum MMA is a marker of vitamin B 12 deficiency. Such deficiency is very frequent in the elderly population and is associated with increase of MMA concentration (205). However, as we excluded participant with vitamin B12 supplementation, the decrease of MMA levels in the elderly control is unexpected and the reason for this decrease is unclear, but it may be attributed to the small size of the young controls in our study (22 vs. 51 elderly controls) than vitamin B12 status.

SM 23:1 concentration was marginally significantly higher in the elderly controls (median 10.5 vs. 8.6 µmol/L, p=0.046). Since the difference in the distribution of statin users between young and elderly controls was not significant, the change in SM 23:1 levels was unexplained by differences in statin use. The information about this SM specie is very rare in literatures, it is only referred that SM 23:1 could be a useful biomarker for predicting treatment response to rosiglitazone in type 2 diabetes (206). Moreover, there is only very poor information about tricosenoic acid (C23:1), the corresponding fatty acid that contains one double bond at an unspecified position.

4.4.2 In the PCa group

The differences in the patient groups were greater than in controls. Several one-carbon metabolites showed a significantly increased tendency with age, including choline, tHcy, Cys, SAH, and SAM **(Table 19&21)**. Choline was significantly higher in the elderly PCa compared to the young PCa patients (median 10.0 vs. 8.4 µmol/L, p=0.001). Our findings agree with the results of Konstantinova *et al* that found an increase in choline concentrations among elderly men

and women with metabolic disorders (159). As we discussed earlier, the lower choline concentrations in the younger PCa might be partly a result of the increased choline use for PtdCho synthesis in the young PCa patients. In the low-grade PCa group, choline, betaine, and DMG concentrations were significantly higher in the low-grade elderly PCa compared to the low-grade young PCa. These three metabolites are positively correlated with each other (74). Since choline/betaine and betaine/DMG ratios showed no differences between these groups, thus this increase may result from the increase of their precursor choline.

Concentrations of tHcy, Cys, SAH, and SAM increased significantly with higher age, while the SAM/SAH ratio decreases in the PCa and low-grade PCa groups. The differences in the high-grade PCa group had a similar tendency, but without a statistical significance. Studies showed that the serum or plasma concentrations of tHcy, SAH, and SAM increase in elderly patients with diseases such as multiple sclerosis, stroke, and peripheral neuropathy, while the SAM/SAH ratio decreases (207;208). We could confirm these findings in our study. The physiological decline in renal function might explain the increase in the tHcy concentrations in the elderly (209). Increased Hcy led to elevated SAH levels and thus decreased SAM/SAH ratios (210), which may explain the differences in SAM/SAH ratio. However, the results in controls group showed similar trends, but without statistical power, suggesting an additional effect of disease on these metabolites.

SM species were almost significantly lower in the elderly compared to the young PCa. In contrary to the controls group, the difference in the distribution of statin users between young and elderly patients was significant. After adjusting for statin use the differences of SM species concentrations between the young and elderly PCa became non-significant. However, since the decrease tendency of SM species is only observed among the PCa patients, thus it seems to be more strongly related to the disease itself. Interestingly, we observed that SM 24:0 concentrations decreased slightly with higher age in the controls and PCa groups. This decrease was significant in the low-grade and high-grade PCa groups. Recently, Collino *et al* reported a similar observation, and noted that SM 24:0 decrease with age (156). Combining this finding with our present data suggesting that SM 24:0 might be only age-dependent variable. As a whole, these results indicate that phospholipid and one-carbon metabolism is altered due to age and disease.

4.5 Metabolites concentrations according to statin use

Statin inhibit 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase, thus block the rate-limiting step of cholesterol biosynthesis (211). The relation between statin use and cancer risk is controversial. Animal studies showed that statins may be carcinogenic (212). In contrast, other evidences found that statin have cancer chemopreventive properties, through inducing apoptosis, inhibiting angiogenesis, and suppressing tumor growth and metastatic process (213;214). The data on statin and PCa risk is inconsistent. Whereas some studies showed no association between statin use and PCa risk (215;216), others observed that statin was associated with elevated overall risk (217). However, a recent meta-analysis of 27 (15 cohort and 12 case-control) studies showed that statins reduce the risk of PCa (218). We examined the effect of statin on phospholipid concentrations among patients and control, as well as the interactions between disease and statin use in the PCa group.

