GLUCONEOGENESIS, LIVER ENERGY METABOLISM AND WEIGHT LOSS IN LUNG CANCER

Dynamic studies using stable isotope tracers and ^{31}P magnetic resonance spectroscopy

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GLUCONEOGENESE, ENERGIESTOFWISSELING IN DE LEVER
EN GEWICHTSVERLIES BIJ PATIENTEN MET LONGKANKER

Dynamische studies met stabiele isotopen
en ^{31}P magnetische resonantie spectroscopie

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Contents

Introduction

Weight loss is a major problem in many types of cancer and is associated with reduced quality of life and a poor prognosis. Weight loss can also interfere with potentially curable treatment [41,56]. Many uncertainties remain about the mechanisms underlying weight loss in patients with cancer. This thesis describes a series of studies aimed at defining alterations in metabolic processes that are potentially related with weight loss in lung cancer patients. Although in literature the terms 'cachexia' (= a syndrome of weight loss and malnutrition) and 'weight loss' are often used interchangeably, in this thesis weight loss will be preferentially used.

In this chapter, current knowledge on weight loss in cancer and its relation with metabolic alterations are reviewed and the aim of this thesis is presented.

Warren already recognised in 1932 that weight loss is a major cause of death in patients suffering from cancer [218]. Several studies have reported negative effects of weight loss on quality of life [161,162], effectiveness of anti-cancer therapy [56,106], and survival [43,93,164,169,198]. Incidences of weight loss vary from 36% in breast cancer to 61% in lung cancer patients [22,37,56,162,198]. The degree of weight loss is independent of the disease stage, suggesting that tumour presence rather than tumour load is responsible [42,154].

A typical feature of weight loss in lung cancer is increased breakdown of not only fat tissue, but also body protein [67,94,154,191,192]. This loss of protein is characterised by the predominant loss of muscle protein, whereas visceral protein tend to be preserved [78,94]. In fact, organs such as liver and spleen tend to be enlarged [94], possibly due to increased organ activity [38] or increased extracellular water [67]. In these respects, weight loss in cancer differs substantially from weight loss caused by simple starvation [21] where weight is predominantly lost as fat tissue. The loss of muscle protein in cancer bears particular clinical significance because it contributes to fatigue, loss of muscle strength, morbidity and mortality.

It is generally assumed that weight loss is caused by an imbalance between energy intake and energy disposal within the body. Decreased nutrient intake caused by altered taste perception [82] or obstruction of the gastrointestinal tract [13,161] is commonly observed in weight-losing cancer patients. The tumour itself or the antitumour therapy could mediate both processes [52,118]. However, hyperalimentation by use of parenteral nutrition often fails to restore muscle mass or to prevent weight loss [35,39,55,191]. Moreover, food intake of weight-losing lung cancer patients was not different from that of weight-stable patients [51,89,142]. Decreased food intake in cancer patients thus cannot fully account for the weight loss [153,162].

Increased energy expenditure in lung cancer patients could also contribute to weight loss. High resting energy expenditure was reported in patients with lung cancer

[74,106,196] or gastrointestinal cancers [73]. However, other investigators did not observe differences in energy expenditure between weight-losing patients with lung cancer and healthy controls [105,142,171] or weight-losing patients with non-malignant illness [66,87,155].

Since involuntary weight loss in cancer patients is not simply due to either a reduction in energy intake or increased energy expenditure, altered host metabolism must play a role. This is particularly true for patients with lung cancer, a tumour type with a high frequency of weight loss, which is usually not accompanied by anorexia or gastrointestinal obstruction. In this thesis, lung cancer is the main tumour type studied. In view of the accelerated loss of muscle protein that distinguishes the weight loss in cancer patients from that in normal starvation, our study is focussed upon the relation between protein metabolism and carbohydrate metabolism.

Protein metabolism

In healthy subjects, protein turnover, including amino acid transport, RNA-synthesis and synthesis of non-essential amino acids, accounts for 20% [221] to 50% [226] of the basal metabolic rate. Protein turnover rates, usually measured using (stable) isotope tracers, give an estimate of the amount of protein that is mobilised within the body as a whole, including both synthesis and breakdown of protein.

Elevated breakdown of whole-body protein has been reported in patients with various tumour types with or without weight loss [62,66,84,107,116,133,141,186, 190,199]. Studies in patients with lung cancer reported elevated protein turnover in weight-stable as well as weight-losing patients as compared to healthy control subjects [89,142,171] or non-cancerous patients without weight loss [66]. However, protein turnover in lung cancer patients was not correlated with the degree of weight loss [66,89]. Because of the close relation between protein turnover and energy expenditure [226], elevation of protein turnover rate will increase metabolic rate. However, in lung cancer patients this relation is not straightforward, since no correlation between whole-body protein turnover and energy expenditure was observed [66]. Thus, elevated whole-body protein turnover has been confirmed in several studies, whereas its relation with weight loss remains unclear.

Protein turnover in specific peripheral tissues of cancer patients has been investigated in a few studies in various tumour types. Muscle protein synthesis was decreased in the leg of patients with mixed tumour types, resulting in a net loss of protein [131]. This loss of protein was characterised by elevated release of amino acids, especially alanine,

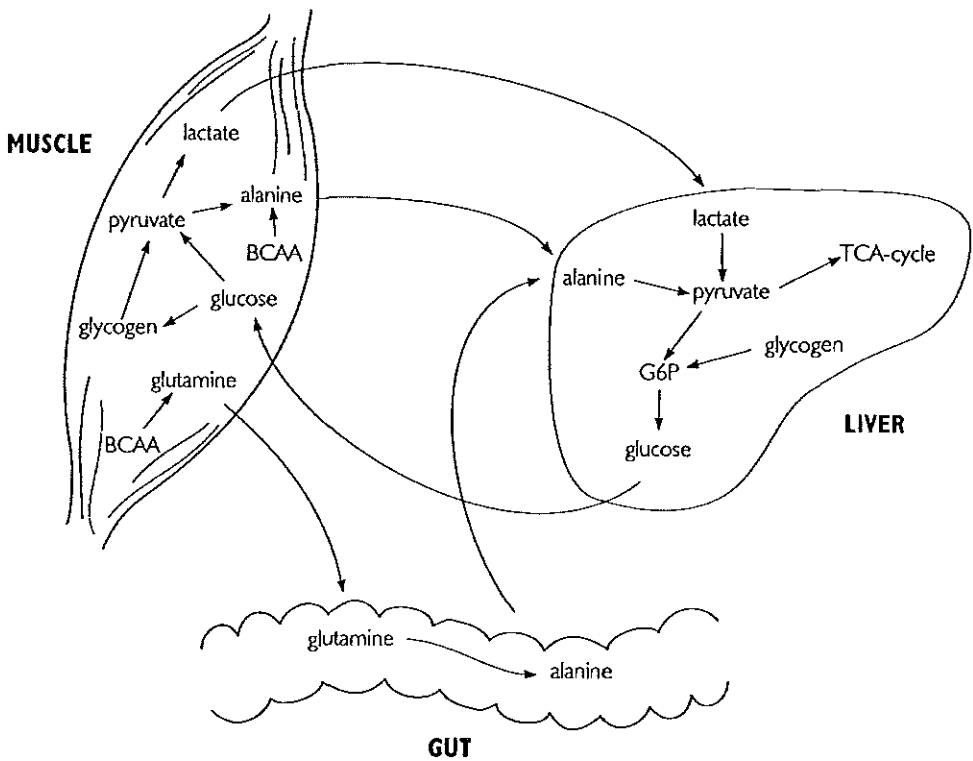

Figure I.1

Schematic pathway of hepatic gluconeogenesis and related metabolic routes in muscle and the intestine. Many of these reactions are reversible, but for clarity only flow directions of gluconeogenesis are indicated, including glucose-alanine cycling and Cori cycling. BCAA, branched-chain amino acids.

as compared to healthy control subjects [24,95]. In one study muscle protein synthesis was measured in percutaneous muscle biopsies from cancer patients in the fed state [64]. This also revealed decreased protein synthesis, whereas no change in whole-body protein turnover was observed.

Carbohydrate metabolism

Altered glucose metabolism in the cancer-bearing host may have been one of the first metabolic alterations recognised in patients with malignancies. Marks et al [136] reported that impaired glucose tolerance was frequent in malignant disease, and this was confirmed by others [33,34,174,180].

Furthermore, elevated glucose turnover was reported in cancer patients with various tumour types [88,96,133,202,204]. In weight-losing cancer patients, levels of glucose turnover were higher than in weight-stable, non-cancer patients [133]. Koea et al [116] found a highly significant correlation between tumour bulk and glucose turnover in patients with various tumour types. In another study it was shown that initially high glucose turnover rates in weight-losing cancer patients with mixed tumour types increased even further within one month [33]. Preferential use of glucose by tumour cells has been reported already around 1930 [217], and was suggested to explain the need for elevated glucose production in the cancer host [79,96,115,122,138,148,208]. It was estimated that the elevated glucose turnover could account for up to 40% of the increase in energy expenditure observed in these patients [133].

Proposed mechanisms underlying elevated glucose production are enhanced gluconeogenesis or glycogenolysis. **Figure 1.1** shows a schematic pathway of hepatic gluconeogenesis and related metabolic routes in muscle and the intestine. Elevated gluconeogenesis from lactate, glycerol, and alanine has been reported in cancer patients. Glucose production from glycerol is only minor, being estimated as ~3% of total endogenous glucose production in cancer [133]. Lactate produced by glycolytic activity of tumour cells [91,98,220] and skeletal muscle in cancer patients [95] is transported to the liver where it is recycled back to glucose, though the gluconeogenic pathway. This cycling of glucose to lactate and back to glucose is called Cori cycling. Since the anaerobic breakdown of glucose to lactate produces 2 ~P and recycling of lactate to glucose costs 6 energy-rich phosphate bonds, the net cost of this pathway is 4 ~P per mole glucose produced. In weight-losing patients with colorectal cancer [97] or solid tumours [96], Cori cycling accounted for 32 - 50% of the total glucose turnover, respectively. Because of the high energy cost of Cori cycling, it was suggested that this could contribute to weight loss in cancer patients [79]. However, Young [226] suggested that less than 10%

of total energy expenditure could be accounted for by Cori cycling.

Protein and carbohydrate metabolism interrelated

Enhanced hepatic glucose production in lung cancer could also be due to increasing rates of gluconeogenesis from muscle-derived amino acids [116,189]. It has been estimated that glucose production from amino acids and glycogen accounts for 20-50% of total glucose production in cancer patients [204].

The main amino acids excreted from skeletal muscle are alanine and glutamine which account for >50% of amino acid nitrogen released by muscle in healthy subjects [69]. Muscle provides ~70% of the alanine released into the systemic circulation and thus is the major source of plasma alanine [40]. Additional amounts of alanine are released from the gut [69,165]. Alanine is synthesised by transamination of pyruvate [95] coupled by breakdown of branched-chain amino acids (BCAA: leucine, valine, isoleucine). Seventy percent of the alanine from muscle is pyruvate-derived, whereas 30% is derived by proteolysis in healthy subjects [36,40,173].

Alanine is preferentially taken up by the liver, whereas a major part of glutamine is taken up by the gut and the kidney (**Figure 1.1**). The liver uses alanine for glucose production; alanine is the principal gluconeogenic amino acid used by the liver [71,109,201]. The conversion of glucose to alanine in muscle and back to glucose in the liver is called the glucose-alanine cycle [68]. It is estimated that glucose-alanine cycling accounts for 5-12% of total gluconeogenesis [68,69].

The first data on elevated gluconeogenesis from alanine in weight-losing cancer patients were obtained in a group with mixed tumour types and compared with an undefined group of weight-losing non-cancerous patients [219]. The contribution of alanine to gluconeogenesis in this study was estimated at ~5% [219]. In patients with localised oesophageal cancer, gluconeogenesis from alanine was increased but not related with weight loss [25]. In lung cancer patients with weight loss, both glucose turnover and protein breakdown were increased [89] suggesting a relation between these two processes. In another study by the same author, decreased plasma alanine levels were reported [88]. The authors suggested that alanine was used as a substrate for endogenous glucose production in these patients [88]. Thus, although gluconeogenesis from alanine is suggested to be elevated in cancer, its significance in lung cancer and the relation with weight loss remains to be determined.

Liver energy metabolism in cancer

Elevated gluconeogenic rates in cancer may put an increased demand on energy metabolism due to the high energy costs of this pathway [100]. As the liver is the main site for gluconeogenesis, especially so for alanine, energy utilisation in this organ may be particularly influenced.

In a study in healthy rats, a close correlation between gluconeogenesis from alanine and hepatic ATP concentrations was reported [147]. In isolated hepatocytes from sarcoma-bearing rats, gluconeogenesis was increased during incubation with lactate as compared to hepatocytes from healthy rats [211]. This increased gluconeogenesis resulted in a 42% decrease in ATP levels, whereas in healthy rats, no change in ATP was observed during incubation with lactate. This suggests that elevated rates of gluconeogenesis in the cancer-bearing host put an increased demand on hepatic energy stores which may contribute to weight loss. Hepatic energy status was reduced in rats bearing prostate carcinomas or sarcomas, as observed by decreased ATP levels and increased P_i/ATP ratios, indicating decreased phosphorylation status [49,181,211]. Changes in hepatic P_i/ATP ratios were already detected before the development of significant weight loss and prior to a decrease in food intake [77,181].

In humans with cancer, information on liver energy status is very limited. In one study in cancer patients with various tumour types, liver ATP levels tended to be lower in patients with weight loss than in healthy controls [51]. As these data were obtained in livers without malignant infiltration, metabolic alterations rather than morphologic changes were suggested to be involved. However, whether decreases in ATP were related with increased gluconeogenic activity within the liver need to be confirmed.

Increasing evidence is emerging for elevated muscle protein degradation and increased endogenous glucose production in cancer. These processes may be interrelated, through muscle-derived alanine as a substrate for gluconeogenesis. No information on gluconeogenesis from alanine is available in lung cancer and it is not known whether this process is related to weight loss. Also, studies in humans with cancer have not provided information regarding the actual localisation of the gluconeogenic process. In addition, it is not known whether changes in hepatic glucose metabolism are associated with alterations in energy metabolism in the liver.

Aim

The present research project is aimed at obtaining quantitative information on gluconeogenesis from alanine and on liver energy metabolism in relation with weight loss in lung cancer patients. The following specific study questions were formulated:

1. Is liver gluconeogenesis increased and ATP concentration decreased in lung cancer patients, and are these alterations related with weight loss?
2. Can depleted liver energy stores in advanced lung cancer patients be restored by ATP infusion, and does this affect glucose metabolism?
3. Do alterations in hepatic gluconeogenesis predict subsequent weight loss in cancer patients?

Techniques used for investigation of substrate metabolism

Substrate metabolism in humans has been extensively investigated using (stable) isotope tracer infusions. More recently, magnetic resonance spectroscopy (MRS), has been used to study metabolism in body tissues *in vivo*. MRS provides information non-invasively on cellular metabolism, such as energetics and substrate turnover [44]. The metabolic state of the liver has been studied using ^{31}P MRS in healthy subjects [18,19,23,46,195] as well as in patients [45,48,51,58,103,143,146,215].

Other applications of ^{31}P MRS have been the dynamic investigation of liver substrate metabolism such as gluconeogenesis. In studies in healthy rats [30] or humans [50,177] bolus infusion of L-alanine as a gluconeogenic substrate induced increasing concentrations of hepatic phosphomonoesters (PME) and phosphodiesters (PDE). The increase in PME was observed to be dose-dependent [50]. Liver metabolite extraction in rats after alanine infusion confirmed these MRS changes to be secondary to increases in 3-phosphoglycerate (3PG) and phosphoenolpyruvate (PEP), respectively (**Figure 1.2**)[50]. Phosphorus MRS has also been applied to study the dynamics of energy metabolism in human liver, by measuring changes in ATP levels or P_i/ATP ratios during a metabolic challenge [16,157,183]. In **Figures 1.3** and **1.4**, examples of an image with the localisation used for ^{31}P MRS and a hepatic ^{31}P MR spectrum of a healthy subject are shown, respectively, as obtained by the technique used in the present thesis.

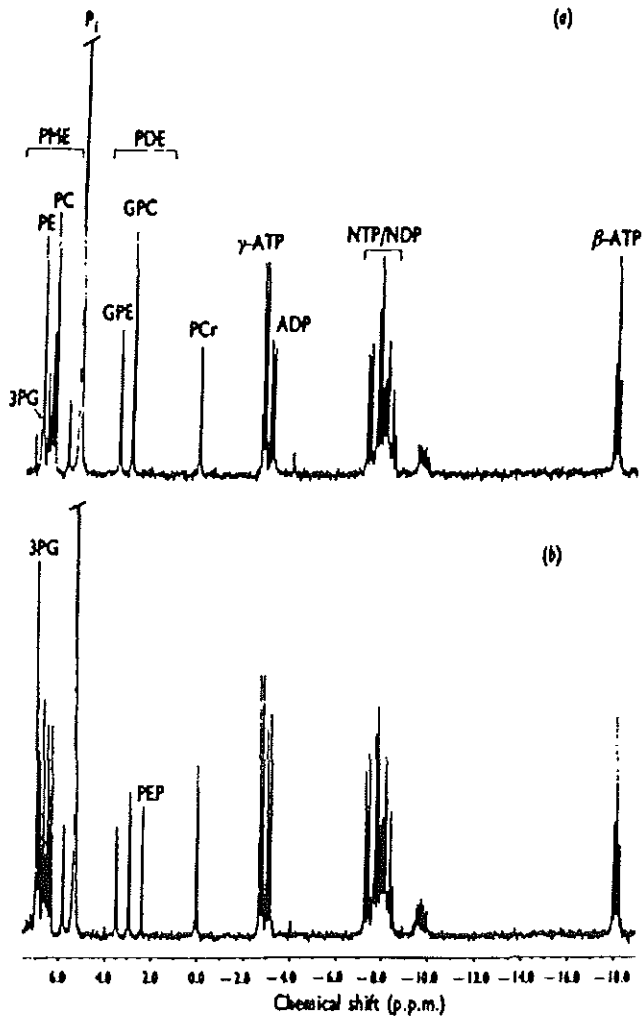

Figure I.2

High-resolution ^{31}P MR spectra of extracts from control rat liver (a) and rat liver after 11.2 mmol/kg L-alanine infusion (b). Proton-decoupled ^{31}P NMR spectra were acquired at 8.4T using a 35° excitation pulse with a repetition time of 10s. Peak assignments: 3PG, 3-phosphoglycerate; PE, phosphorylethanolamine; PC, phosphorylcholine; P_i, inorganic phosphate; GPE, glycerolphosphorylethanolamine; GPC, glycerphosphorylcholine; γ-ATP and β-ATP, γ- and β-phosphate groups of ATP, respectively; NTP/NDP, nucleotide tri- and diphosphates; PCr, phosphocreatine (added as an internal chemical shift and concentration standard); PEP, phosphoenolpyruvate. The approximate positions of the multicomponent PME and PDE peaks are indicated [50].