4.5.1 In the control group

32.7 percent of the controls used statin in the present study, age and one-carbon metabolism metabolites did not significantly differ between statin and non-statin users. Statin users showed a significant decrease in the sum concentrations of PtdCho and SM compared to non-statin user. Furthermore, many phospholipid species were significantly lower in statin users among the controls including; four PtdCho species (34:2, 36:2, 36:3, and 38:3), one Lyso-PtdCho specie (18:0), and most SM species (SM 16:0, 16:1, 22:0, 22:1, 23:0, 23:1, 23:2, 24:0, 24:1 and 24:2). In general, we observed a decrease tendency in the concentrations of most SM, PtdCho and Lyso-PtdCho species. PtdEth concentrations showed no significant differences with statin use.

Statin exert multiple effects on lipid profile, their predominant actions are inhibition cholesterol synthesis, increased LDL catabolic rate, and decreased LDL and VLDL production (219;220). Phospholipid constitute 20-30 % of the lipids of LDL and VLDL (97). By far, the main phospholipid constituents of these lipoprotein classes are PtdCho and SM (90-93%) (99). Whereas Lyso-PtdCho constitutes about 4 % and PtdEth less than 2% of LDL and VLDL phospholipid (99). Therefore, we can argue that the decrease in LDL and VLDL levels due to statin therapy resulting in a subsequent decrease of phospholipid concentrations, especially PtdCho and SM. Furthermore, SM content is higher in LDL and VLDL than other lipoprotein classes (2-2.5 fold) (97;99), thus SM are likely to be more affected by statin than other phospholipid and this might explain the stronger observed variation in SM species.

4.5.2 In the PCa group

The PCa statin users (38.1% of PCa patients) displayed significant increases in concentrations of betaine, DMG, Cys, SAH, and SAM compared to non-statin users **(Table 27)**. The statin users in this group had a higher median age than the non-statin users with significant difference (68.6 vs. 64.3, p=0.047), which may explain the cause of these differences. Moreover, various factors such as comorbidities and decline in renal function play roles in this tendency as discussed earlier.

Similar to controls, the sum concentrations of PtdCho and SM decreased also significantly in the PCa statin users. But, unlike controls, the decrease of Lyso-PtdCho sum concentrations was significant among patients who used statin. Furthermore, these decreases were accompanying with the decrease of additional phospholipid species, including PtdCho (34:3, 36:4, and 38:4), Lyso-PtdCho (16:0, and 20:4), and SM (14:0, 18:0, and 20:0). Moreover, two PtdEth species (38:4, and 38:5) displayed a significant increase with the use of statin. As discussed earlier, such variations may be not only related to statin effect but also to the age and disease itself.

However, distinct alterations in the phospholipid profile of plasma and membrane lipids were observed in diseases. For example, cancer patients showed changes in the phospholipid composition in the blood cells (221). Alterations in phospholipid species pattern of lipids and lipoproteins in several diseases were also documented (222-224). Hiukka *et al* attributed these alterations to the increased hydrolysis susceptibility of SM by sphingomyelinase (SMase) (223). These observations suggest possible disease-related factors behind the decrease in phospholipid concentrations among the PCa patients. In accordance, the non-statin user with PCa in our study showed a decrease tendency in SM levels compared to the controls without statin treatment.

SM 16:0 and 18:0 concentrations decreased significantly in the elderly PCa in both the statin user and the non-statin user groups compared to the controls in each group, suggesting that this decrease is independent of statin use. This confirm our previous conclusion that SM 16:0 and 18:0 have the potential to distinguish between the elderly PCa and BPH patients. Collectively, our findings suggest that PCa affect the circulating phospholipid profile. Furthermore, a synergic interaction effect between statin use and disease on phospholipid concentrations was observed in this study.