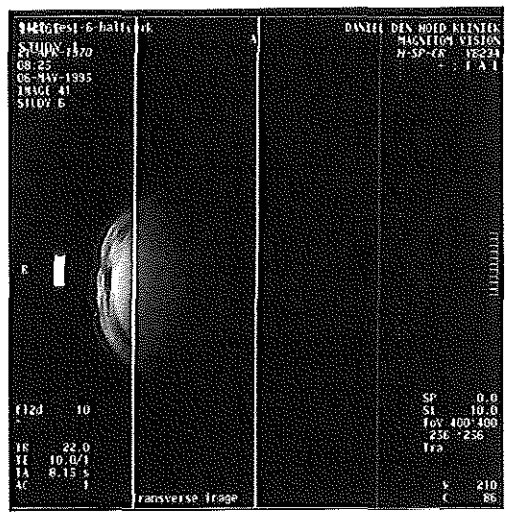

Figure 1.3

Transverse one-dimensional chemical shift image (1D-CSI) of the liver of a healthy subject obtained with a 16 cm surface coil at 2.0 Tesla showing 1×4 phase-encoding steps (volume 40×10×4 cm^3). A methylene diphosphonate (MDP) reference sample in the centre of the coil is visible (left). The region of interest (second slice from the left) is used for measurements.

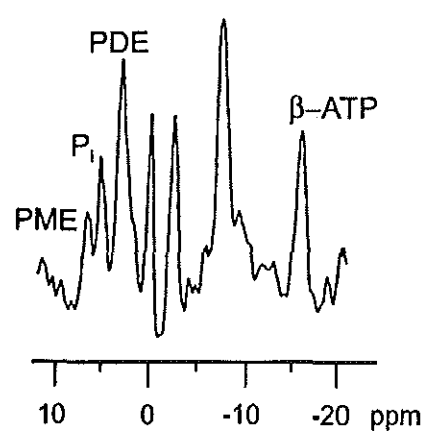

Figure 1.4

Representative ^{31}P MR spectrum of a normal human liver acquired at 2.0 Tesla using a 135° excitation pulse (60° weighted average in the liver volume) with a repetition time of 1 s. Peak assignments: PME, phosphomonoesters; P_i, inorganic phosphate; PDE, phosphodiesters; β-ATP, β-phosphate group of ATP. The scale represents chemical shift in ppm.

Outline of thesis

In **Chapter 2**, a study on whole body glucose and alanine turnover, and gluconeogenesis from alanine in patients with lung cancer is reported, and their respective relation with the presence and degree of weight loss is analysed. **Chapter 3** explores the question of whether phosphomonoesters in the liver of lung cancer patients, as observed by ^{31}P MRS, reflect gluconeogenic intermediates. In **Chapter 4**, hepatic gluconeogenesis from alanine in lung cancer patients with or without weight loss is investigated dynamically during intravenous infusion of L-alanine, with special emphasis on changing concentrations of gluconeogenic intermediates within the liver. Hepatic energy metabolism and phosphorylation status in lung cancer patients before and during L-alanine infusion is described in **Chapter 5**. Effects of intravenous ATP infusion in lung cancer patients on hepatic energy and phosphorylation status, and glucose metabolism are described in **Chapter 6** and **Chapter 7**, respectively. **Chapter 8** reports on gluconeogenesis and liver metabolism in patients with breast cancer. In **Chapter 9**, the prognostic value of elevated hepatic concentrations of MRS-observed gluconeogenic intermediates for the development of weight loss is investigated. The results of these studies are discussed and placed in a broader perspective of altered metabolism and weight loss in lung cancer in **Chapter 10**.

2

Weight loss and gluconeogenesis from alanine in lung cancer patients

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Susanne Leij-Halfwerk,[1] Pieter C Dagnelie,[1,2] J Willem O van den Berg,[1]
J Darcos L Wattimena,[1] Christien H Hordijk-Luijk,[1] JH Paul Wilson[1]

[1]Department of Internal Medicine II, Erasmus University Medical Center Rotterdam, Rotterdam
[2]Department of Epidemiology, University Maastricht, Maastricht, The Netherlands

Abstract

The role of gluconeogenesis from protein in the pathogenesis of weight loss in lung cancer has remained unclear. The aim was to study gluconeogenesis from alanine in lung cancer patients and to analyze the relation with the degree of weight loss. In a cross-sectional study primed-constant infusions of [6,6-$^{2}H_{2}$]-D-glucose and [3-^{13}C]-L-alanine were used to assess whole body glucose and alanine turnover, and gluconeogenesis from alanine in weight-losing (CaWL, n=9) and weight-stable (CaWS, n=10) lung cancer patients, and healthy subjects (C, n=15).

Energy intake and plasma alanine concentrations were not significantly different in CaWL, CaWS and C. Whole body glucose production was significantly higher in CaWL when compared with CaWS and C (0.74 ± 0.06, mean ± SEM, *vs.* 0.55 ± 0.04 and 0.51 ± 0.04 mmol/kg/h, respectively, $P<0.01$). Alanine turnover was significantly elevated in CaWL (0.57 ± 0.04 *vs.* 0.42 ± 0.05 (CaWS) and 0.40 ± 0.03 (C) mmol/kg/h, respectively, $P<0.01$). Also, gluconeogenesis from alanine was significantly higher in CaWL (0.47 ± 0.04 mmol/kg/h *vs.* 0.31 ± 0.04 and 0.29 ± 0.04, respectively, $P<0.01$). The degree of weight loss was positively correlated with glucose and alanine turnover, as well as with gluconeogenesis from alanine (r= 0.45, r= 0.45, and r= 0.45, respectively, all $P<0.01$).

Aberrant glucose and alanine metabolism exists in weight-losing lung cancer patients. These changes are related with the degree of weight loss and not with the presence of lung cancer per se.

Introduction

Weight loss is frequently observed in patients with cancer. There is evidence showing that weight loss is related to poor therapy outcome and reduced survival [43,56,198]. Weight loss may occur in early stages of cancer, even before other signs of the presence of a tumor appear. Although animal studies suggest that the development of weight loss in cancer is closely related to both the presence and the size of the tumor [5], in humans relations with the size, extent, stage, and type of the tumor are less clear [115].

When cancer patients lose weight, they lose both fat and muscle mass [94]. In comparison with anorectic patients with the same degree of weight loss, cancer patients lose relatively more muscle mass [60]. Although there is no doubt that anorexia is a frequent phenomenon in cancer patients, reduced food intake by itself does not explain the loss in lean body mass observed in these patients, implicating that derangements in host metabolism must be a major contributing factor [43,64].

Metabolic alterations which have been previously reported in cancer patients include

elevated glucose turnover and increased endogenous glucose production [88,96,115,133,204]. Other studies reported net protein catabolism in weight-losing cancer patients, mainly from muscle, whereas in weight-stable cancer patients no change in protein catabolism was observed [186]. It has been hypothesized that increased amounts of alanine, originating from muscle degradation in cancer patients, are used for gluconeogenesis [5,26], with loss of nitrogen through urea production as a consequence. However, studies on gluconeogenesis from alanine in tumor-bearing animals [3] and cancer patients [25,219] are inconclusive as far as the relation with weight loss is concerned. Furthermore, studies so far do not account for anorexia as a potential confounder since information on food intake was usually not acquired.

Lung cancer is a tumor type with a high frequency of weight loss in which anorexia or obstruction are less likely to be the cause of the observed weight loss. Although elevated protein turnover rates have been reported in lung cancer patients with weight loss, the role of gluconeogenesis from protein in the pathogenesis of weight loss in this type of cancer has remained unclear [142].

The aim of the present study was to study protein-derived gluconeogenesis in a well defined group of lung cancer patients. For this purpose alanine was selected as a precursor of gluconeogenesis because alanine is the key protein-derived glucose precursor utilized by the liver [201]. Patients with and without weight loss were included in order to differentiate between possible effects of lung cancer and the degree of weight loss on gluconeogenesis in these patients.

Subjects and methods

Subjects

Patients with histologically proven non-small cell lung cancer attending the outpatient department of the University Hospital Rotterdam, Rotterdam, The Netherlands, were recruited. Two groups of lung cancer patients were studied: patients, who had ≥ 5% weight loss in the previous 6 months (weight-losing, WL), and patients without weight loss in the previous 6 months (weight-stable, WS). Patients were only included after an interval of at least 3 months after surgery, or at least 4 weeks after chemotherapy or radiotherapy. Patients who were in remission or apparently cured were excluded. Other exclusion criteria were pregnancy, metabolic disease, corticosteroid treatment, liver metastases as verified by CT or ultrasound, alcohol consumption of more than 10 glasses per week, and use of a slimming diet. Healthy control subjects without weight loss were included as a reference. The study was approved by the Medical Ethical Committee of the Erasmus University Medical Center Rotterdam, Rotterdam, the Ne-

therlands. All participants signed informed consent.

Dietary intake

Food intake was recorded using a standard food diary during seven days preceding the experiment. The subjects received oral and written information from a trained nutritionist (SL) about the procedures for filling in the diary and registering all consumed drinks and foods using household measures. Subjects were asked to maintain their usual dietary habits and none reported any significant changes in their diet. Subjects abstained from alcohol during 5 days prior to the experiment. All medication taken was noted.

Household measures were converted into weights using a standard food conversion table (Department of Human Nutrition, Wageningen, The Netherlands) and a nutrition software program (Komeet version 2.0, B-Ware, Arnhem, The Netherlands). Average daily intake of energy, fat, carbohydrate and protein was calculated.

Study design

Body weight, height, upper-arm circumference, and thickness of four skinfolds (triceps, biceps, subscapular, supra iliac, using a standard skinfold caliper (Holtain Ltd., London, Great Britain)) were measured and the body mass index was calculated. The upper-arm muscle circumference was calculated as described by Frisancho [75].

The subjects were studied in the morning after an overnight fast. A cannula (0.8×25 mm) was positioned in the left cubital vein for the infusion of stable isotope tracers. In the contralateral cubital vein, an identical cannula was positioned for blood sampling. To study gluconeogenesis, a solution was prepared containing [6,6-$^{2}H_{2}$]-D-glucose, 98 atom%, and [3-^{13}C]-L-alanine, 99 atom% (Mass Trace, Woburn, USA), in water and this was sterilized by autoclavating in glass vials. A priming dose of 30 µmol/kg [6,6-$^{2}H_{2}$]-D-glucose was administered followed by a continuous infusion of 10 µmol/kg/h [6,6-$^{2}H_{2}$]-D-glucose for 90 minutes. Simultaneously, a priming dose of 80 µmol/kg [3-^{13}C]-L-alanine was given followed by a continuous infusion of 40 µmol/kg/h [3-^{13}C]-L-alanine during 90 minutes. Both tracer solutions were infused using calibrated syringe pumps (Perfusor® fm, Braun, Germany).

Venous blood samples were drawn immediately before the isotope infusions were started, and at 10 min intervals from 60 to 90 min., when steady state conditions during the tracer infusions had been achieved. Plasma concentrations of glucose, alanine, insulin, glucagon, and thyroid hormones as well as isotopic enrichment of glucose and alanine were determined.

Analytical methods

Blood samples were collected in tubes containing lithium heparin (Becton Dickinson Vacutainer®, Meylan Cedex, France) and immediately stored on ice. After centrifugation (10 min., 1200*g*, 4°C), the plasma was collected and stored at -20°C until analyzed. An aliquot of the infusate was analyzed to document actual concentrations of the tracers in each study.

Blood glucose concentrations were determined enzymatically with glucose-oxidase and peroxidase (Boehringer Mannheim, Mannheim, Germany). Plasma alanine was measured enzymatically as described by Williamson [223]. Plasma concentrations of insulin and glucagon were determined by radio immuno assay techniques (Biosource, Fleurus, Belgium and Euro-Diagnostica, Sweden, respectively). Serum total T_4 and T_3 were measured by radio immuno assay, and total rT_3 was measured according to Bauer [11].

Isotopic enrichments were determined using the following procedures. Plasma was deproteinized by adding 0.3 M barium hydroxide (Sigma Diagnostics, St.Louis) and 0.3 M zinc sulphate (Merck, Darmstadt, Germany). After centrifugation (8 min.,15000*g*, 4°C) the supernatant was applied to an ion exchange column (mixed bed: AG50W-X8 and AG1-X8, 200-400 mesh, 0.2 g each; BioRad, California). Glucose and alanine were eluted from the column using water and 4 M ammonium hydroxide (Merck, Darmstadt, Germany), respectively, and dried under nitrogen. A glucose derivate (aldonitril penta acetate) was made according to Varma et al [216]. An alanine t-butyldimethylsilyl derivate was prepared as described by Chaves Das Neves et al [32].

Isotopic enrichments were measured by injecting 1 µl samples with a split ratio of 50:1 on a fused silica capillary column of 25 m × 0.22 mm, coated with 0.11 µm HT5 (SGE, Victoria, Australia). The relative isotopic enrichments of deuterated glucose and carbon-13 alanine were determined using a Carlo Erba GC8000 gas chromatograph coupled to a Fisons MD800 mass spectrometer (GC-MS) (Interscience B.V., Breda, The Netherlands) in electron impact ionization mode. The variation coefficient in enrichment was 0.2 mole% for both [6,6-$^2H_2$]-glucose and [3-^{13}C]-alanine, and no concentration effect was observed at this level of mole% enrichment. Ions were selectively monitored at mass per unit charge (*m/z*) 187 for natural glucose and 189 for the deuterated molecule. The isotopic enrichment of [3-^{13}C]-alanine was determined at the *m/z* ratios 260 and 261 for carbon-12 and carbon-13 alanine, respectively [137].

Total enrichment of carbon-13 glucose was measured separately (aldonitril penta-acetate derivation) using a gas chromatograph combustion isotope ratio mass spectrometer (GC-IRMS) (Optima, Micromass UK, Middlewich, Cheshire, Great Britain). The [^{13}C]-glucose enrichment in atom% excess (APE) was monitored after combustion to CO_2 at mass 44 for carbon-12 and 45 for carbon-13, respectively.

Calculations and statistics

Whole body rate of appearance (R_a) of glucose and alanine were calculated during steady state following a one-compartment model, using the formula:

$$R_a = F_i \times ((IE_i / IE_{ecf}) - 1), \quad (1)$$

where F_i is the isotope infusion rate (mmol/kg/h), IE the isotopic enrichment of the infusate (mole% excess, MPE), and IE_{ecf} the isotopic enrichment of the extracellular fluid (plasma)(MPE)[224]. The percentage glucose produced from alanine equals:

$$(IE\ ^{13}C\text{-glucose}_{plasma} / (IE\ ^{13}C\text{-alanine}_{plasma} \times 0.33)) \times 100\% \quad (2)$$

where IE ^{13}C-glucose$_{plasma}$ is the isotopic enrichment in plasma (expressed as $^{13}CO_2$ atom% excess, APE). Since alanine has three carbon atoms, 100% enrichment of [3-^{13}C]-alanine (mole percent excess, MPE) is equivalent to 33% enrichment of $^{13}CO_2$ from alanine (atom percent excess, APE). Therefore, the enrichment of alanine (MPE) is multiplied by 0.33 in order to obtain comparable enrichments (i.e. expressed as $^{13}CO_2$ atom% excess) for both alanine and glucose. Gluconeogenesis from alanine (mmol/kg/h) is then obtained as:

$$\%\ \text{glucose from alanine} \times R_a\ ([^2H_2]\text{-glucose}). \quad (3)$$

Finally, the percentage of alanine converted into glucose was calculated by dividing the rate of gluconeogenesis from alanine by the rate of appearance of alanine [40].

Results are presented as means ± standard error of the mean (SEM). Differences between group means were compared simultaneously using analysis of variance, adjusting for age. Bonferroni correction was applied to allow for multiple testing. The correlation between variables was analyzed using Pearson's correlation coefficient. Multiple linear regression was used to analyze interrelationships with adjustment for possible confounders. Differences were stated statistically significant at P values < 0.05.

Results

Study population

Nineteen lung cancer patients, nine weight-losing (7 males, 2 females) and ten weight-stable (6 males, 4 females), and fifteen healthy control subjects (3 males, 12 females) were included in the study. The lung cancer patients had been diagnosed with non-small cell lung cancer stage IIIA (one WS, three WL), IIIB (three WS, two WL) or IV (six WS, four WL) (WHO grading system). The characteristics of the study population are described in **Table 2.1**. Age was significantly higher in the lung cancer patients compared to the healthy control subjects (P<0.05). The WL patients had lost 9.0 ± 1.4 kg or 12% (range 6 - 22%) of their pre-illness stable body weight within the six months preceding the study, in contrast with WS lung cancer patients and healthy subjects. Body weight,

percent of ideal body weight, body mass index, mid-upper arm circumference, and the sum of four skinfolds were significantly lower in WL lung cancer patients compared with WS patients and control subjects ($P<0.05$). Upper-arm muscle circumference was slightly, but not significantly, lower in the WL cancer patients than in WS cancer patients and healthy control subjects. Albumin and transthyretin (prealbumin) concentrations were lower in the WL than in WS patients and healthy control subjects ($P<0.01$).

Dietary intake

None of the subjects reported any changes in food intake compared with pre-illness food intake. Carbohydrate intake preceding the experiment was at least 200 g/d in all subjects (**Table 2.2**). No significant differences in energy, carbohydrate, protein or fat intake were found among the groups.

Glucose and alanine metabolism

Baseline plasma glucose and alanine concentrations were not significantly different between the lung cancer patients and healthy control subjects, but glucose levels in WL patients were significantly higher than in WS patients ($P<0.05$) (**Table 2.3**). Steady state was reached within 60 minutes after the start of the isotope tracer infusions as judged from visual inspection of isotope enrichment profiles. Relative isotopic enrichments of [$^2H_2$]-glucose, [^{13}C]-alanine and [^{12}C]-glucose in plasma during steady state were not statistically different between the groups.. Turnover rates of glucose and alanine are shown in **Figure 2.1**. Whole-body glucose turnover was significantly higher in the WL lung cancer patients than in WS patients or healthy control subjects ($P<0.01$). Similarly, alanine turnover was significantly higher in the WL lung cancer patients than in the two other groups ($P<0.01$). Gluconeogenesis from alanine was significantly elevated in the WL lung cancer patients when compared with both WS lung cancer patients and healthy controls ($P<0.01$). The percentage glucose derived from alanine varied from 64.6 ± 4.2 in the WL and 60.2 ± 11.8 in WS lung cancer patients to 55.0 ± 4.8% in healthy control subjects (N.S.). The percentage of alanine converted into glucose was 84.2 ± 6.9, 70.4 ± 7.1, and 70.0 ± 7.1%, respectively (N.S.). Glucose and alanine turnover, and gluconeogenesis from alanine were significantly correlated with the degree of weight loss (r=0.45, 0.45, and 0.45, respectively, all $P<0.01$) and inversely correlated with percentage of ideal body weight (r= -0.38, -0.44, and -0.50, respectively, all $P<0.05$). The relation between gluconeogenesis from alanine in lung cancer patients and percentage of weight loss is shown in **Figure 2.2.**

Table 2.1

Characteristics of the study population[1]

	Control	Lung cancer	
		WS	WL
	(n=15)	(n=10)	(n=9)
Age (y)	52 (29-72)[a,b,2]	63 (38-75)[a]	69 (53-81)[b]
Height (m)	1.74 ± 0.02	1.69 ± 0.03	1.71 ± 0.03
Weight (kg)	77.2 ± 2.5[a]	73.5 ± 3.8[b]	60.9 ± 3.5[a,b]
% Ideal body weight (%)	123.0 ± 4.1[a]	119.3 ± 6.4[b]	96.0 ± 5.0[a,b]
Weight loss (%; 6 mo)	-	-2.0 ± 1.0[a]	12 ± 2[a]
Body mass index (kg/m^2)	25.7 ± 0.8[a]	25.6 ± 1.3[b]	20.9 ± 1.1[a,b]
Arm circumference (cm)	32.1 ± 1.0[a]	30.3 ± 1.4[b]	26.1 ± 1.2[a,b]
Arm muscle circumference (cm)	26.1 ± 0.9	26.0 ± 0.9	24.4 ± 1.4
Sum of skinfolds (mm)[3]	69.6 ± 4.0[a]	64.6 ± 8.4[b]	29.8 ± 3.8[a,b]
Albumin (g/L)	46 ± 1[a]	44 ± 1[b,4]	38 ± 2[a,b,4]
Transthyretin (g/L)[5]	0.29 ± 0.01[a]	0.28 ± 0.02[b,4]	0.17 ± 0.02[a,b]

[1] Mean ± SEM. Differences between group means were appraised by analysis of variance; WS, weight-stable; WL, weight-losing. Means within a row with a superscript letter in common are significantly different, $P<0.05$.

[2] Mean (range).

[3] Calculated as the sum of the biceps, triceps, subscapular, and suprailiac skinfolds (mm).

[4] One value missing.

[5] Prealbumin.

Turnover measurements were not correlated with gender or age. Multivariate analysis showed that only the degree of weight loss was a significant predictor of glucose and alanine turnover as well as of gluconeogenesis from alanine. Neither energy, nor carbohydrate or protein intake on the day prior to the experiment were related with plasma glucose and alanine concentrations nor with turnover measurements.

Hormone concentrations

Fasting insulin and glucagon concentrations, and insulin:glucagon ratios were not significantly different between lung cancer patients and healthy controls (**Table 2.4**). Glucagon levels in WL patients were lower than in WS patients ($P<0.01$). Insulin / glucagon ratios were significantly correlated with alanine turnover (r= -0.36, $P=0.04$).

Table 2.2

Daily intake of energy and selected nutrients in healthy control subjects, weight-stable and weight-losing lung cancer patients.[1]

		Control	Lung cancer	
			WS	WL
		(n=15)	(n=10)	(n=9)
Energy	(kJ/day)	7838 ± 401	8260 ± 405	8170 ± 644
Fat	(g/day)	77 ± 5	80 ± 5	74 ± 9
	(energy %)	37 ± 1	37 ± 1	34 ± 2
Carbohydrate	(g/day)	206 ± 13	224 ± 12	231 ± 16
	(energy %)	45 ± 2	46 ± 1	48 ± 1
Protein	(g/day)	79 ± 4	85 ± 5	81 ± 5
	(energy %)	17 ± 1	18 ± 1	17 ± 1

[1] Mean ± SEM. Based on 7-day food record. Differences between group means were appraised by analysis of variance, adjusted for age. WS, weight-stable; WL, weight-losing. Means within a row with a superscript letter in common are significantly different, $P<0.05$.

No other significant correlations between insulin or glucagon concentrations and turnover measure- ments were observed. Thyroxine (T_4) and T_3 levels did not differ between any of the groups. In contrast, rT_3 and rT_3 / T_3 ratios were significantly elevated in WL lung cancer patients ($P<0.05$) and were correlated with the degree of weight loss (r=0.47 and r=0.53, respectively, $P<0.01$). Reverse T_3 concentrations were also correlated with glucose turnover (r=0.36, $P=0.03$).

Discussion

The present study was aimed at quantifying gluconeogenesis from alanine in both weight-losing and weight-stable patients with advanced lung cancer compared to healthy control subjects. Although several authors have reported abnormal glucose metabolism in patients with advanced malignant disease [60,96,116,133,204], there are few studies in lung cancer patients. Heber et al [88] reported elevated glucose turnover rates in 65% of non-small cell lung cancer patients with weight loss.

Furthermore, elevated whole-body protein turnover has been reported in cancer patients, using different amino acids [62,64,116,133,186], but data in lung cancer

Table 2.3

Plasma glucose and alanine concentrations, and isotopic enrichments (IE) of plasma glucose and alanine during turnover measurements in healthy control subjects, weight-stable and weight-losing lung cancer patients.[1]

	Control	Lung cancer	
		WS	WL
	(n=15)	(n=10)	(n=9)
Glucose (mmol/L)	5.9 ± 0.2	5.2 ± 0.2[a]	5.8 ± 0.3[a]
Alanine (μmol/L)	347 ± 6	368 ± 8	349 ± 13
Isotopic enrichment of plasma:			
$^2H_2$-glucose (MPE)[2]	2.19 ± 0.25	1.88 ± 0.19	1.55 ± 0.13
^{13}C-alanine (MPE)[2]	8.78 ± 0.78	8.32 ± 0.62	7.22 ± 0.60
^{13}C-glucose (APE)[3]	1.52 ± 0.14	1.49 ± 0.18	1.53 ± 0.13

[1] Mean ± SEM. Isotopic enrichments were determined under steady state conditions during primed-constant infusions of [6,6-$^2H_2$]-D-glucose and [3-^{13}C]-L-alanine. Differences between group means were appraised by analysis of variance, adjusted for age; WS, weight-stable; WL, weight-losing. Means within a row with a superscript letter in common are significantly different, $P<0.05$.

[2] Mole% excess.

[3] $^{13}CO_2$ Atom% excess.

patients are sparse and inconclusive regarding the role of weight loss. Richards et al [171], using a primed-constant infusion of [^{15}N]-glycine, reported increased protein turnover rates in patients with advanced lung cancer with weight loss. Melville et al [142] also reported elevated protein turnover in newly diagnosed, non-metastatic lung cancer patients using [^{13}C]-leucine, but these authors did not observe any relation with weight loss. Fearon et al [66] studied lung cancer patients (stage II/III) both with and without weight loss; whole body protein turnover rates as measured by [^{15}N]-glycine were elevated in all cancer patients, but again, no relation with weight loss was observed. Heber et al [89] reported elevated protein turnover in lung cancer patients as measured by [^{14}C]-lysine, which was inversely correlated with percent of ideal body weight but not with the degree of weight loss. In the present study in lung cancer patients alanine turnover was significantly correlated with both the degree of weight loss and percent ideal body weight.

So far, gluconeogenesis from alanine in cancer patients has only been studied using a single bolus infusion of [^{14}C]-alanine, i.e. in non-steady state conditions. Waterhouse et

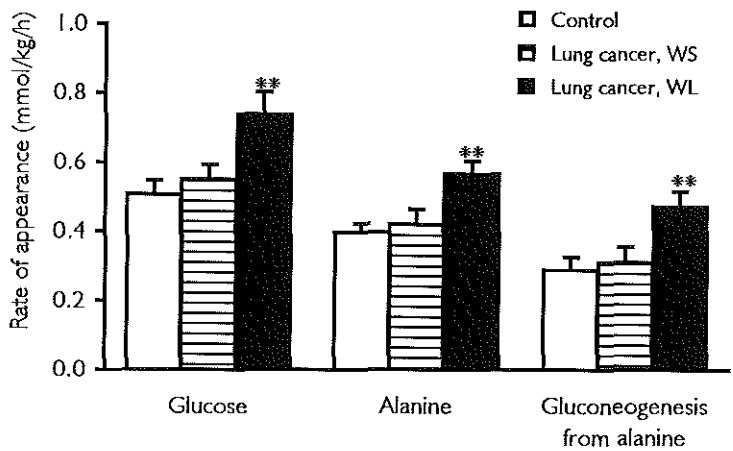

Figure 2.1

Whole body rate of appearance (turnover) of glucose and alanine, and gluconeogenesis from alanine in healthy control subjects (n=15), weight-stable (WS, n=10), and weight-losing (WL, n=9) lung cancer patients. Turnover rates were assessed using primed-constant infusions of [6,6-$^{2}H_{2}$]-glucose and [3-^{13}C]-alanine. Bars and error bars represent means ± SEM. **Significantly different from WS lung cancer patients and healthy controls, $P<0.01$ (ANOVA, adjusted for age).

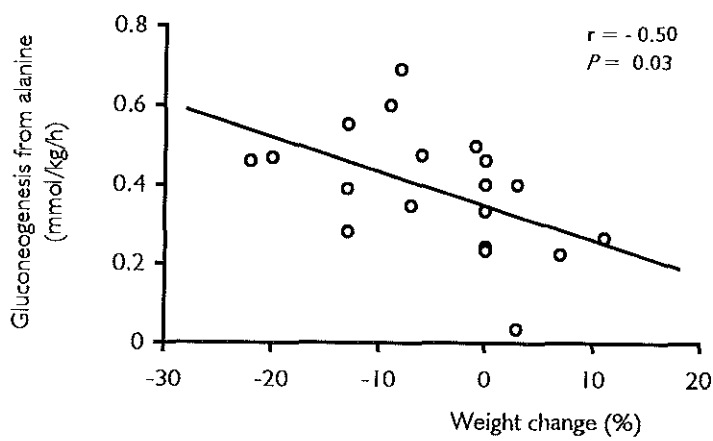

Figure 2.2

Gluconeogenesis from alanine in lung cancer patients plotted against the degree of weight change from pre-illness stable weight in the previous 6 months (n=19). Gluconeogenesis from alanine was assessed using primed-constant infusions of [6,6-$^{2}H_{2}$]-glucose and [3-^{13}C]-alanine. r, Pearson's correlation coefficient.

Table 2.4

Fasting hormone concentrations in healthy control subjects, weight-stable and weight-losing lung cancer patients.[1]

	Control	Lung cancer	
		WS	WL
	(n=15)	(n=10)	(n=9)
Insulin (mU/L)	9.3 ± 1.2	11.2 ± 2.9	8.3 ± 2.2[2]
Glucagon (ng/L)	20 ± 2	28 ± 3[a]	22 ± 5[a]
Insulin / Glucagon	0.5 ± 0.1	0.4 ± 0.1	0.8 ± 0.3
T_4 (nmol/L)	100 ± 10	107 ± 5	103 ± 8
T_3 (nmol/L)	1.85 ± 0.12	1.87 ± 0.06	1.63 ± 0.17
rT_3 (nmol/L)	0.26 ± 0.02[a]	0.30 ± 0.03[b]	0.40 ± 0.05[a,b]
rT_3/T_3	0.15 ± 0.02[a]	0.16 ± 0.02	0.27 ± 0.05[a]

[1] Mean ± SEM. Differences between group means were appraised by analysis of variance, adjusted for age; WS, weight-stable; WL, weight-losing. Means within a row with a superscript letter in common are significantly different, $P<0.05$.

[2] One value missing.

al [219] reported elevated rates of alanine-to-glucose conversion in WL cancer patients with mixed tumor types and metastatic disease when compared with undernourished subjects without cancer. Dietary intake was not reported in that study. Burt et al [25] reported elevated alanine turnover in patients with localized esophageal cancer, which was not related with the degree of weight loss. The present study, which was performed under steady state conditions with infusion of [$^2H_2$]-glucose and [^{13}C]-alanine, demonstrated that gluconeogenesis from alanine was elevated in weight-losing but not in weight-stable lung cancer patients.

It should be noted that the approach using [^{13}C]-alanine underestimates gluconeogenesis due to dilution of ^{13}C in intracellular pyruvate pools and at oxaloacetate, caused by exchange with the TCA cycle [224]. We assumed that the the yield of transfer of ^{13}C from alanine to glucose via the oxaloacetate turnover in liver and the extent of $^{13}CO_2$ reincorporation via gluconeogenesis into carbons 3 and 4 of glucose are identical in all three groups. Furthermore, due to rapid exchange of ^{13}C between alanine and pyruvate/lactate, gluconeogenesis may include a contribution from Cori cycling. We were able to measure ^{13}C enrichment of lactate and found no differences between healthy subjects, weight-stable or weight-losing lung cancer patients. Food intake was carefully

monitored in all subjects by means of a dietary record on the seven days preceding the turnover measurements. Results revealed no significant differences in energy intake between weight-losing and weight-stable lung cancer patients, or healthy control subjects; in fact lung cancer patients tended to have higher energy intakes than healthy controls. In other words, weight loss and the difference in turnover measurements in our study population of lung cancer patients were unrelated with reduced food intake.

Several mechanisms may be responsible for an elevated gluconeogenesis from alanine in weight-losing lung cancer patients. Increased gluconeogenesis could be caused by decreased insulin or increased glucagon concentrations [101,174]. However, in our study and studies by others [60,88,89] fasting insulin concentrations were not decreased in weight-stable or weight-losing lung cancer patients. Furthermore, no difference in glucagon concentrations between lung cancer patients and healthy controls was observed. Rofe et al [174] reported an altered insulin response and insulin: glucagon ratio in cancer patients with various tumor types, although there was no consistent relationship with the degree of weight loss in these patients. Clearly, it is not possible to define mechanisms on the basis of plasma concentrations of insulin or glucagon alone; however, our data do not support a predominant role for insulin or glucagon concentrations in the observed alterations in gluconeogenesis in weight-losing lung cancer patients.

A second hypothesis is that elevated gluconeogenesis from alanine is due to increased substrate availability or increased transport of alanine into the liver. In animal studies *in vivo*, alanine concentrations in the liver of tumor-bearing hosts were elevated [172], but plasma concentrations were normal [4] or decreased [101], suggesting elevated uptake of alanine by the liver. Reduced plasma alanine concentrations in weight-losing lung cancer patients have been reported by some authors [28,88]. However, in the present study we did not detect differences in plasma alanine concentrations between weight-losing and weight-stable lung cancer patients and healthy controls, despite a 36% increase in alanine flux in the weight-losing cancer patients; moreover, no correlation between plasma alanine concentrations and alanine turnover was observed (r= -0.08, P=0.67). Based on our results, it would therefore appear that elevated alanine turnover would be simply caused by increased plasma alanine concentrations; however, based on our data the possibility of an increased alanine uptake by the liver cannot be ruled out.

Yet other possible mechanisms are the acute-phase response and circulating cytokines [135,182,207] and/or elevated activity of one or more gluconeogenic enzymes within the liver, as demonstrated for phosphoenolpyruvate carboxykinase, glucose-6-phosphatase [83], and pyruvate carboxylase [128] in *in vitro* models. In weight-losing lung cancer patients, hydrazine sulphate reduced glucose production [34] and increased

plasma alanine levels [203], probably by inhibiting phosphoenolpyruvate carboxykinase. However, no data on hepatic gluconeogenic enzyme activities in humans with lung cancer are available.

Gluconeogenesis from alanine is an energy consuming process and enhanced conversion of alanine into glucose could act as an energy depleting mechanism. Though elevated gluconeogenesis may be an important factor in the pathology of weight loss in lung cancer, this does not preclude contributions from other energy-wasting processes to weight loss in lung cancer. Nevertheless, increased gluconeogenesis from alanine could result in loss of body protein. It is thought that loss of muscle mass contributes to impaired muscle function and decreased survival [56,154].

In summary, elevated rates of glucose and alanine turnover as well as gluconeogenesis from alanine have been detected in advanced lung cancer patients with weight loss. These metabolic abnormalities are neither related with the presence of lung cancer nor with reduced energy intake. Prospective studies on the relation between elevated gluconeogenesis from alanine and weight loss in lung cancer and the underlying mechanisms are warranted.

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3

Hepatic sugar phosphate levels reflect gluconeogenesis in lung cancer: simultaneous turnover measurements and ^{31}P magnetic resonance spectroscopy *in vivo*

Submitted for publication

Susanne Leij-Halfwerk,[1] Pieter C Dagnelie,[1,2] J Willem O van den Berg,[1]
J H Paul Wilson,[1] Paul E Sijens[2]

[1] Department of Internal Medicine II, Erasmus University Medical Center Rotterdam, Rotterdam,
[2] Department of Diagnostic Radiology, Daniel Den Hoed Cancer Center, Rotterdam and
[3] Department of Epidemiology, University Maastricht, Maastricht, The Netherlands

Abstract

Stable isotope tracers were used to assess whether levels of phosphomonoesters (PME) and phosphodiesters (PDE) in the liver of lung cancer patients, as observed by ^{31}P magnetic resonance (MR) spectroscopy, reflect elevated whole body glucose turnover and gluconeogenesis from alanine. Patients with advanced non-small cell lung cancer without liver metastases (n=24), weight loss 0-24%, and healthy control subjects (n=13) were studied after an overnight fast. ^{31}P MR spectra of the liver *in vivo* were obtained and glucose turnover and gluconeogenesis from alanine were determined simultaneously using primed-constant infusions of [6,6-$^{2}H_2$]-glucose and [3-^{13}C]-alanine.

Liver PME concentrations were 6% higher in lung cancer patients compared to controls (NS); PME levels in patients with ≥5% weight loss were significantly higher than in patients with <5% weight loss (P<0.01). PDE did not differ between the groups. In lung cancer patients, whole body glucose production was 19% higher (NS) and gluconeogenesis from alanine 42% higher (P<0.05) compared to healthy subjects; turnover rates in lung cancer patients with ≥5% weight loss were significantly elevated compared to both patients with <5% weight loss and healthy subjects (P<0.05). PME levels were significantly correlated with glucose turnover and gluconeogenesis from alanine in lung cancer patients (r=0.48 and r=0.48, respectively, P<0.05).

In conclusion, elevated PME levels in lung cancer patients appear to reflect increased glucose flux and gluconeogenesis from alanine. These results are consistent with the hypothesis that elevated PME is due to contributions from gluconeogenic intermediates.

Introduction

Weight loss is a common phenomenon in patients with cancer and significantly contributes to the high morbidity and mortality in this disease [43,56,198]. Since it was suggested that anorexia alone cannot fully account for the occurrence of weight loss in several tumor types including lung cancer, attempts have been made to investigate underlying mechanisms in the cancer-bearing host. Profound alterations in host metabolism including elevated protein breakdown [171], increased glucose turnover [89] and endogenous glucose production [219] have been reported and were suggested to contribute to the observed weight loss in cancer.