5. Conclusions

Abnormal choline metabolism has been reported in malignant transformation; therefore there is much interest in choline metabolic pathways related either to one-carbon metabolism or phospholipid metabolism as a tumor marker and for therapeutic objectives. Our main aim was to investigate plasma concentrations of choline and its related metabolites in patients with PCa and those with BPH. The novel findings of this study are:

- Plasma choline showed no significant differences between PCa patients and BPH controls. Further explanations include that selection of the control group (not healthy) may not be appropriate or to the fact that choline pool is large and free plasma choline may not be the best marker for tissue choline.
- Younger men (age ≤65 years) diagnosed with PCa showed significantly higher plasma concentrations of SM species 14:0 and 20:0 compared to those with BPH.
- Elderly low-grade, high-grade, and PCa patients (age >65 years) had significantly lower plasma concentrations of SM species 16:0 and 18:0 compared to the elderly BPH controls. Therefore, SM 16:0 and 18:0 can distinguish between the elderly PCa and BPH patients.
- PCa was associated with a specific phospholipid profile pattern.
- Age was associated with significantly higher choline, tHcy, Cys, SAH, and SAM levels in the PCa patients.
- Statin use was associated with lower plasma phospholipid levels, especially PtdCho and SM. This lowering effect is synergized by the presence of PCa.

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Appendix

Appendix A: Standards and chemicals

Chemical	Purity	Company	CAS number	Catalogue number
Choline related metabolites: standards and internal standards				
Betaine anhydrous	ultra; ≥ 99.0%	Sigma Aldrich, Munich, Germany	107-43-7	61962
Choline chloride	≥ 99%	Sigma Aldrich, Munich, Germany	67-48-1	C7017
N,*N*,-Dimethylglycine (= DMG)	99%	Sigma Aldrich, Munich, Germany	1118-68-9	D1156
N,*N*,*N*-Trimethyl-d_9-glycine hydrochloride (= d_9-betaine)	98 atom % D	Isotec, Sigma Aldrich, Munich, Germany	285979-85-3	616656
Choline chloride-trimethyl-d_9 (=d_9-choline)	98 atom % D	Isotec, Sigma Aldrich, Munich, Germany	61037-86-3	492051
N,*N*-Dimethyl-d_6-glycine HCl (= d_6-DMG)	99 atom % D	CDN Isotopes, Quebec, Canada	347840-03-3	D-3509
SAH and SAM: standards and internal standards				
S-(5'-Adenosyl)-*L*-homocysteine, crystalline (= SAH)	≥98% (HPLC)	Sigma Aldrich, Munich, Germany	979-92-0	A9384
S-(5′-Adenosyl)-*L*-methionine *p*-toluenesul-fonate salt, from yeast (L-methionine enriched) (= SAM)	≥80% (HPLC)	Sigma Aldrich, Munich, Germany	17176-17-9	A2408
S-Adenosyl-*L*-methionine-d_3 (S-methyl-d_3) tetra(*p*-toluenesulfonate) salt (= [$^2H_3$]-SAM)	85% chemical purity; 99 atom % D	CDN Isotopes, Quebec, Canada	17176-17-9 (unlabeled compound)	D-4093
S-(5'-Adenosyl)-*L*-homocysteine (= [$^{13}C_5$]-SAH)		Henkjan Gellekink group, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands	979-92-0 (unlabeled compound)	

Appendix A: Standards and chemicals (continued)