It has been argued that the liver may play an important role in the metabolic alterations which contribute to weight loss in cancer [20,115]. Altered enzyme activities [6,83,92,175] as well as decreased phosphorylation and energy status [6,49] were reported in the liver of tumor-bearing rats. Increasing tumor burden was shown to be

correlated with decreasing phosphorylation status [181] and increasing gluconeogenic enzyme activity in the liver [156]. In contrast, in humans information on liver metabolism in cancer is extremely scarce. In human hepatomas, activities of pyruvate carboxylase, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, all regulatory enzymes of gluconeogenesis, were elevated compared to normal liver tissue [86].

In order to understand alterations in liver metabolism in cancer patients it is essential to obtain information on liver *in vivo*. Although non-specific changes in ^{31}P magnetic resonance (MR) spectra have been detected in various liver diseases including primary and secondary hepatic cancer, systemic effects of cancer on tumor-free host liver have rarely been investigated in humans *in vivo*. In a previous ^{31}P MRS study markedly elevated phosphomonoesters (PME) and reduced phosphodiesters (PDE) in the tumor-free, i.e. non-metastatic, liver of weight-losing cancer patients with various tumor types were observed [51].

Since PME and PDE resonances contain contributions from phospholipid intermediates, membrane precursors, as well as sugar phosphates such as glucose-6-phosphate (G6P), 3-phosphoglycerate (3PG), and phosphoenolpyruvate (PEP) [145], it has been difficult to interpret changes in ^{31}P MRS-detected components in terms of metabolic alterations.

We recently observed elevated glucose turnover and gluconeogenesis from alanine in weight-losing (WL) lung cancer patients [126]. Since gluconeogenesis from alanine occurs predominantly in the liver [201] it was suggested that elevated concentrations of gluconeogenic intermediates caused the increased PME levels in these patients. In the present study ^{31}P MRS of the liver and turnover measurements were combined in order to relate hepatic and systemic alterations in lung cancer patients. The aim was to investigate whether elevated PME and PDE levels in tumor-free liver as observed with ^{31}P MRS are correlated with elevated glucose flux and gluconeogenesis from alanine.

Methods

Subjects

The study was approved by the Medical Ethical Committee of the Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands. Patients with non-small cell lung cancer stage IIIA/B or IV (WHO grading system) attending the outpatient department of the University Hospital Rotterdam, The Netherlands, were recruited. Patients who were in remission or apparently cured were excluded. Additional exclusion criteria were: liver metastases (as checked for by CT/ultrasound), metabolic disease, corticosteroid treatment, elective surgery <3 months, chemo- or radiotherapy <4 weeks prior to

study, alcohol consumption of more than 100 g/week (=10 glasses); pregnancy; extreme anorexia or artificial weight reduction by dieting. Healthy subjects without weight loss were included as a control group. All participants signed informed consent.

Experimental design

The subjects were studied in the morning after an overnight fast. A cannula (0.8×25 mm) was placed in the left cubital vein for the infusion of stable isotope tracers. In the contralateral cubital vein, an identical cannula was introduced for blood sampling. To study gluconeogenesis, a solution was prepared containing [6,6-$^{2}H_{2}$]-D-glucose, 98 atom% and [3-^{13}C]-L-alanine, 99 atom% (Mass Trace, Woburn, USA) in water and this was sterilized by autoclaving in glass vials. A priming dose of 30 µmol/kg [6,6-$^{2}H_{2}$]-D-glucose was administered followed by a continuous infusion of 10 µmol/kg/h [6,6-$^{2}H_{2}$]-D-glucose for 90 minutes. Simultaneously, a priming dose of 80 µmol/kg [3-^{13}C]-L-alanine was given followed by a continuous infusion of 40 µmol/kg/h [3-^{13}C]-L-alanine during 90 minutes. Both tracer solutions were infused using calibrated syringe pumps (Perfusor® fm, Braun, Germany).

Venous blood samples were drawn immediately before the isotope infusions were started, and at 10 min intervals after steady state conditions during the tracer infusions had been reached. Based on observations by others [102] and in our laboratory steady state was obtained between 60-90 min of the tracer infusions. Isotopic enrichments of deuterium and carbon-13 glucose, and carbon-13 alanine in plasma were determined.

Biochemistry and turnover measurements

Blood samples were collected in tubes containing lithium heparin (Becton Dickinson Vacutainer®, Meylan Cedex, France) and immediately stored on ice. After centrifugation (10 min, 1200 *g*, 4°C), the plasma was collected and stored at -20°C until analysed. An aliquot of the infusate was analysed to document actual concentrations of the tracers in each study.

Isotopic enrichments were determined using the following procedures. Plasma was deproteinized by adding 0.3 M barium hydroxide (Sigma Diagnostics, St.Louis, USA) and 0.3 M zinc sulphate (Merck, Darmstadt, Germany). After centrifugation (8 min.,15000 *g*, 4°C) the supernatant was applied to an ion exchange column (mixed bed: AG50W-X8 and AG1-X8, 200-400 mesh, 0.2 g each; BioRad, California, USA). Glucose and alanine were eluted from the column using water and 4 M ammonium hydroxide (Merck, Darmstadt, Germany), respectively, and dried under nitrogen. A glucose derivate (aldonitril penta acetate) was made according to Varma et al [216]. An alanine t-butyldimethylsilyl derivate was prepared as described by Chaves Das Neves et al [32].

Isotopic enrichments were measured by injecting 1 µl samples with a split ratio of

50:1 on a fused silica capillary column of 25 m × 0.22 mm, coated with 0.11 µm HT5 (SGE, Victoria, Australia). The relative isotopic enrichments of deuterated glucose and carbon-13 alanine were determined using a Carlo Erba GC8000 gas chromatograph coupled to a Fisons MD800 mass spectrometer (GC-MS) (Interscience B.V., Breda, The Netherlands) in electron impact ionization mode. The variation coefficient in enrichment was 0.2 mole% for both [6,6-$^{2}H_{2}$]-glucose and [3-^{13}C]-alanine, and no concentration effect was observed at this level of mole% enrichment. Ions were selectively monitored at mass per unit charge (*m/z*) 187 for natural glucose and 189 for the deuterated molecule. The isotopic enrichment of [3-^{13}C]-alanine was determined at the *m/z* ratios 260 and 261 for carbon-12 and carbon-13 alanine, respectively [137].

Total enrichment of carbon-13 glucose was measured separately (aldonitril pentaacetate derivation) using a gas chromatograph combustion isotope ratio mass spectrometer (GC-IRMS) (Optima, Micromass UK, Middlewich, Cheshire, Great Britain). The [^{13}C]-glucose enrichment, as atom% excess (APE) was monitored after combustion to CO_2 at mass 44 for carbon-12 and 45 for carbon-13, respectively.

Whole body rate of appearance (Ra) of glucose and gluconeogenesis from alanine were calculated during steady state assuming an one-compartment model as described by Wolfe [224] and were expressed as mmol.kg^{-1}. h^{-1}. It was assumed that the dilution of ^{13}C in intracellular pyruvate pools and at oxaloacetate, caused by exchange with the TCA cycle [224], would be similar in lung cancer patients and healthy subjects.

^{31}P MR spectroscopy of the liver

Hepatic 31-phosphorus MR spectra were obtained during steady state of the isotope tracers. Spectroscopy studies were performed with a whole-body MR system equipped with a Helicon magnet operating at 2.0 Tesla (Vision Magneton, Siemens AG, Erlangen, Germany). A 16 cm diameter transmit/receive ^{1}H/^{31}P surface coil was used for MRI localization, shimming, and ^{31}P MR spectroscopy. Elastic bands were used for positioning the coil lateral to the liver in the mid-axillary plane. Field homogeneity achieved in shimming resulted in water peak line widths which were usually less than 40 Hz (≈0.5 ppm). After obtaining an image of the region of interest, an one-dimensional chemical shift imaging (1D-CSI) sequence was applied on a transverse slice of 4 cm centered on the surface coil and the liver (1×4 phase-encoded matrix, field of view 40×40 cm^{2}), yielding volumes of 40×10×4 cm^{3} [195]. Five spectra were collected with a 640 µs Hanning-sinc shaped radio frequency pulse resulting in a flip angle of 135° in the center of the coil, and 60° (weighted average) in the liver volume with a repetition time (TR) of 1 s (40 acquisitions). We previously demonstrated [51] that use of TR=1 s and TR=20 s gives similar differences for PME in weight-losing or weight-stable cancer patients, and healthy control subjects. Furthermore, the saturation at TR=1 s and a pulse angle of 60°

used in our study is maximally 15% for PME (relative to β-ATP) and 30% for PDE (relative to β-ATP)[195].

Time domain data were Fourier transformed after Gaussian multiplication (center: 0 ms, width 30 ms) and phase corrected. Quantification of spectral peak areas was performed using Numaris-3 software package (Siemens AG, Erlangen, Germany) including polynomial baseline correction followed by frequency domain curve fitting [194]. Metabolite concentrations were calculated from peak areas and expressed relative to total MR-detectable phosphate as described elsewhere [51]. In each experiment the average of five subsequent ^{31}P MR spectra was used for calculations.

Statistics

Results are presented as means ± standard error of the mean (SEM). Differences between group means were compared using Student's t-test for independent groups. Reported correlations between variables are Pearson's correlation coefficients. Differences were considered statistically significant at P values < 0.05.

Results

Twenty-four patients with non-small cell lung cancer, stage IIIA/B or IV (WHO grading system), and thirteen healthy control subjects were included in the study. In lung cancer the mean age was 66 y (range 38-85y) which was significantly higher than in controls (46 y, range 25-69) (P<0.05). Body weight was significantly less in cancer patients than in healthy controls (65.7 ± 2.5 *vs.* 75.3 ± 2.7 kg, respectively, P<0.05). The patients had lost 5 ± 1 kg or 7% (range 0 -24%) of their pre-illness body weight in the previous 6 months.

Whole-body turnover rates measured in lung cancer patients and healthy controls are shown in **Table 3.1.** Since it was previously shown that turnover rates are correlated with the degree of weight loss [126], data of all lung cancer patients are presented together as well as data of patients with <5% (weight-stable, WS) or ≥5% of weight loss (weight-losing, WL) separately. Gluconeogenesis from alanine was significantly higher in lung cancer patients than in control subjects (P<0.05). Both turnover rates of glucose and gluconeogenesis from alanine were significantly elevated in WL lung cancer patients compared with WS patients and healthy controls (P<0.01). Age did not significantly influence these results (data not shown).

Examples of MR spectra from a healthy control subject, a weight-stable and a

Table 3.1

Rate of appearance (turnover) of glucose and gluconeogenesis from alanine in healthy control subjects and lung cancer patients.[1]

	Glucose (mmol/kg/h)	Gluconeogenesis from alanine (mmol/kg/h)
Control (n=13)	0.52 ± 0.05	0.28 ± 0.04
Lung cancer (n=24)	0.62 ± 0.04	0.41 ± 0.04 *
< 5% weight loss (n=12)[2]	0.53 ± 0.05	0.32 ± 0.05
≥ 5% weight loss (n=12)	0.71 ± 0.06 **†	0.51 ± 0.04 **‡

[1] Mean ± SEM.

[2] Weight loss was defined as percentage weight loss from pre-illness stable weight in the six months prior to the study.

Statistically significant differences (Student's t-test for independent groups): compared to healthy subjects *P<0.05, **P<0.01; compared to lung cancer patients with < 5% weight loss †P<0.05; ‡P<0.01.

Table 3.2

Hepatic metabolite levels as observed by ^{31}P magnetic resonance spectroscopy in healthy control subjects and lung cancer patients.[1]

	PME/P_{total}[2]	PDE/P_{total}	PME/PDE
Control (n=13)	0.079 ± 0.007	0.298 ± 0.018	0.275 ± 0.029
Lung cancer (n=24)	0.084 ± 0.005	0.293 ± 0.015	0.319 ± 0.027
< 5% weight loss (n=12)[3]	0.069 ± 0.004	0.279 ± 0.024	0.289 ± 0.037
≥ 5% weight loss (n=12)	0.098 ± 0.008 ‡	0.306 ± 0.019	0.349 ± 0.040

[1] Mean ± SEM.

[2] Metabolite levels are expressed as percentage of total MR-detectable phosphate.

[3] Weight loss was defined as percentage weight loss from pre-illness stable weight in the six months prior to the study.

Statistical significant different from lung cancer patients with < 5% weight loss: ‡P<0.01 (Student's t-test for independent groups).

weight-losing lung cancer patient are shown in **Figure 3.1.** Phosphomonoesters (PME) were significantly elevated in WL lung cancer patients when compared with WS patients (P<0.01) (**Table 3.2**). No significant differences in PDE levels were observed between the groups. Although PME/PDE ratios were higher in lung cancer patients than

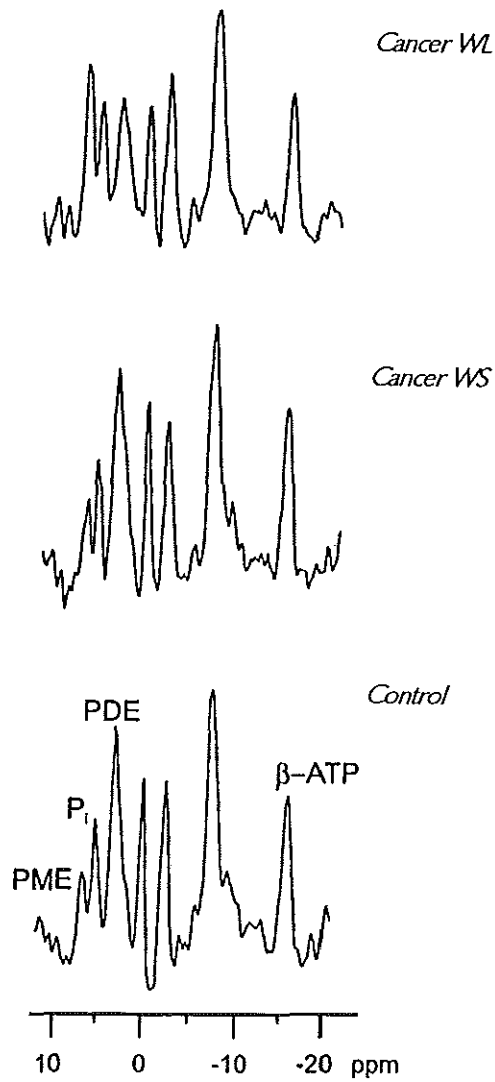


Figure 3.1

Phosphorus MR spectra of the liver of a healthy control subject ("control"), a weight-stable lung cancer patient ("cancer WS") and a weight-losing lung cancer patient ("cancer WL"). Spectra were acquired at 2.0 Tesla using a 135° excitation pulse (60° weighted average in the liver volume) with a repetition time of 1 s. PME, phosphomonoesters; P_i, inorganic phosphate; PDE, phosphodiesters; β-ATP, β-phosphate group of adenosine triphosphate. The scale represents chemical shift in ppm.

in controls the difference did not reach statistical significance. Again, age did not significantly influence the results.

In **Figure 3.2**, correlations between liver metabolites and turnover measurements in lung cancer patients are shown. PME was significantly correlated with glucose turnover and gluconeogenesis from alanine in lung cancer patients (r=0.48 and r=0.48, respectively, $P<0.05$). In healthy subjects no significant correlations were observed (r= -0.19 and r= -0.24, respectively, $P>0.42$). PDE was neither correlated with glucose turnover nor with gluconeogenesis from alanine in lung cancer patients (r= -0.30 and r= -0.39, respectively, $P>0.05$) or control subjects (r = -0.04 and r= -0.22, respectively, $P>0.47$). Positive correlations between PME/PDE ratio and glucose turnover or gluconeogenesis from alanine were observed in lung cancer patients (r= 0.47 and r=0.55, respectively, $P<0.05$), but not in healthy controls (r= -0.18 and r= -0.02, respectively).

Discussion

Phosphorus MR spectroscopy is a promising tool for the non-invasive study of diseased states [104,146]. However, one limitation to its application in human disease has been the difficulty in interpretating ^{31}P MR spectra in terms of metabolic alterations. To our knowledge, no previous studies combining ^{31}P MR spectroscopy with turnover measurements have been reported, in contrast to studies using ^{13}C MRS [166,178]. In the present study, quantitative information on hepatic substrate metabolism in lung cancer patients was obtained by simultaneous application of ^{31}P MRS and turnover measurements. Specifically, the relation between hepatic concentrations of PME and PDE as observed by ^{31}P MR spectroscopy and glucose turnover and gluconeogenesis from alanine using stable isotope tracers was assessed.