Chemical	Purity	Company	CAS number	Catalogue number
Phospholipid: standards and internal standards				
1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (=PtdCho 28:0)	≥99%	Sigma Aldrich, Munich, Germany	18194-24-6	P2663
1,2-dioleoyl-*sn*-glycero-3-phosphocholine (=PtdCho 36:2)	>99%	Avanti	4235-95-4	850375P
N-nervonoyl-D-*erythro*-sphingosylphosphorylcholine (=SM 24:1)	>99%	Avanti	94359-13-4	860593P
N-palmitoyl-D-*erythro*-sphingosylphosphorylcholine (=SM 16:0)	>99%	Avanti	254-89-3	860584P
1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (=PtdEth 36:2)	>99%	Avanti	4004-5-1	850725P
1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (=PtdEth 16:0/16:0)	≥97%	Sigma Aldrich, Munich, Germany	923-61-5	P1348
1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (=Lyso-PtdCho 18:0)	>99%	Avanti	19420-57-6	855775P
1-palmitoyl-*sn*-glycero-3-phosphocholine (=Lyso-PtdCho 16:0)	≥99%	Sigma Aldrich, Munich, Germany	17364-16-8	L5254
1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine (=PtdCho 15:0/15:0)	>99%	Avanti	3355-27-9	850350P
N-hexanoyl-D-*erythro*-sphingosylphosphorylcholine (=SM 6:0)	>99%	Avanti	182493-45-4	860582P
1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine (=PtdEth 15:0/15:0)	>99%	Avanti	109032-52-2	850704P
1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (=Lyso-PtdCho 17:0)	>99%	Avanti	50930-23-9	855676P

Appendix A: Standards and chemicals (continued)

Chemical	Purity	Company	CAS number	Catalogue number
Homocysteine, methylmalonic acid, cystathionine: internal standards				
DL-(2-Amino-2-carboxyethyl)-homocysteine-3,3,4,4-d_4 (= d_4-Cys)	98.0 atom % D	CDN Isotopes, Quebec, Canada	146764-57-0	D-3349
DL-Homocystine-3,3,3',3',4,4,4',4'-d_8 (= d_8-Hcy)	98.0 atom % D	CDN Isotopes, Quebec, Canada	108641-82-3	D-3030
Methyl-d_3-malonic acid (= d_3-MMA)	99.7 atom % D	CDN Isotopes, Quebec, Canada	42522-59-8	D-2810
Other chemicals				
2-propanol	ULC/MS grade	Biosolve, Valkenswaard, The Netherlands	67-63-0	162641
Acetic acid, glacial	+99.99%	Sigma Aldrich, Munich, Germany	64-19-7	338826
Acetonitrile	ULC/MS grade	Biosolve, Valkenswaard, The Netherlands	75-05-8	01204102
Ammonia solution analaR NORMAPUR	25%	BDH Prolabo (VWR International GmbH, Darmstadt, Germany)	1336-21-6	1133.2500
Ammonium acetate	ULC/MS grade	Biosolve, Valkenswaard, The Netherlands	631-61-8	01244156
Ammonium formate	ULC/MS grade	Biosolve, Valkenswaard, The Netherlands	540-69-2	01984156
Butylhydroxytoluol (BHT)	≥99.0% (GC)	Sigma Aldrich, Munich, Germany	128-37-0	34750 Fluka
Chloroform	≥99.8% (GC)	Merck, Darmstadt, Germany	67-66-3	1024442500

Appendix A: Standards and chemicals (continued)

Chemical	Purity	Company	CAS number	Catalogue number
Other chemicals				
Dithiothreitol (= DTT)	≥ 99.5%	AppliChem GmbH, Darmstadt, Germany	27565-41-9	A2948
Ethylenediaminetetraacetic acid disodium salt dihydrate (= EDTA)	99.6%	Sigma Aldrich, Munich, Germany	6381-92-6	E4884
Formic acid	ULC/MS grade; 99%	Biosolve, Valkenswaard, The Netherlands	64-18-6	06914131
Hydrochloric acid	fuming, pro analysis; 37%	Merck Chemicals, Darmstadt, Germany	7647-01-0	100317
Methanol, absolute	ULC/MS grade	Biosolve, Valkenswaard, The Netherlands	67-56-1	13684102
N-Methyl-N-tert-butyldimethylsilyltrifluoro-acetamid (= MBDSTFA)		Machery and Nagel, Düren, Germany	77377-52-7	701440.201
β-Mercaptoethanol	cell culture tested	Sigma Aldrich, Munich, Germany	60-24-2	M7522
Sodium hydroxide, solid	pro analysis; ≥ 99%	Merck Chemicals, Darmstadt, Germany	1310-73-2	106495