Several authors have used ^{31}P MR spectroscopy for the characterization of various disease states in humans and animal models. Elevated PME levels have been reported in diseased liver, for instance in patients with liver cirrhosis [103,149,205,206], chronic alcohol abuse [143], or hepatic malignancies [45]. It was suggested that this was caused by contributions of glycerol-3-phosphate or intermediates in the pathway of phospholipid biosynthesis, phosphorylcholine (PC), or phosphorylethanolamine (PE). Furthermore, ^{31}P MRS studies revealed reduced hepatic PDE levels in cirrhotic patients [103,205,206] and hepatic malignancies [45], and elevated PDE levels in subjects with alcohol abuse depending on the type and severity of the disease [143]. Decreased levels of glycerophosphorylcholine (GPC) and glycerophosphorylethanolamine (GPE), both products of phospholipid breakdown, or decreased levels of endoplasmic reticulum within the

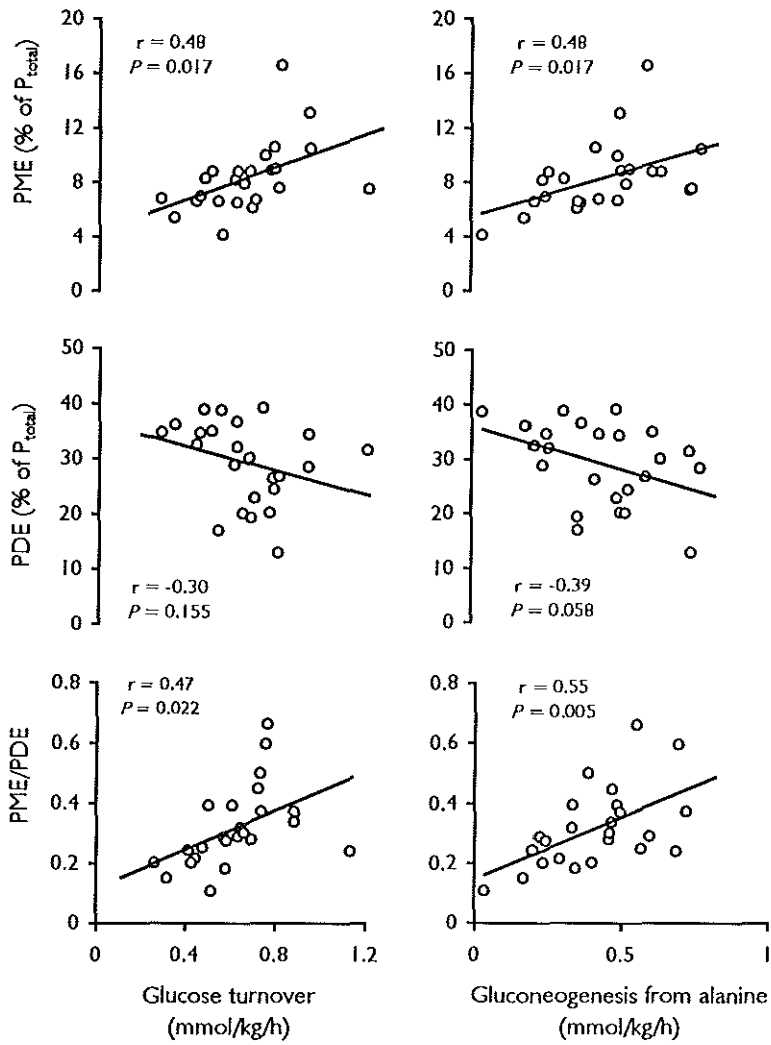
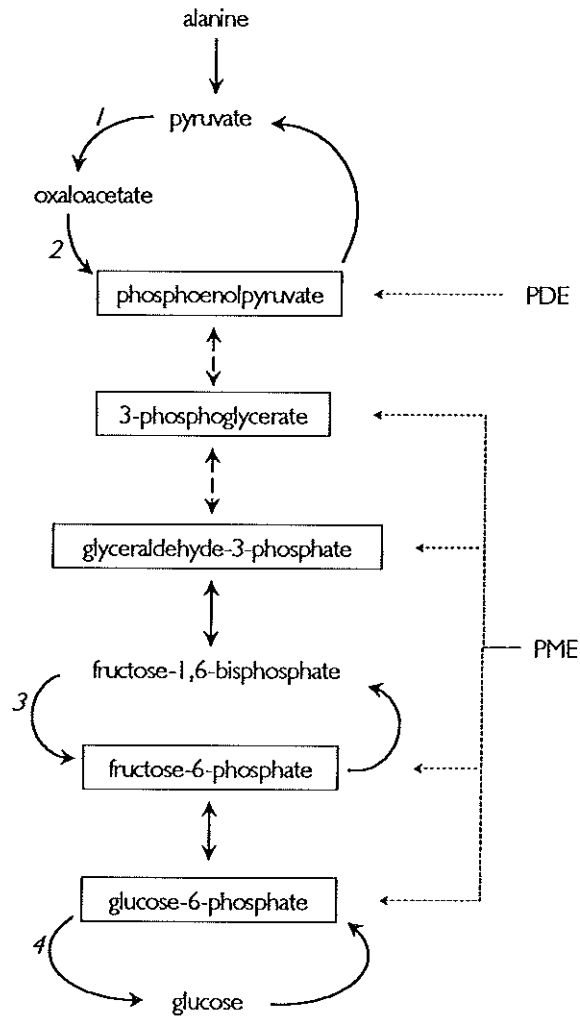


Figure 3.2

Metabolite concentrations as observed by ^{31}P magnetic resonance spectroscopy of the liver plotted against flux measurements as assessed by primed-constant infusion of stable isotope tracers in lung cancer patients (n=24). r, Pearson's correlation coefficient; PME, phosphomonoesters; PDE, phosphodiesters.



Scheme 3.1

Pathway of hepatic gluconeogenesis from alanine with some of the intermediates contributing to the phosphomonoester (PME) and phosphodiester (PDE) resonances in ^{31}P MR spectra indicated. Numbers are gluconeogenic key-enzymes: 1, pyruvate carboxylase; 2, phosphoenolpyruvate carboxykinase; 3, fructose-1,6-bisphosphatase; 4, glucose-6-phosphatase.

hepatocytes were suggested as possible contributors to these changes. In the present study ^{31}P MR spectroscopy was performed in lung cancer patients with healthy livers, thus comprising a distinct metabolic situation. The absence of liver metastases in all cancer patients was verified by CT / ultrasound; furthermore, all patients had normal liver function tests. Hepatic PME levels were increased in lung cancer patients with weight loss, confirming previous studies in mixed tumor types [51], but no difference in PDE levels was observed between lung cancer patients and healthy controls. Even though mean age in lung cancer patients was higher than in healthy subjects, the age-ranges largely overlapped. Furthermore, we checked our data for significant correlations between metabolite levels and age. No significant correlations were observed between age and PME (r=0.16, P=0.33), PDE (r=-0.01, P=0.98) or PME/PDE (r=0.21, P=0.22). In addition, in a study by Bourdel-Marchasson et al. [19] it was reported that hepatic PME, PDE and PME/PDE in young healthy subjects (age 30.5 ± 2.1y) and elderly healthy subjects (age 80.4 ± 6.3 y) were not significantly different. Glucose turnover and gluconeogenesis from alanine were elevated in WL lung cancer patients as reported previously [126].

The pathway of gluconeogenesis from alanine is shown in **Scheme 3.1.** In the cytosol, alanine is converted into pyruvate, which in turn is transported into the mitochondria and converted into oxaloacetate by pyruvate carboxylase. Liu et al., using ^{13}C MRS, suggested that the activity of pyruvate carboxylase was increased in the liver of tumor-bearing rats [128]. The next step is the conversion of oxaloacetate into phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxykinase (PEPCK), a regulatory enzyme in the gluconeogenic pathway, in humans present in the mitochondria and in the cytosol [139]. In rats bearing sarcomas the activity of PEPCK was about twice that of healthy rats [83,156] and increased with increasing tumor burden [211]. When PEP enters the gluconeogenic pathway it is converted into glucose via a number of intermediates including 3-phosphoglycerate (3PG), fructose-1,6-bisphosphate (F1,6P), fructose-6-phosphate (F6P), and glucose-6-phosphate (G6P). Two other regulatory enzymes of the gluconeogenic pathway, fructose-1,6-bisphosphatase and glucose-6-phosphatase, catalyse the reactions from F1,6P to F6P and G6P to glucose, respectively. Elevated activity of glucose-6-phosphatase was detected in the liver of sarcoma-bearing rats, whereas the activity of fructose-1,6-bisphosphatase was only slightly increased [83].

Since the interpretation of PME or PDE concentrations alone is not straightforward, in the present study a comparison with turnover measurements was made. PME was correlated with glucose turnover and gluconeogenesis from alanine in lung cancer patients, but not in healthy controls. The elevated PME levels observed in WL lung cancer patients could be a result of accumulation of MR-detectable gluconeogenic

intermediates within the liver. It has previously been shown that infusion of a gluconeogenic substrate caused ^{31}P MRS-detectable changes in the PME and PDE resonances due to increased concentrations of 3PG and PEP, respectively, within the liver of healthy humans and rats [30,49,177]. So far, MR spectroscopy data on tumour-free livers in humans or animal models are extremely limited. ^{31}P MRS metabolite profiles of biopsy samples of histologically normal liver tissue from patients bearing hepatomas showed elevated contributions of PE and PC; however, changes in sugar phosphate concentrations could not be quantified in that study due to the significant period of ischemia which arises during the collection of human biopsies [12]. In tumour-free liver of rats bearing the Dunning prostate tumour, decreased levels of G6P and F6P compared to control rats were reported [49]. It was suggested that the decreased values of G6P could have been caused by elevated G6P-ase activity.

In contrast to PME levels, PDE levels in lung cancer patients in the present study were inversely correlated with gluconeogenesis from alanine, possibly indicating increased conversion of PEP into 2-phosphoglycerate and further. PME/PDE ratios were significantly correlated with whole-body glucose turnover and gluconeogenesis from alanine in lung cancer. We cannot exclude contributions of PC, PE, GPC, GPE or endoplasmic reticulum to the PME and PDE resonances. However, bearing in mind that the liver of the lung cancer patients examined in the present study was apparently healthy (verified by CT/ultrasound and liver function tests), elevated phospholipid turnover would not be likely. Moreover, the elevated PME levels were specific for weight-losing lung cancer patients, they were absent in weight-stable patients. There is no hypothesis present in literature linking PC, PE, GPC or GPE to weight loss in cancer. In contrast, there is a clear hypothesis linking weight loss and elevated gluconeogenesis in cancer. Therefore, it seems unlikely that elevated PME and PDE were caused by PC, PE, GPC or GPE. The fact that in healthy control subjects hepatic PME levels are not significantly correlated with glucose flux or gluconeogenesis, would suggest that metabolites other than gluconeogenic intermediates, such as PC and PE, are the predominant contributors to the PME resonance in these subjects.

In summary, this combined turnover and ^{31}P MRS study in humans *in vivo* would suggest that elevated hepatic PME levels in lung cancer patients reflect increased glucose flux and gluconeogenesis from alanine in these patients. Our data are consistent with the hypothesis that elevated PME is due to high contributions from gluconeogenic intermediates, possibly 3-phosphoglycerate. In healthy controls no such correlation was observed. Dynamic studies could provide further information on hepatic metabolism and enzyme activities in lung cancer patients *in vivo*.

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4

Altered hepatic gluconeogenesis during L-alanine infusion in weight-losing lung cancer patients as observed by ^{31}P MR spectroscopy and turnover measurements

Submitted for publication

Susanne Leij-Halfwerk,[1,2] J Willem O van den Berg,[1] Paul E Sijens,[2] J H Paul Wilson,[1] Matthijs Oudkerk,[2] Pieter C Dagnelie[1,3]

[1] Department of Internal Medicine II, Erasmus University Medical Center Rotterdam, Rotterdam,
[2] Department of Diagnostic Radiology, Daniel Den Hoed Cancer Center, Rotterdam and
[3] Department of Epidemiology, University Maastricht, Maastricht, The Netherlands

Abstract

Profound alterations in host metabolism in lung cancer patients with weight loss have been reported, including elevated phosphomonoesters (PME) as detected by ^{31}P magnetic resonance spectroscopy (MRS). In healthy subjects, infusion of L-alanine induced significant increases in hepatic PME and phosphodiesters (PDE) due to rising concentrations of 3-phosphoglycerate and phosphoenolpyruvate, respectively. The aim of the present study was to monitor these changes in the tumor-free liver of lung cancer patients during L-alanine infusion by means of simultaneous ^{31}P MRS and turnover measurements. Twenty-one lung cancer patients without liver metastases with (CaWL) or without weight loss (CaWS), and twelve healthy control subjects (C) were studied during an i.v. L-alanine challenge of 1.4-2.8 mmol/kg followed by 2.8 mmol/kg/h for 90 min.

Plasma L-alanine concentrations increased during alanine infusion, from 0.35 - 0.37 mM at baseline to 5.37 ± 0.14 mM in CaWL, 6.67 ± 0.51 mM in CaWS, and 8.47 ± 0.88 mM in C (difference from baseline and between groups during alanine infusion, all $P<0.001$). Glucose turnover and liver PME levels at baseline were significantly elevated in CaWL. Alanine infusion increased whole-body glucose turnover by 8 ± 3% in CaWS ($P=0.03$), whereas no significant change occurred in CaWL and C. PME levels increased by 50 ± 16% in C (area under the curve, $P<0.01$) and by 87 ± 31% in CaWS ($P<0.05$) after 45-90 min. In contrast, no significant change in PME was observed in CaWL. Plasma insulin concentrations increased during L-alanine infusion in all groups to levels which were lower in CaWL than in CaWS and C ($P<0.05$). In lung cancer patients, but not in C, changes in PME and PDE levels during alanine infusion were inversely correlated with their respective baseline levels ($r= -0.82$ and $r= -0.86$, respectively, $P<0.001$). Also, changes in PME during alanine infusion in lung cancer patients were inversely correlated with the degree of weight loss ($r= -0.54$, $P<0.05$).

In conclusion, this study demonstrates the presence of major alterations in the pathway of hepatic gluconeogenesis in weight-losing lung cancer patients, as shown by elevated glucose flux before and during L-alanine infusion, and by the increased PME and PDE levels which reflect accumulation of gluconeogenic intermediates in these patients. Weight-stable lung cancer patients show accelerated rise in PME and PDE levels during L-alanine infusion, suggesting enhanced induction of the gluconeogenic pathway. Our results suggest altered gluconeogenic enzyme activities and elevated alanine uptake within the liver of WL/WS lung cancer patients.

Introduction

Weight loss in lung cancer is associated with both impaired therapy outcome [56] and reduced survival [37,43,56,198]. Characteristic features of weight loss in lung cancer are breakdown of both fat mass and skeletal muscle, whereas visceral organs are typically spared or even enlarged [38,94]. Although profound alterations in host substrate metabolism in cancer patients have been reported, mechanisms responsible for the observed weight loss are yet poorly understood. Isotope tracer studies showed elevated protein breakdown and glucose turnover in lung cancer patients using isotope tracers [89,142,171]. Increased gluconeogenesis from alanine was observed in tumor-influenced hepatocytes [175], in tumor-bearing animals *in vivo* [129], and in cancer patients with various tumor types [219]. We recently reported increased whole-body gluconeogenesis from alanine in lung cancer patients with weight loss [126]. A significant correlation between gluconeogenesis from alanine and the degree of weight loss was also observed.

Since the liver is the main site for gluconeogenesis [201], the observed increase in gluconeogenesis form alanine as observed in weight-losing lung cancer patients is likely to be partly related with altered liver metabolism [20,115]. In animal models, altered hepatic enzyme activities [6,92] and decreases in liver phosphorylation status [49] and energy balance [6] were correlated with tumor burden [181]. Furthermore, elevated concentrations of gluconeogenic intermediates such as glucose-6-phosphate were observed within the liver of these animals [6]. Another experimental study revealed altered hepatic metabolism in response to fructose infusion even in rats with minimal tumor burden [77]. These alterations preceded the onset of cachexia and it was suggested that they were related to elevated hepatic gluconeogenesis in these animals.

Due to lack of non-invasive techniques, data on altered liver metabolism in humans with lung cancer are limited. In recent studies using ^{31}P magnetic resonance spectroscopy (MRS), elevated concentrations of phosphomonoesters (PME) were observed in the liver of weight-losing cancer patients with various tumor types [51] and lung cancer [125]. In contrast, liver PME levels in weight-stable cancer patients were not significantly different from those in healthy subjects. Furthermore, hepatic PME levels were significantly correlated with the rate of gluconeogenesis from alanine in lung cancer patients, but not in healthy subjects [125]. MRS studies have also been used to obtain dynamic information on liver metabolism by monitoring changes in hepatic metabolite concentrations during infusion of a gluconeogenic substrate. Changes in PME and ATP levels were reported using ^{31}P MRS with L-alanine infusion *in vivo* in healthy rats [30] as well as in rats after ischemia [147] or surgery [200]. In healthy humans, ^{31}P MRS with either a bolus [50] or continuous [177] infusion of L-alanine was shown to provide information

on changes in concentrations of gluconeogenic intermediates within the liver. However, information on liver gluconeogenic intermediates during a metabolic challenge in lung cancer patients is lacking.

The aim of the present study was to monitor glucose metabolism in tumor-free liver of lung cancer patients with and without weight loss by means of ^{31}P MRS, with infusion of L-alanine as a gluconeogenic substrate. Data were compared with flux measurements using stable isotope tracers before/during alanine infusion.

Materials and methods

Subjects

The study was approved by the Medical Ethical Committee of the Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands. Patients with non-small cell lung cancer stage IIIA/B or IV (WHO grading system) attending the outpatient department of the University Hospital Rotterdam, The Netherlands, were recruited. Patients who were in remission or apparently cured were excluded. Additional exclusion criteria were: liver metastases (as checked for by CT/ultrasound), metabolic disease, corticosteroid treatment, elective surgery <3 months, chemo- or radiotherapy <4 weeks prior to study, alcohol consumption of more than 100g/week (=10 glasses), pregnancy, extreme anorexia or artificial weight reduction by dieting. Healthy subjects without weight loss were included as a control group. All participants signed informed consent.

Experimental design

All subjects kept a dietary record during 7 days and refrained from alcoholic drinks for three days prior to the MRS measurements. Data on pre-illness stable weight, current weight, and weight loss over the previous 6 months were taken from hospital records supplemented with oral information from patients. The subjects were studied between 7:30 a.m. and 1:00 p.m. after an overnight fast (12-14 h). Body weight was measured to the nearest 0.1 kg on an electrical weighing scale (Seca 707, Hamburg, Germany), height was measured to the nearest 0.1 cm, and thickness of four skinfolds (triceps, biceps, subscapular, supra iliac) were measured to the nearest 0.2 mm using a standard skinfold caliper (Holtain Ltd., London, Great Britain). A cannula (0.8×25 mm) was placed in the left cubital vein for the infusion of the stable isotope tracer and unlabeled L-alanine. In the contralateral cubital vein, an identical cannula was positioned for blood sampling. To determine whole-body glucose turnover a solution was prepared containing [6,6-$^{2}H_{2}$]-D-glucose, 98 atom% (Mass Trace, Woburn) in water, sterilized by autoclaving in glass vials. A solution of (unlabeled) L-alanine in water (100g per liter, Bufa B.V.,

Uitgeest, The Netherlands) was prepared, sterilized by autoclaving in glass bottles, and warmed to ~30°C.

The study consisted of two phases. During the first phase (baseline), a priming dose of 30 μmol/kg of [6,6-$^{2}H_{2}$]-glucose was administered followed by a continuous infusion of 10 μmol/kg/h for 90 min. During the second phase, a priming dose of 1.4 - 2.8 mmol/kg unlabeled L-alanine was administered in 5-8 min and followed by a continuous infusion of 2.8 mmol/kg/h L-alanine for 90 min in order to reobtain a steady state; simultaneously the isotope tracer infusion was continued. Venous blood samples were collected as follows: phase 1) one sample immediately before the isotope tracer infusion was started (for determination of background enrichment of ^{2}H-glucose) and at 10 min intervals from 30 to 90 min during isotope tracer infusion, after steady state was reached; phase 2) at 15 min intervals during continuous L-alanine infusion. Phosphorus MR spectra of the liver were obtained at baseline and at 3 min intervals during L-alanine infusion.

^{31}P MR spectroscopy of the liver

Spectroscopy studies were performed with a whole-body MR system equipped with a Helicon magnet operating at 2.0 Tesla (Vision Magneton, Siemens AG, Erlangen, Germany). A 16 cm diameter transmit/receive ^{1}H/^{31}P surface coil was used for MRI localization, shimming and ^{31}P MR spectroscopy. Elastic bands were used for positioning the coil lateral to the liver in the mid-axillary plane. Field homogeneity achieved in shimming resulted in water peak line widths which were usually less than 40Hz (≈0.5 ppm). After obtaining an image of the region of interest, a one-dimensional chemical shift imaging (1D-CSI) sequence was applied on a transverse slice of 4 cm centered on the surface coil and the liver (1×4 phase-encoded matrix, field of view 40×40 cm^{2}), yielding volumes of 40×10×4 cm^{3} [195]. Spectra were collected with a 640 μs Hanning-sinc shaped radio frequency pulse resulting in a flip angle of 135° in the center of the coil, and 60° (weighted average) in the liver volume with a repetition time (TR) of 1 s (40 acquisitions).