Appendix B: Equipment

Equipment	Description	Company
Analytical balance	Sartorius CP224S-0CE Sartorius ME215P-0CE	Sartorius AG, Göttingen, Germany
Blood collection	Citrate: S-Monovette 5 mL 9NC, 92 x 11 mm EDTA: S-Monovette 2.7 mL K3E, 66 x 11 mm; S-Monovette 9 mL K3E, 92 x 16 mm Li-Hep: S-Monovette 4.7 mL LH-Gel, 75 x 15 mm Needle: Safety-Multifly 21G tube 200 mm Serum: S-Monovette 4.7 mL Z-Gel, 75 x 15 mm	Sarstedt, Nümbrecht, Germany
Centrifuge	Eppendorf centrifuge 5810 R, A-4-62 Rotor	Eppendorf AG, Hamburg, Germany
	Hettich Mikro 20	Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany
	Sigma 3K12,	Sigma Laborzentrifugen GmbH, Osterode, Germany
	Optima LE-80K Preparative Ultracentrifuge, Type 50.2 Ti Rotor	Beckman Coulter GmbH, Krefeld, Germany
Cooling	Ziegra Ice machine ZBE 70-35	Ziegra Eismaschinen GmbH, Isernhagen, Germany
	Heraeus HERA freeze HFU-Basic Series, -86°C	Thermo Fischer Scientific, Waltham, USA
Concentrator	Eppendorf concentrator 5301	Eppendorf AG, Hamburg, Germany
DNA isolation	QIAamp DNA blood mini kit	Qiagen GmbH, Hilden, Germany
Gases	ALPHAGAZ 1 Argon	AIR LIQUIDE Deutschland GmbH, Düsseldorf, Germany
	Cmc instruments NGM nitrogen-membrane-generator (LCMS)	Cmc instruments GmbH, Eschborn, Germany
Heating	Thermo Scientific Haake Open-Bath Circulators C10-W19	Thermo Fischer Scientific, Waltham, USA
HPLC system	Waters 2795 alliance HT	Waters Corporation, Milford, USA
Mass spectrometer	MicroMass Quattro Micro API (coupled to HPLC system) MicroMass Quattro Premier XE (coupled to UPLC system)	Waters Corporation, Milford, USA
Microwave	NN-5256, 900 watts	Panasonic Deutschland GmbH, Hamburg, Germany