Time domain data were Fourier transformed after Gaussian multiplication (center: 0 ms, width 30 ms) and phase corrected. Quantification of spectral peak areas was performed using Numaris-3 software package (Siemens AG, Erlangen, Germany) including polynomial baseline correction followed by frequency domain curve fitting [194]. Metabolite concentrations were calculated from peak areas and expressed relative to total MR-detectable phosphate as previously described [51]. Total MR-detectable phosphate did not change during L-alanine infusion (data not shown).

Substrate concentrations and glucose turnover

Blood samples were collected in tubes containing lithium heparin (Becton Dickinson Vacutainer®, Meylan Cedex, France) and immediately stored on ice. After centrifugation (10 min, 1200 *g*, 4°C), the plasma was collected and stored at -20°C until analyzed. Blood glucose concentration was measured enzymatically with a glucose-oxidase/peroxidase assay (Boehringer Mannheim, Mannheim, Germany). Plasma alanine concentration was determined enzymatically as described by Williamson [223]. Isotopic enrichment of deuterium-glucose (mole% excess, MPE) in plasma was determined by gas chromatography-mass spectrometry as described previously [126]. Plasma concentrations of insulin and glucagon were determined at two time points during baseline and two time points during L-alanine infusion by radio immuno assay techniques (Biosource, Fleurus, Belgium and Euro-Diagnostica, Sweden, respectively).

Statistical analysis

Results are reported as means ± standard error of the mean (SE). In each experiment the average of five subsequent MR-spectra was used as baseline value for calculations. Mid-time points of MRS data acquisition at 15 min intervals during L-alanine infusion (7.5, 22.5, 37.5 min, etc.) were used for graphical representation, with values being expressed relative to the baseline value of healthy control subjects (=100%). As a measure of overall spectral response, integrals of time-response curves (area under the curve, AUC) of peak areas over 0-45 min, 45-90 min, and 0-90 min intervals during L-alanine infusion were calculated and expressed relative to the baseline values. Between-group differences in baseline values and response to alanine infusion were analyzed using ANOVA. Changes from baseline values were analyzed using Student's paired *t*-test. Differences between groups were appraised by multiple regression analysis, including age and priming dose of L-alanine as covariates. Pearson's correlation coefficients were calculated between baseline metabolite concentrations (expressed relative to total MR-detectable phosphate) and absolute metabolite change (AUC) per minute during L-alanine infusion. *P* values <0.05 indicate statistical significance.

Results

Study population

Twenty-one patients with non-small cell lung cancer were included in the study: nine weight-losing (≥ 5% weight loss, CaWL) and twelve weight-stable patients (< 5% weight loss, CaWS). Twelve healthy subjects were included as controls (C). Characteristics of the study population are listed in **Table 4.1.** Mean age of lung cancer patients was

Table 4.1

Characteristics of study population.[1]

	Control	Lung cancer		P Value	
		WS	WL	Ca vs.	WL vs.
	(n = 12)	(n = 12)	(n = 9)	C	WS
Age (y)	37-69 [2]	38-76	53-81	<0.01	0.20
Gender (m/f)	2 / 10	7 / 5	7 / 2		
Disease stage (IIIA/IIIB/IV)	-	1 / 5 / 6	3 / 2 / 4		
Previous therapy (n)[3]					
Surgery	0	2	2		
Radiotherapy	0	6	4		
Chemotherapy	0	6	0		
Weight (kg)	77.0 ± 3.0	72.8 ± 3.4	60.9 ± 3.5	0.36	0.02
Weight change (kg)	0	1 ± 1	-9.0 ± 1.4	0.56	<0.001
Weight change (%)	0	1 ± 1	-12 ± 2	0.59	<0.001
% Ideal body weight	124 ± 5	117 ± 5	96 ± 5	0.33	<0.01
Body mass index (kg/m^2)	25.8 ± 1.0	25.1 ± 1.0	20.9 ± 1.1	0.62	0.01
Sum of skinfolds (mm)[4]	71 ± 6	63 ± 6	30 ± 4	0.29	<0.001
Albumin (g/liter)	46 ± 1[5]	43 ± 1[5]	38 ± 2[5]	0.34	<0.01
Prealbumin (g/liter)	0.30 ± 0.01	0.29 ± 0.01[5]	0.17 ± 0.02[5]	0.47	<0.001

[1] Mean ± SE.

[2] Range

[3] Antitumor treatment: surgery > 3 months, radio- and chemotherapy . 4 weeks prior to the MRS study (number of patients)

[4] Sum of four skinfolds: biceps, triceps, supra iliac, subscapular (mm)

[5] One value missing

higher than in C, although age ranges largely overlapped. Disease stage was similar in the WL/WS lung cancer patients. The previous antitumor treatment was also comparable in both groups, except for chemotherapy which had been given as a previous treatment in six CaWS but in none of the CaWL. Note that none of the patients received any antitumor therapy at the time of the study. The WL lung cancer patients had lost 9.0 ± 1.4 kg (mean ± SE) or 12% (range 6 - 22%) of their pre-illness stable body weight within the six months preceding the study. Body weight, body mass index and sum of skinfolds were significantly lower in CaWL when compared with CaWS and C (P<0.05). Albumin and prealbumin levels were also significantly decreased in CaWL. Liver function tests were normal in all subjects. All patients had a history of smoking compared to 42%

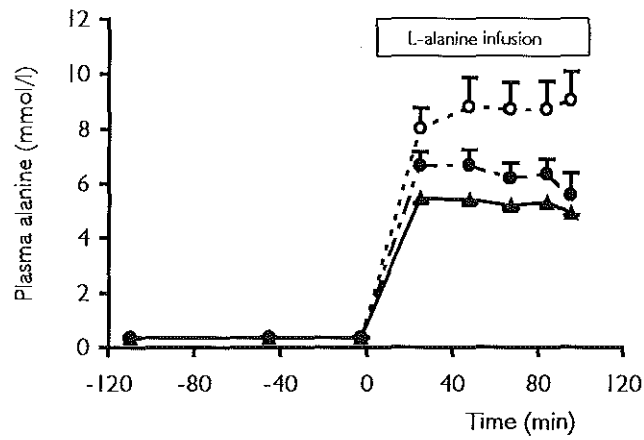

Figure 4.1

Plasma alanine concentrations in healthy control subjects (--o--; n = 11), weight-stable (–●–; n = 11) and weight-losing (–▲– ; n = 9) lung cancer patients before and during a primed-constant infusion of 1.4-2.8 mmol/kg + 2.8 mmol/kg/h L-alanine. Curves and error bars represent means ± SE.

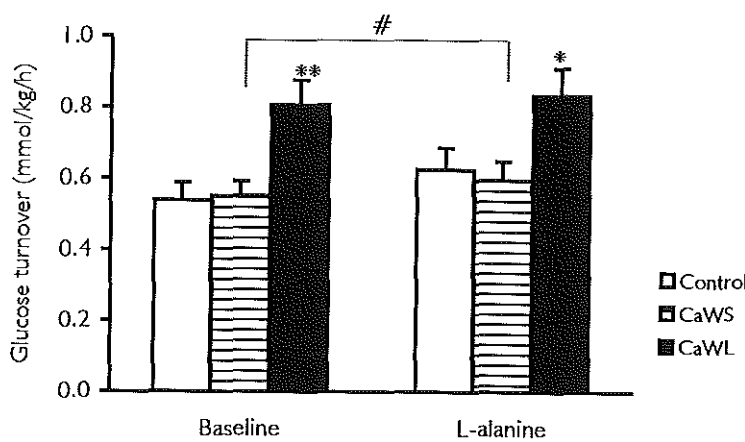

Figure 4.2

Whole body rate of appearance (turnover) of glucose before and during primed-continuous L-alanine infusion in healthy control subjects (Control, n=10), weight-stable (CaWS, n=12), and weight-losing (CaWL, n=6) lung cancer patients. Turnover rates were assessed using a primed-constant infusion of [6,6-$^2H_2$]-glucose. Bars and error bars represent means ± SE. Significantly different from WS lung cancer patients and healthy controls: *$P<0.05$, **$P<0.01$ (ANOVA, adjusted for age). #Significantly different from baseline, $P<0.05$ (Paired t-test).

of the healthy subjects. Actual smokers at the time of study were 38% of WL, 33% of WS cancer patients, and 33% of healthy control subjects. No differences in energy intake were detected between any of the groups. Because of the difference in age between the groups, all data were checked for potential confounding by age. Although in no case significant confounding by age was observed, all presented statistical analyses are adjusted for age.

Plasma substrate concentrations and flux measurements

Fasting blood glucose levels were similar in lung cancer patients (CaWL, 5.8 ± 0.3 mM (mmol/liter); CaWS, 5.3 ± 0.2 mM) and healthy subjects (5.7 ± 0.2 mM) and did not change during L-alanine infusion (CaWL, 5.7 ± 0.4 mM; CaWS, 5.0 ± 0.1 mM; C, 5.5 ± 0.2 mM). Baseline plasma alanine concentrations were similar in lung cancer patients and healthy controls (0.35 - 0.37 mM, **Figure 4.1**). L-Alanine infusion caused a sharp and highly significant rise in plasma alanine concentrations to an average of 5.37 ± 0.14 mM in CaWL, 6.67 ± 0.51 mM in CaWS, and 8.47 ± 0.88 mM in C (CaWL *vs.* CaWS and CaWS *vs.* C: $P<0.001$). These post-alanine plasma concentrations were significantly different between all groups ($P<0.001$). Turnover rates of glucose at baseline and during L-alanine infusion are presented in **Figure 4.2.** Whole-body glucose turnover at baseline was 35% higher in CaWL as compared to both CaWS and C ($P<0.01$). During alanine infusion glucose turnover slightly increased by 0.03 ± 0.02 mmol/kg/h in CaWL ($P=0.19$), 0.05 ± 0.02 mmol/kg/h in CaWS ($P<0.05$), and 0.09 ± 0.04 mmol/kg/h in C ($P=0.08$). Glucose turnover during alanine infusion was still 36% higher in CaWL than in CaWS and C ($P<0.05$).

Hepatic concentrations of gluconeogenic intermediates

Baseline phosphomonoesters (PME) were significantly elevated in CaWL (10.5 ± 1.0 %) when compared with CaWS and C (6.7 ± 0.5 and 7.9 ± 0.7, respectively, $P<0.01$, corrected for age), as previously reported. No differences in PDE (CaWL, 30.6 ± 1.9; CaWS, 27.8 ± 2.2; C, 31.4 ± 2.0) were observed between the groups.

In **Figure 4.3** changes in hepatic metabolite concentrations during L-alanine infusion are shown. PME increased gradually in C and reached statistical significance at 60 min ($P<0.01$). In CaWS, PME showed a sharp and highly significant rise during the first 30 min and was still significantly elevated at 75 min of L-alanine infusion ($P<0.05$). The slope of PME from 0-30 min was significantly steeper in CaWS than in C (2.48 ± 0.50% / min *vs.* 0.77 ± 0.45% / min; $P=0.01$). In contrast, PME concentrations in CaWL did not change significantly during alanine infusion. Phosphodiesters (PDE) initially decreased in C but increased in both CaWS and CaWL, with significantly different slopes of PDE curves between 0-30 min between WL/WS lung cancer patients *vs.*

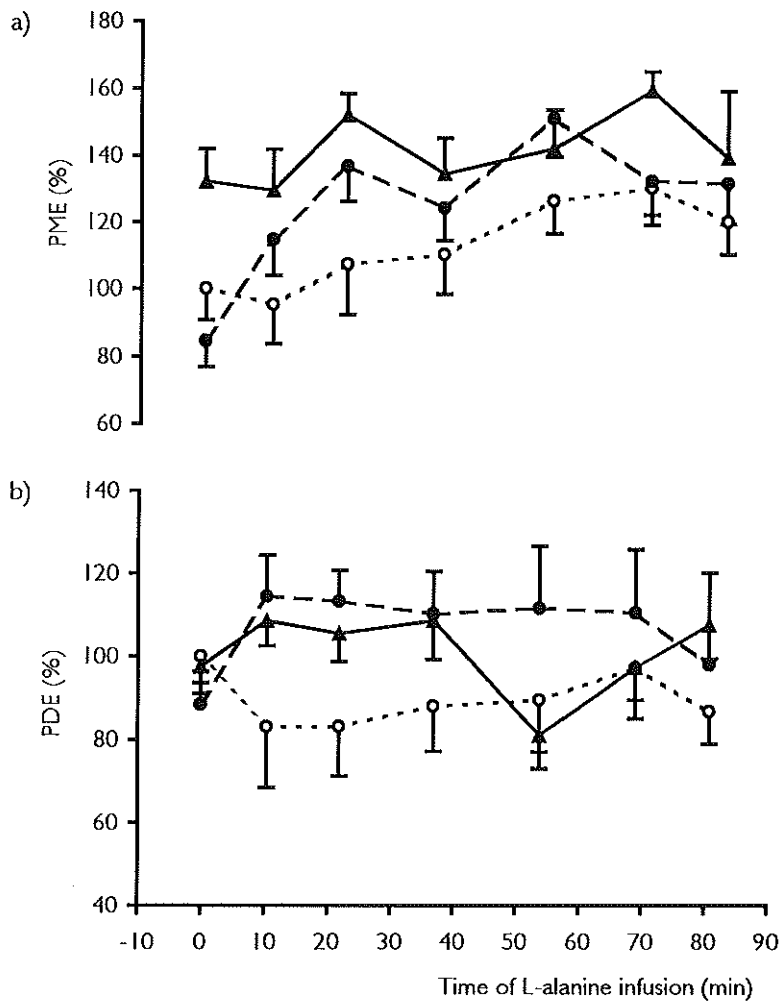

Figure 4.3

Metabolite concentrations in the liver of healthy control subjects (--o--; n = 9), as well as weight-stable (–●–; n = 10) and weight-losing (–▲– ; n = 7) lung cancer patients during a primed-constant infusion of 1.4-2.8 mmol/kg + 2.8 mmol/kg/h L-alanine: a) phosphomonoesters (PME), b) phosphodiesters (PDE). Curves and error bars represent means ± SE. Baseline values are means from 5 spectra acquired in each subject before L-alanine infusion. Values are expressed as percentage of mean baseline value of healthy subjects (= 100%). Times during L-alanine infusion are mid-time points of ^{31}P MRS data collection referenced to the start of the L-alanine infusion (0=baseline). Significance of changes during L-alanine infusion compared to baseline (Student's paired t-test): *P<0.05, **P<0.01.

healthy subjects (0.62 ± 0.33% / min and 1.20 ± 0.77 % / min *vs.* -0.57 ± 0.33% / min, respectively; P=0.02). PDE levels as such were not significantly different from baseline at any time point in either of the three groups.

Overall changes during L-alanine infusion: area under the curve

Overall changes in metabolite concentrations during L-alanine infusion relative to baseline are presented in **Table 4.2.** During the first 45 min of alanine infusion the increase in PME concentrations was significantly less in CaWL than in CaWS and C (P=0.02). At 45-90 min of alanine infusion this difference in response between CaWL and CaWS/C remained although it was no longer statistically significant.

Table 4.2

Hepatic phosphoric metabolite levels after primed-constant infusion of L-alanine in healthy control subjects and lung cancer patients.[1]

	Time of alanine infusion	Control (n = 9)		Lung cancer WS (n = 10)		Lung cancer WL (n = 7)	P Value[2] Ca vs. C	P Value[2] WL vs. WS
PME	0-45 min	21 ± 9		58 ± 14	**	7 ± 8	0.29	0.02
	45-90 min	50 ± 16	**	87 ± 31	*	7 ± 9	0.54	0.17
	0-90 min	33 ± 11	*	69 ± 19	**	11 ± 10	0.41	0.09
PDE	0-45 min	-5 ± 7		32 ± 20		23 ± 15	0.58	0.16
	45-90 min	-3 ± 10		40 ± 33		9 ± 12	0.51	0.14
	0-90 min	-8 ± 5		30 ± 22		12 ± 11	0.55	0.06

[1] Area under the curve during L-alanine infusion expressed as change from baseline (%, mean ± SE).
[2] For between group differences in response to alanine infusion adjusted for age and L-alanine prime dose. Difference from baseline values (Student's paired t-test): *P<0.05, **P<0.01.

Hormone levels

Plasma insulin and glucagon levels are presented in **Figure 4.4.** Baseline insulin concentrations were lower in CaWL than in CaWS and C (P<0.05). Insulin levels showed a strong increase at 45 min of L-alanine infusion (P<0.01) and were still significantly elevated from baseline at 90 min in all groups (P<0.01). In CaWL, insulin levels remained significantly lower than in both CaWS and C during the 90 min of alanine infusion (P<0.01). Baseline glucagon levels were similar in WL/WS lung cancer patients and control subjects. L-alanine infusion caused a substantial rise in plasma glucagon at 45-

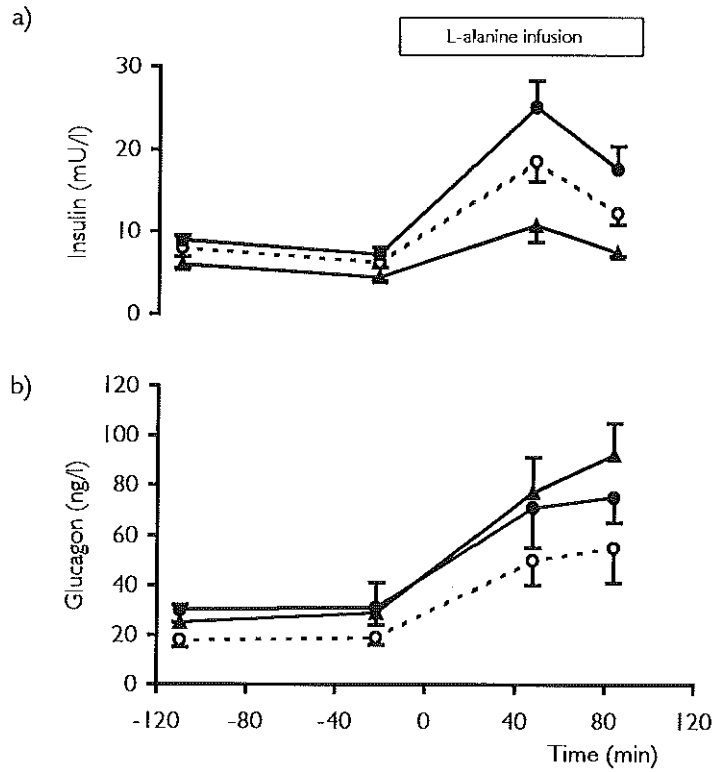

Figure 4.4

Plasma insulin (a) and glucagon (b) concentrations in healthy control subjects (--o--; n = 10), weight-stable (–●–; n = 10) and weight-losing (–▲– ; n = 6) lung cancer patients before and during a primed-constant infusion of 1.4-2.8 mmol/kg + 2.8 mmol/kg/h L-alanine. Curves and error bars represent means ± SE.

90 min in all groups ($P<0.01$). No significant differences in glucagon concentrations during alanine infusion were observed between any of the groups.

Correlations

Spectral changes in PME and PDE during alanine infusion were strongly dependent on their respective baseline concentrations in lung cancer patients (r=-0.82 and r=-0.86, respectively; $P<0.001$) but not in C (r=-0.07 and r=-0.30, respectively) (**Figure 4.5**). Furthermore, patients with a higher degree of weight loss showed less increase in PME levels during alanine infusion (r= -0.54, $P<0.05$).

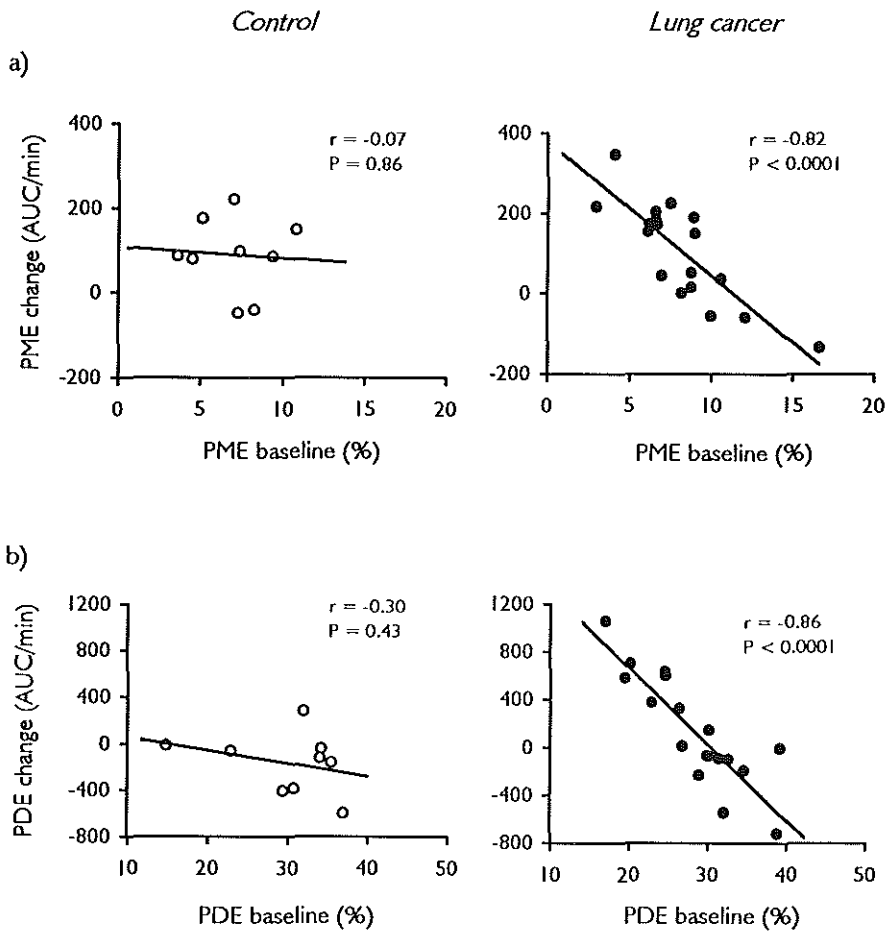

Figure 4.5

Changes in metabolite concentrations in the liver of healthy control subjects (n=9) and lung cancer patients (n=17) during a primed-constant infusion of L-alanine plotted against their respective baseline values for: a) PME, b) PDE, both expressed relative to total MR-detectable phospate (%). r, Pearon's correlation coefficient.

Discussion

In the present study, hepatic gluconeogenesis from alanine in lung cancer patients was monitored by means of ^{31}P MR spectroscopy during an i.v. L-alanine challenge, and information on gluconeogenic intermediates was obtained non-invasively. Simultaneously, glucose turnover before and during L-alanine infusion was measured using stable isotope tracers.

Glucose flux was found to be significantly elevated in WL lung cancer patients at baseline as compared to WS lung cancer patients and control subjects, confirming other studies [89,126]. Changes in glucose turnover during L-alanine infusion were minimal, as could be expected in view of the autoregulatory mechanisms which control hepatic glucose output [57,108,225]. Liver PME levels increased during alanine infusion both in CaWS and C, confirming studies performed in healthy animals [30] and humans [50,177] in which this rise in PME was attributed to increased concentrations of 3-phosphoglycerate (3PG) [30,50]. Our finding in the present study that in WS lung cancer patients PME increased significantly faster and reached twice as high levels as in healthy subjects may reflect a more rapid rise in concentrations of 3PG in the liver of these patients. In contrast, in weight-losing lung cancer patients, PME was already elevated at baseline and did not increase any further during alanine infusion. Moreover, a strong negative correlation between baseline PME levels and rise in PME during alanine infusion was observed in lung cancer patients, but not in healthy controls, suggesting that levels of 3PG in WL patients were maximal at baseline and could not be increased any further by an i.v. alanine challenge.

Even though mean PDE concentrations were similar in lung cancer patients and healthy controls both at baseline and during alanine infusion, a significant difference in slope between patients (increase) and healthy subjects (decrease) was detected in the first 30 minutes of alanine infusion. As for PME, changes in PDE during alanine infusion were negatively correlated with baseline PDE levels in lung cancer patients but not in healthy subjects. The PDE resonance contains components of phospholipid membranes, such as glycerophosphorylethanolamine and glycerophosphorylcholine, and the gluconeogenic intermediate phosphoenolpyruvate [146]. In liver extracts of healthy rats, post-alanine infusion phosphoenolpyruvate concentrations were significantly elevated [30,50], suggesting that the increase in PDE in lung cancer patients observed in the present study is most likely due to elevated accumulation of phosphoenolpyruvate.

The mechanisms involved in the increasing levels of PME before and during alanine infusion in lung cancer patients could be enhanced uptake of alanine within the hepatocytes and/or elevated gluconeogenic enzyme activity. In animal studies *in vivo*, alanine concentrations in the liver of tumor-bearing hosts were elevated [172], whereas plasma

alanine concentrations were decreased [101], suggesting elevated uptake of alanine by the liver. Some authors reported reduced plasma alanine concentrations in weight-losing lung cancer patients [28,88]. In the present study, we did not detect any differences in baseline plasma alanine levels between WL/WS lung cancer patients and healthy subjects. This indicates that elevated alanine flux previously reported in weight-losing lung cancer patients [126] is counterbalanced by increased alanine uptake in the liver, resulting in similar plasma levels in CaWL, CaWS, and C. It is noteworthy that, even though plasma alanine concentrations increased significantly in all groups during infusion of a standardized alanine dose, they did not increase to the same extent but in the order CaWL < CaWS < C (P<0.001). This would imply that alanine uptake by the liver during alanine infusion is increased in lung cancer patients, and especially so in weight-losing patients.

Elevated activities of gluconeogenic key enzymes in the liver of tumor-bearing hosts have been reported by several authors, which could explain the elevated PME levels in WL lung cancer patients at baseline, as well as the faster and larger increase in PME observed in CaWS during alanine infusion. Increased PDE and PME levels during alanine infusion, most likely reflecting elevated phosphoenolpyruvate and 3PG concentrations, could be explained by enhanced activities of pyruvate carboxylase (converting pyruvate into oxaloacetate) and/or phosphoenolpyruvate carboxylase (converting oxaloacetate into phosphoenolpyruvate). The observed increase in glucose production could also be the result of enhanced glucose-6-phosphatase activity. Indeed, animal studies showed elevated activities of pyruvate carboxylase in the liver of rats bearing mammary adenocarcinomas [128], and phosphoenolpyruvate carboxykinase [156] and glucose-6-phosphatase [83] in the liver of sarcoma-bearing rats. Factors which may be involved in the enhanced activities of gluconeogenic enzymes are decreased insulin or increased glucagon concentrations [10,101,174], stimulating gluconeogenic key enzymes [160]. Relatively higher glucagon:insulin ratios were observed in CaWL in comparison to WS patients.

In summary, this study demonstrates the presence of major alterations in gluconeogenesis in the tumor-free liver of lung cancer patients both with and without weight loss. Weight-losing lung cancer patients have markedly elevated glucose flux before as well as during L-alanine infusion. This is also confirmed by elevated PME and PDE levels within the liver which reflect accumulation of gluconeogenic intermediates in these patients both before and during alanine infusion. Neither glucose flux nor concentrations of gluconeogenic intermediates within the liver show any change during alanine infusion in WL cancer patients, suggesting that gluconeogenesis is already maximally induced at baseline in these patients. Weight-stable lung cancer patients, having a normal glucose flux, show an accelerated rise in PME and PDE levels during L-alanine infusion. Our

results suggest that both altered gluconeogenic enzyme activities within the liver and elevated alanine uptake are involved in these abnormalities.

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5

Decreased energy and phosphorylation status in the liver of lung cancer patients with weight loss

Submitted for publication

Susanne Leij-Halfwerk,[1,2] Pieter C Dagnelie,[1,3] Matthijs Oudkerk,[2] Paul E Sijens[2]

[1] Department of Internal Medicine II, Erasmus University Medical Center Rotterdam, Rotterdam,
[2] Department of Diagnostic Radiology, Daniel Den Hoed Cancer Center, Rotterdam and
[3] Department of Epidemiology, University Maastricht, Maastricht, The Netherlands

Abstract

Altered energy status has been reported in the liver of tumour-bearing animals, but data on energy status in humans are scarce. Therefore, bioenergetics in tumour-free liver of lung cancer patients were monitored using ^{31}P magnetic resonance spectroscopy (MRS) with infusion of L-alanine as a gluconeogenic challenge.

Twenty-one overnight-fasted lung cancer patients without liver metastases, with (CaWL) or without weight loss (CaWS), and twelve healthy control subjects (C) were studied. Hepatic energy status was monitored before and during an i.v. L-alanine infusion of 1.4-2.8 mmol/kg + 2.8 mmol/kg/h for 90 min. by ^{31}P MR spectroscopy.

Baseline levels of ATP in WL lung cancer patients, expressed relative to total MR-detectable phosphate, were reduced (CaWL, 9.5 ± 0.9% vs. CaWS, 12.6 ± 0.8% and C, 12.4 ± 0.8%; $P<0.05$) and inversely correlated with the degree of weight loss in lung cancer patients ($r = -0.46$, $P=0.03$). P_i /ATP ratios were increased ($P<0.05$), indicating reduced liver phosphorylation status. During L-alanine infusion, ATP levels decreased in all groups ($P<0.05$); in CaWL, ATP levels were lower at all time-points between 0-90 min as compared to both CaWS and C ($P<0.05$). P_i/ATP ratios were significantly higher after 70-90 min of L-alanine infusion in CaWL compared to CaWS and C ($P<0.05$).

Hepatic ATP and phosphorylation status are reduced in weight-losing lung cancer patients, in contrast with WS patients and healthy subjects, and continue to decrease during infusion of a gluconeogenic substrate, suggesting impaired energy regenerating capacity in these patients.

Introduction

Weight loss is a common phenomenon in advanced cancer patients which affects both outcome of anti-tumour therapy [56] and survival [43,198]. Alterations in intermediary host metabolism have been frequently described, including elevated protein breakdown [89], increased glucose production [26,96,133], and enhanced Cori cycle activity [5,116]. Since these processes put a continuous demand on energy stores of the cancer-bearing host, energy reserves may become depleted which could contribute to weight loss. Although the mechanisms are not fully understood, it has been suggested that the liver is involved with metabolic alterations causing weight loss in cancer [20,115].

In animals bearing experimental tumours, reduced liver phosphorylation status has been reported [49]. In sarcoma-bearing rats, liver ATP levels progressively decreased [211] and P_i /ATP ratios increased [181] with increasing tumour-burden, suggesting intracellular energy depletion. Moreover, changes in intracellular P_i /ATP ratios were

already detected before the development of significant weight loss and prior to a decrease in food intake [77,181]. It was suggested that elevated P_i release during fructose infusion was a result of increased gluconeogenic [77]. Hepatocytes from tumour-rats also showed elevated gluconeogenesis from lactate and a dramatically decreased energy regenerating capacity during this elevated metabolic process [211].

Due to lack of non-invasive techniques, data on altered energy metabolism in the liver of humans with cancer are scarce. In a recent study, ^{31}P magnetic resonance spectroscopy (MRS) was used as a first attempt to measure hepatic energy status in cancer patients *in vivo* [51]. Results suggested reduced concentrations of ATP and P_i depending on the technique utilised, in cancer patients with various tumour types, even though P_i/ATP ratios were not different from healthy subjects [51].

The aim of the present study was to monitor ATP and P_i levels in tumour-free liver of a well-defined group of lung cancer patients with or without weight loss using ^{31}P MRS. L-alanine was infused in order to assess the energy generating capacity of the liver during a gluconeogenic challenge.

Subjects and methods

Subjects

The study was approved by the Medical Ethical Committee of the Erasmus University Medical Centre Rotterdam, Rotterdam, The Netherlands. Patients with non-small cell lung cancer stage IIIA/B or IV (WHO grading system) attending the outpatient department of the University Hospital Rotterdam, The Netherlands, were recruited. Patients who were in remission or apparently cured were excluded. Additional exclusion criteria were: liver metastases (as checked for by CT/ultrasound), metabolic disease, corticosteroid treatment, elective surgery <3 months, chemo- or radiotherapy <4 weeks prior to study, alcohol consumption of more than 100g/week (=10 glasses), pregnancy, extreme anorexia or artificial weight reduction by dieting. Healthy subjects without weight loss were included as a control group (C). All participants signed informed consent.

Twenty-one patients with non-small cell lung cancer were included in the study: nine weight-losing (weight loss 9.0 ± 1.4 kg (mean ± SEM) or 12% (range 6-22%), CaWL) and twelve weight-stable patients (< 5% weight loss, CaWS). Twelve healthy subjects without weight loss were included as a control group (C). In lung cancer patients mean age was higher (CaWL: 69 y (mean), range 53-81; CaWS: 63 y, range 38-76) as compared to C (49 y, range 37-69) although age ranges largely overlapped. Similar disease stages (CaWL: 3 IIIA, 2 IIIB, 4 IV; WS: 1 IIIA, 5 IIIB, 6 IV) and previous anti tumour treatment (surgery: 2 WL, 2 WS; radiotherapy: 4 WL, 6 WS; chemotherapy: 0 WL, 6 WS) were

observed in the WL/WS lung cancer patients. Minimal time period between the previous therapy and MRS study was 3 months for surgery and 4 weeks for radio- and chemotherapy. None of the patients received any therapy at the time of the study. Data on pre-illness stable weight, current weight, and weight loss over the previous 6 months were taken from hospital records supplemented with oral information form patients. Body weight was measured to the nearest 0.1 kg on an electrical weighing scale (Seca 707, Hamburg, Germany), height was measured to the nearest 0.1 cm, and thickness of four skinfolds (triceps, biceps, subscapular, supra iliac) were measured to the nearest 0.2 mm using a standard skinfold caliper (Holtain Ltd., London, Great Britain). Body weight, body mass index, and sum of skinfolds, as well as albumin and prealbumin levels were significantly decreased in CaWL as compared to CaWS and C ($P<0.01$). Liver function tests were normal in all subjects. No differences in energy intake between the lung cancer patients and healthy controls were detected as calculated from a dietary record during 7 days prior to the study.

Experimental design

All subjects refrained from alcoholic drinks for three days prior to the MRS measurements. The subjects were studied between 7:30 a.m. and 1:00 p.m. after an overnight fast (12-14 hours). A cannula (0.8×25 mm) was positioned in the left cubital vein for the infusion of L-alanine. In the contralateral cubital vein, an identical cannula was positioned for blood sampling. Blood samples were obtained for the determination of plasma substrate concentrations just before the L-alanine infusion was started and during alanine infusion. A solution of L-alanine in water (10% w/v) was prepared (Bufa B.V., Uitgeest, The Netherlands), sterilised by autoclavating in glass bottles, and warmed to ~30°C. After obtaining five baseline ^{31}P MR spectra, a priming dose of 1.4 - 2.8 mmol/kg L-alanine was administered in 5-8 min and followed by a continuous infusion of 2.8 mmol/kg/h L-alanine for 90 minutes in order to obtain steady state conditions. Plasma alanine concentrations were determined enzymatically as described by Williamson [223]. Blood glucose concentrations were measured enzymatically with glucose-oxidase and peroxidase (Boehringer Mannheim, Mannheim, Germany).

^{31}P MR spectroscopy of the liver

Spectroscopy studies were performed with a whole-body MR system equipped with a Helicon magnet operating at 2.0 Tesla (Vision Magneton, Siemens AG, Erlangen, Germany). A 16 cm diameter transmit/receive ^{1}H/^{31}P surface coil was used for MRI localization, shimming and ^{31}P MR spectroscopy. Elastic bands were used for positioning the coil lateral to the liver in the mid-axillary plane. Field homogeneity achieved in shimming resulted in water peak line widths which were usually less than 40Hz (≈0.5 ppm). After

obtaining an image of the region of interest, a one-dimensional chemical shift imaging (1D-CSI) sequence was applied on a transverse slice of 4 cm centered on the surface coil and the liver (1×4 phase-encoded matrix, field of view 40×40 cm^2), yielding volumes of 40×10×4 cm^3 [195]. Spectra were collected with a 640 μs Hanning-sinc shaped radio frequency pulse resulting in a flip angle of 135° in the centre of the coil, and 60° (weighted average) in the liver volume with a repetition time (TR) of 1 s (40 acquisitions). Spectra were obtained at baseline and sequentially at 3 min intervals during the continuous L-alanine infusion.

Time domain data were Fourier transformed after Gaussian multiplication (centre: 0 ms, width 30 ms) and phase corrected. Quantification of spectral peak areas was performed using Numaris-3 software package (Siemens AG, Erlangen, Germany) including polynomial baseline correction followed by frequency domain curve fitting [194]. Metabolite concentrations were calculated from peak areas and expressed relative to total MR-detectable phosphate as previously described [51]. Total detectable MR-phosphate did not change during L-alanine infusion (data not shown). Intracellular pH values were calculated from the chemical shift difference between the P_i and α-ATP resonances [134].