Equipment	Description	Company
Mixer/Shaker/Vortex	IKA MS2 Minishaker	IKA – Werke GmbH & Co. KG, Staufen, Germany
	RM5-40 Horizontal Mixer	Bennett Scientific Ltd., Newton Abbot, UK
	VARIOMAG Monotherm Heatable Magnetic Stirrer	VARIOMAG-USA, Daytona Beach, USA
PCR	Eppendorf Mastercycler ep gradient S	Eppendorf AG, Hamburg, Germany
	PSQ 96MA instrument	Biotage AB, Uppsala, Sweden
pH meter	Schott Intruments Lab 870 pH meter (N 6000 A electrode)	SI Analytics GmbH, Mainz, Germany
Photometer	Aurius CE2041 Spectrophotometer	CECIL Instruments Ltd., Cambridge, UK
Pipetting	Biohit m10 (0.5-10 µL), m200 (20-200 µL), m1000 (100-1000 µL) pipette	Biohit Deutschland GmbH, Rosbach v. d. Höhe, Germany
	Combitips plus; 0.5, 1, 5, 10, 25 mL	Eppendorf AG, Hamburg, Germany
	Multipette plus	
	Pipette tips 20 µL, 200 µL, 1000 µL	Sarstedt, Nümbrecht, Germany
	Serological Pipette 10 mL, 25 mL	
Reaction tubes	PCR Tubes 0.2 mL, PCR clean Microtube 1.5 mL	Eppendorf AG, Hamburg, Germany
	Tube 12 mL, 105x16.8 mm, PS; Tube 15 ml, 120x17 mm, PP; Tube 50 mL, 114x28 mm, PP	Sarstedt, Nümbrecht, Germany
	OptiSeal Polyallomer Centrifuge Tubes 1 x 3 ¼ in. (26 x 77 mm), 29.9 mL	Beckman Coulter GmbH, Krefeld, Germany
Sample preparation	Oasis MAX (1 ccm/30 mg and 3 ccm/60 mg) columns	Waters Corporation, Milford, USA
	Varian Bond Elut PBA columns	Varian Inc., Palo Alto, USA
Ultrasonic cleaner	VWR USC600T	VWR International GmbH, Darmstadt, Germany
UPLC columns	Acquity UPLC BEH HILIC column (100 mm x 2.1 mm (i.d.); 1.7 µm particle size)	Waters Corporation, Milford, USA
	Acquity UPLC BEH C_{18} column (50 mm x 2.1 mm (i.d.); 1.7 µm particle size)	
	Acquity UPLC BEH Shield RP18 column (100×2.1 mm (i.d.); 1.7 µmol/L particle size)	
UPLC in-line filter	0.2 µm in-line filter	Waters Corporation, Milford, USA

Equipment	Description	Company
UPLC precolumns	Acquity HILIC VanGuard pre-column (5 mm x 2.1 mm (i.d.); 1.7 µm particle size) Acquity BEH C_{18} VanGuard pre-column (5 mm x 2.1 mm (i.d.); 1.7 µm particle size) Acquity UPLC BEH Shield RP18 VanGuard pre-column (5×2.1 mm (i.d.); 1.7 µmol/L particle size)	Waters Corporation, Milford, USA
UPLC system	Waters Acquity UPLC	Waters Corporation, Milford, USA
Vials and glassware	5-SV – EPA Screw Top Vials, 5 mL glass tubes	Chromacol, Herts, UK
	11 mm Snap Cap with PTFE/Silicone septa	SUN-Sri (Thermo Fischer), Rockwood, USA
	12 x 32 Maximum Recovery glass vials including Snap Caps with locked-in, Pre-slit PTFE/Sil Septa	Waters Corporation, Milford, USA
	Snap/Crimp V-Vial 8002-SC-D/V15µ	Glastechnik Graefenroda GmbH, Gräfenroda, Germany
	Wheaton Sample Vial Clear, with Screw Thread, 19x65 mm, 12 mL, with Screw Caps	neoLab Migge Laborbedarf-Vertriebs GmbH, Heidelberg, Germany
	DURAN GL 45 glass bottles, 200 mL, 500 mL, 1000 mL	DURAN Group GmbH, Wertheim/Main, Germany
Water purification	Milli-Q Academic	Millipore, Molsheim, France

Appendix C: Reference ranges for laboratory parameters

Parameter	Sample material	Method of determination	Reference range
Creatinine	Lithium-heparin plasma	Jaffé determination (kinetic colorimetric assay)	males: 0.7-1.2 mg/dL (61.9-106.1 µmol/L[a]) females: 0.5-0.9 mg/dL (44.2-79.6 µmol/L)
GFR	Lithium-heparin plasma	MDRD equation	80-140 ml/min
Urea	Lithium-heparin plasma	Kinetic test with urease and glutamate dehydrogenase	16.6-48.5 mg/dL
Uric Acid	Lithium-heparin plasma	Enzymatic colorimetric assay	males: 3.4-7 mg/dL females: 2.4-5.7 mg/dL
Glucose	Lithium-heparin plasma	UV test (hexokinase/G6P-DH)	60-100 mg/dL
ASAT	Lithium-heparin plasma	IFCC (37°C)	males: 10-50 U/L females: 10-35 U/L
ALAT	Lithium-heparin plasma	IFCC (37°C)	males: 10-50 U/L females: 10-35 U/L
Gamma-GT	Lithium-heparin plasma	Enzymatic colorimetric assay	males: < 60 U/L females: < 40 U/L
ALP	Lithium-heparin plasma	Colorimetric assay	males: 40-129 U/L females: 35-104 U/L
Blood count	EDTA whole blood	SYSMEX SF 3000 or XE 5000	males: Hb: 14.0-18.0 g/dL Hct: 41-53% females: Hb: 12.0-16.0 g/dL Hct: 36-46%
CRP	Lithium-heparin plasma	Immunoturbidimetric determination	< 5 mg/L
Total PSA	Serum	Elecsys® test based on sandwich principle	0-4 ng/mL

[a] Conversion factor creatinine: 88.4

Reference values were retrieved November 03, 2014 from: http://www.uniklinikum-saarland.de/de/einrichtungen/kliniken_institute/zentrallabor/referenzwerte_verfahrensliste/.

Appendix D: List of figures

Appendix E: List of tables

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List of Publications

Original publications and reviews

1. ***Awwad HM***, Ohlmann CH, Stoeckle M, Aziz R, Geisel J, Obeid R. Choline-phospholipids inter-conversion is altered in elderly patients with prostate cancer. Biochimie. 2016 (in press).
2. Obeid R, ***Awwad HM***, Rabagny Y, Graeber S, Herrmann W, Geisel J. Plasma trimethylamine N-oxide concentration is associated with choline, phospholipids, and methyl metabolism. Am J Clin Nutr. 2016 (in press).
3. ***Awwad HM***, Kirsch SH, Geisel J, Obeid R. Measurement of concentrations of whole blood levels of choline, betaine, and dimethylglycine and their relations to plasma levels, J Chromatogr B Analyt Technol Biomed Life Sci. 2014;957:41-5.
4. ***Awwad HM***, Geisel J, Obeid R., The role of choline in prostate cancer, Clin Biochem. 2012; 45:1548-53.
5. ***Awwad HM***, Abokhamis I. Diagnostic Performances of Anti-Cyclic Citrullinated Peptide Antibodies type IgM, IgA and IgG in Syrian Patients with Rheumatoid Arthritis, J Clin. Lab. 2010; 56:95-102.

Posters and presentations:

1. Obeid R, ***Awwad HM***, Rabagny Y, Graeber S, Herrmann W, Geisel J. Trimethylamine N-oxide is related to choline, phospholipids and methyl metabolisms. 45. Jahrestagung Klinischer Lipidstoffwechsel, Maikammer / Pfalz, Germany, 26-28 November, 2015.
2. ***Awwad HM***, Ohlmann CH, Stoeckle M, Aziz R, Geisel J, Obeid R. Choline and related phospholipids in patients with prostate cancer. ^{10}th International Conference - One Carbon Metabolism, Vitamins B and Homocysteine, Medical School, University of Lorraine, Nancy, France, 7-11 July, 2015.
3. ***Awwad HM***, Kirsch SH, Geisel J, Obeid R. Whole blood levels of choline, betaine, and dimethylglycine and their relations to plasma levels. German Conference for Laboratory Medicine (DKLM), Mannheim, Germany, 24-27 September, 2014. Published in Clin Chem Lab Med 2014; 52(11):eA103–eA203.
4. ***Awwad HM***, Abokhamis I. Diagnostic Performances of Anti-Cyclic Citrullinated Peptide Antibodies type IgM, IgA and IgG in Syrian Patients with Rheumatoid Arthritis. First International Congress on Controversies in Rheumatology and Autoimmunity, Florence, Italy, March 10-12, 2011.
5. ***Awwad HM***, Abokhamis I. Diagnostic Performances of Anti-Cyclic Citrullinated Peptide Antibodies type IgM, IgA and IgG in Syrian Patients with Rheumatoid Arthritis. 1st Symposium of the Asia Pacific League of Associations for Rheumatology, Taipei, Taiwan, April 15-17, 2011.

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