Statistical analysis

Results are reported as means ± standard error of the mean (SEM). In each experiment the average of five subsequent spectra was used as baseline value for calculations. Mid-time points of MRS data acquisition at 15 min intervals during L-alanine infusion (7.5, 22.5, 37.5 min, etc.) were used for graphical representation, and values expressed relative to the mean baseline value of healthy control subjects (=100%). Integrals of time-response curves (area under the curve) of absolute peak areas over 0-45 min, 45-90 min, and 0-90 min intervals during L-alanine infusion were calculated as a measure of overall spectral response and expressed relative to baseline values (AUC). Directions of response were analysed by calculation of the slope of sequential spectra between 0-30 min of L-alanine infusion. Changes from baseline values were appraised using Student's paired *t*-test. Between-group differences in baseline values and in response to alanine infusion were analysed by ANOVA. Multiple regression analysis was used to analyse interrelationships with adjustment for age and L-alanine priming dose. Pearson's correlation coefficients between baseline metabolite concentrations (expressed relative to total MR-detectable phosphate) for baseline values and absolute metabolite change (AUC) per minute during L-alanine infusion were calculated. *P* values <0.05 indicate statistical significance.

Results

Fasting blood glucose levels were similar in lung cancer patients and did not change during L-alanine infusion. Baseline plasma alanine concentrations were similar between lung cancer patients and healthy controls (0.35 - 0.37 mM) and increased significantly during L-alanine infusion to 5.4 ± 0.1 mM in CaWL, 6.7 ± 0.5 mM in CaWS, and 8.5 ± 0.9 mM in C (change from baseline and difference between all groups: $P<0.001$).

^{31}P MRS at baseline and during L-alanine infusion

Table 5.1 shows hepatic baseline values of ATP and inorganic phosphate (P_i) in lung cancer patients and healthy control subjects. Liver ATP concentrations were significantly lower in CaWL than in CaWS and C ($P<0.01$, adjusted for age). ATP levels in CaWS and C were not significantly different. No significant differences in P_i levels were observed between the groups, although P_i was slightly higher in CaWL. P_i/ATP ratios were significantly increased in CaWL when compared with both CaWS and C ($P<0.05$), indicating a decreased liver phosphorylation status. Again, P_i/ATP ratios in CaWS and C did not differ significantly.

Table 5.1

Baseline hepatic metabolite levels expressed relative to total MR-detectable phosphate in healthy control subjects and lung cancer patients.[1]

	Control	Lung cancer		P Value[2]	
		WS	WL	Ca vs.	WL vs.
	(n = 12)	(n = 12)	(n = 9)	C	WS
ATP [3]	12.4 ± 0.8	12.6 ± 0.8	9.5 ± 0.9	0.60	0.01
P_i [3]	8.4 ± 0.7	8.5 ± 0.4	9.1 ± 1.1	0.90	0.98
P_i/ATP	0.72 ± 0.08	0.72 ± 0.06	1.07 ± 0.19	0.68	0.03

[1] Mean ± SEM.

[2] Differences between group means were analysed by ANOVA adjusted for age; Ca, lung cancer; C, healthy control; WS, weight-stable; WL, weight-losing

[3] Percent of total MR-detectable phosphate.

Changes in ATP and P_i concentrations during L-alanine infusion are presented in **Figure 5.1**. ATP levels showed a slow but steady decrease in all groups which became statistically significant from baseline values after 20 min of alanine infusion in CaWL ($P<0.05$)

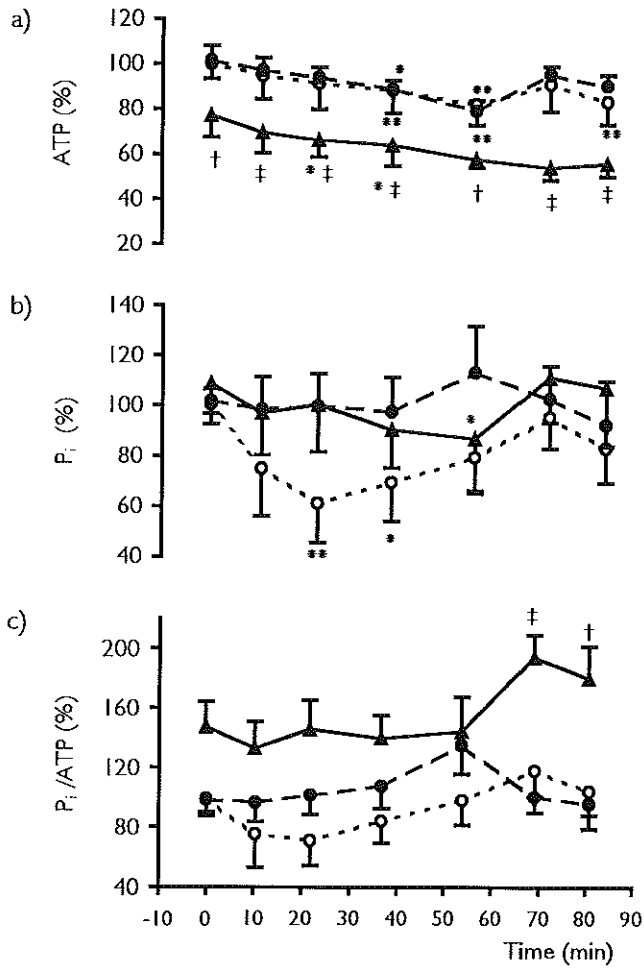

Figure 5.1

Energy metabolite concentrations in the liver of healthy control subjects (–o–; n = 9), as well as weight-stable (–●–; n = 10) and weight-losing (–▲– ; n = 7) lung cancer patients during a primed-constant infusion of 1.4-2.8 mmol/kg + 2.8 mmol/kg/h L-alanine; a) ATP, b) P_i, c) P_i /ATP. Curves and error bars represent means ± SEM. Baseline values are means from 5 spectra acquired in each subject before L-alanine infusion. Values are expressed as percentage of mean baseline value of healthy subjects (=100%). Times during L-alanine infusion are mid-time points of ^{31}P MRS data collection referenced to the start of the L-alanine infusion (0=baseline). Significance of changes during L-alanine infusion compared to baseline (Student's paired t-test): *P<0.05, **P<0.01. Significant difference between CaWL and CaWS/C: †P<0.05, ‡P<0.01 (ANOVA).

Table 5.2

Overall response of hepatic metabolite levels to primed-constant infusion of L-alanine in healthy control subjects and lung cancer patients.[1]

		Control		Lung cancer			
				WS		WL	
		(n = 9)		(n = 10)		(n = 7)	
ATP	0-45 min	-6 ± 3	**	-5 ± 4		-16 ± 5	*
	45-90 min	-12 ± 5	**	-13 ± 5	*	-21 ± 7	*
	0-90 min	-12 ± 3	**	-9 ± 4	*	-17 ± 5	*
P_i	0-45 min	-21 ± 6	*	1 ± 10		-11 ± 11	
	45-90 min	-9 ± 13		9 ± 18		-16 ± 5	*
	0-90 min	-18 ± 8		-1 ± 11		-9 ± 9	
P_i/ATP	0-45 min	-12 ± 8		11 ± 14		4 ± 14	
	45-90 min	9 ± 16		30 ± 24		26 ± 15	
	0-90 min	-6 ± 11		13 ± 15		21 ± 12	

[1] Area under the curve during L-alanine infusion expressed as change from baseline (%, mean ± SEM). Difference from baseline values (Student's paired t-test): *P<0.05, **P<0.01.

and after 35-40 min in CaWS (P<0.05) and C (P<0.01). In CaWL, ATP levels were significantly lower at all time points between 0-90 min when compared with both CaWS and C (P<0.05). Between 60-90 min of alanine infusion, partial recovery of ATP was observed in both C and CaWS, but not in CaWL where ATP continued to decrease. P_i levels in C decreased sharply during the first 30 min (P<0.01), but recovered to baseline values between 60 and 90 min of alanine infusion. In contrast, no significant change in P_i was observed in CaWS throughout alanine infusion, whereas in CaWL P_i showed a slow but continuous decrease which became statistically significant at 60 min of alanine infusion, with a sudden recovery thereafter. P_i /ATP ratios did not change significantly during the first 60 min of alanine infusion in either patients or healthy subjects, even though P_i /ATP tended to decrease in C during the first 30 min. After 60 min of alanine infusion P_i /ATP ratios showed a sudden significant rise in CaWL when compared with CaWS and C (P<0.01). This difference remained statistically significant (P<0.05) until 90 min when the alanine infusion was discontinued.

Overall changes during L-alanine infusion

Overall changes in metabolite concentrations during L-alanine infusion relative to baseline are presented in **Table 5.2**. Although ATP decreased relatively more in CaWL and

almost three times as much as in CaWS and C between 0-45 min, the difference compared to CaWS and C was not statistically significant. Changes in P_i after alanine infusion were also not significantly different between lung cancer patients and healthy subjects at either 0-45 or 45-90 of alanine infusion. P_i/ATP ratios in lung cancer patients tended to increase during alanine infusion whereas in control subjects a trend to decrease was observed (NS), but again the changes were not significantly different between the groups. Alanine infusion did not change liver pH in any of the groups (data not shown).

Correlations

Baseline levels of ATP were inversely correlated with the degree of weight loss in lung cancer patients (r=-0.46, P=0.03). Furthermore, baseline ATP levels were inversely correlated with P_i at baseline (r=-0.38, P=0.03). Changes in ATP during L-alanine were significantly correlated with baseline P_i (r=0.50, P<0.01), but no significant correlation between changes in ATP and P_i during alanine infusion was detected (r=-0.168, P=0.41).

Discussion

In the present study, bioenergetics in the tumour-free liver of lung cancer patients during an i.v. L-alanine challenge were monitored using ^{31}P MR spectroscopy. Alanine infusion is known to stimulate gluconeogenesis [57,59,225], and a standardised dose can be used to investigate gluconeogenic and energy metabolism within the liver by ^{31}P MRS directly and non-invasively [50]. In the present study, primed-constant infusion of 1.4-2.8 mmol/kg L-alanine + 2.8 mmol/kg/h induced a steady decrease in ATP levels in healthy controls. This decrease in liver ATP was also reported after bolus infusion of 5.6 mmol alanine/kg in healthy rats [30], but was not observed at a lower dose of 2.8 mmol/kg alanine in healthy rats [30] or humans [50].

Baseline ATP levels were significantly reduced in weight-losing lung cancer patients, whereas ATP levels in weight-stable lung cancer patients were not different from those in healthy control subjects. Furthermore, phosphorylation status was significantly decreased in weight-losing cancer patients, as shown by elevated P_i /ATP ratios. During L-alanine infusion, ATP levels in WL cancer patients decreased to a greater extent than in WS patients and healthy control subjects. It is remarkable that, between 60-90 min of L-alanine infusion, ATP levels in WS cancer patients and healthy subjects showed some recovery, whereas in WL cancer patients the decrease in ATP concentrations continued thoughout the 90-min infusion period.

In sarcoma-bearing rats progressive decrease in liver ATP levels was detected be-

tween 5 and 20% of tumour-burden [211], whereas significant weight loss and anorexia were only detected at 20% tumour-burden. In liver from rats bearing sarcomas, decreased phosphorylation potential was detected biochemically [6] and by ^{31}P MRS *in vivo* [181]. Phosphorylation status was also negatively correlated with tumour-burden, and reduced phosphorylation status was most prominent at a high tumour-burden when also weight loss and anorexia were present [181]. A study in cancer patients with mixed tumour types suggested apparently decreased ATP levels in the tumour-free liver [51].

The loss of hepatic ATP during alanine infusion could be caused by different mechanisms. Since gluconeogenesis from alanine is an energy consuming process which requires 6 moles of ATP per mole glucose produced, an increase in gluconeogenesis puts a demand on the energy stores in the hepatocyte. If ATP is utilised at a higher rate than ATP is resynthesised, an energy deficit in the hepatocyte would occur. Furthermore, complete metabolism of alanine includes the urea cycle in addition to gluconeogenesis, requiring an additional 4 ~P per mole of glucose produced [151]. The energy-dependent uptake of alanine into the hepatocyte could also be involved in the decrease in ATP levels during alanine infusion [170,214].

The observed bioenergetic derangements in lung cancer patients may be related to the presence of a distant tumour [211] causing increased metabolic activity in the liver [26]. Elevated gluconeogenesis from alanine has previously been reported in weight-losing lung cancer patients [85]. When compared with healthy rats, P_i /ATP ratios during fructose infusion were increased in the liver of rats with minimal tumour burden [77], prior to the onset of cachexia. It was suggested that the increase in P_i was caused by elevated hepatic gluconeogenesis in these rats resulting in increased release of P_i in the conversion of glucose-6-phoshate to glucose [77]. In tumour-influenced rat hepatocytes it was demonstrated that elevated gluconeogenesis during incubation with lactate resulted in a 42% drop in ATP levels, whereas in hepatocytes from healthy animals no change in ATP levels was observed, suggesting decreased energy regenerating capacity in tumour-bearing liver [211]. Furthermore, this progressive loss of liver ATP levels was correlated with decreased insulin levels [211]. Brauer et al. showed that the tumour-induced reduction of phosphorylation in the host liver of rats bearing sarcomas was prevented by short-term as well as chronic insulin administration [20]. Moreover, insulin-treated rats did not develop weight loss, possibly due to inhibition of gluconeogenesis [20]. This would suggest that in weight-losing lung cancer patients the reduced ATP levels within the liver may be correlated with the increased gluconeogenesis, and that an impaired adaptation to the metabolic stress induced by infusion of a gluconeogenic substrate exists in these patients.

In summary, this study has shown decreased ATP levels and phosporylation status in

weight-losing lung cancer patients. During alanine infusion, ATP levels decrease to a greater extent in WL lung cancer patients, and do not show the recovery seen in WS patients and healthy subjects. Further studies on the mechanisms responsible for the observed alterations in liver energy metabolism are warranted.

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6

Adenosine triphosphate infusion increases liver energy status in advanced lung cancer patients: an *in vivo* ^{31}P magnetic resonance spectroscopy study

Submitted for publication

Susanne Leij-Halfwerk,[1,2] Hendrik J Agteresch,[1] Paul E Sijens,[2] Pieter C Dagnelie[1,3]

[1] Department of Internal Medicine II, Erasmus University Medical Center Rotterdam, Rotterdam,
[2] Department of Diagnostic Radiology, Daniel Den Hoed Cancer Center, Rotterdam and
[3] Department of Epidemiology, University Maastricht, Maastricht, The Netherlands

Abstract

Decreased liver ATP levels and phosphorylation status have recently been observed in lung cancer patients with weight loss. The aim of the present study was to investigate whether ATP infusion restores liver energy status in advanced lung cancer patients using ^{31}P magnetic resonance spectroscopy (MRS).

Nine patients with advanced non-small cell lung cancer were studied one week before (baseline) and at 22-24 hours of continuous ATP infusion (37-75 µg/kg/min). After an overnight fast, localised hepatic ^{31}P MR spectra (repetition time 15 s) were obtained and analysed for ATP and P_i content. Ten healthy subjects (without ATP infusion) were studied as a control.

Liver ATP levels in lung cancer patients increased from 8.8 ± 0.7 % (of total MR-detectable phosphate; mean ± SE) at baseline to 12.2 ± 0.9 % during ATP infusion ($P<0.05$). These levels were similar to those in healthy subjects (11.9 ± 0.9%). In patients with >5% weight loss, the increase in ATP levels was most prominent (from 7.9 ± 0.7 to 12.8 ± 1.0%, $P<0.01$).

In conclusion, ATP infusion restores hepatic energy levels in patients with advanced lung cancer, especially in weight-losing patients. These changes could have beneficial effects on the nutritional status of weight-losing lung cancer patients.

Introduction

Weight loss is a common phenomenon in lung cancer patients and contributes significantly to the high morbidity and mortality in this disease [56,198]. Alterations in intermediary host metabolism have been frequently described, including elevated protein turnover [89,131], glucose production [116,204] and Cori cycle activity [96]. In an *in vitro* study, gluconeogenesis in isolated hepatocytes from sarcoma-bearing rats was increased during incubation with lactate as compared to hepatocytes from healthy rats [211]. This increased gluconeogenesis resulted in a 42% decrease in ATP levels, whereas in healthy rats no change in ATP was observed [211]. This suggests that elevated rates of gluconeogenesis in the cancer-bearing host may put an increased demand on the energy stores and may contribute to weight loss.

Alterations in hepatic energy status have been well documented in animal models of various tumors. Decreased liver phosphorylation status, as observed by increased P_i/ATP ratios, was detected in rats bearing prostate tumors [49] or sarcomas [6,20,181] and was correlated with increasing tumor burden [181]. It is noteworthy that these alterations in liver energy status were already detected before the development of weight loss [77].

Decreased liver ATP levels as detected by ^{31}P magnetic resonance spectroscopy (MRS) were reported in patients with various tumor types [51]. Recently, we reported decreased hepatic ATP and phosphorylation status in weight-losing lung cancer patients, when compared with weight-stable patients and healthy controls [124].

In mice bearing colon tumors daily intraperitoneal ATP injections increased total liver and erythrocyte ATP pools [168]. These increases were associated with a significant inhibition of host weight loss [168]. The present study was aimed at investigating whether continuous ATP infusion increases hepatic ATP levels and phosphorylation status in advanced lung cancer patients.

Subjects and methods

Subjects

The study was approved by the Medical Ethical Committee of the Erasmus University Medical Center Rotterdam. All patients signed informed consent. Eligible for the study were patients with histological or cytologically proven non-small cell lung cancer, stage IIIB or IV (WHO grading system) without curative options, and Karnofsky index of 60% or more. Patients with cognitive dysfunction or liver, renal, respiratory, or heart failure, and patients undergoing surgery, concurrent chemotherapy, or radiotherapy involving all lesions were excluded. Ten healthy subjects (age range 37-69 y; body weight 76.4 ± 3.6 kg (mean ± SE)) where included as a control.

Study design

In nine patients (7 males, 2 females) liver energy and phoshorylation status were studied using ^{31}P magnetic resonance spectroscopy (MRS) one week before (baseline) and during continuous ATP infusion, i.e. 22-24 hours after starting ATP infusion. The patients received ATP infusion doses varying from 37 to 75 µg/kg/min. Clinical characteristics of these patients are listed in **Table 6.1.** Healthy control subjects were only studied at baseline. ATP infusions (6.1 mg ATP-$Na_2 \cdot 3H_2O$ in 1 ml NaCl 0.9%)(Merck, Darmstadt, Germany) were sterilised by filtration and given with initial dose of 20 µg/kg/min and increased by increments of 10 µg/kg/min every 30 min until a maximum dose of 75 µg/kg/min, or until the maximally tolerated dose had been reached. If any side effects occurred, the dose was reduced to the last given dose or further until side effects disappeared, usually within minutes after lowering the ATP dose. Thereafter, ATP was infused at a continuous rate. The most frequently occurring side effects were chest discomfort and "needing to take a deep breath".

Table 6.1

Characteristics of non-small cell lung cancer patients.

Patient (no.)	Gender (m/f)	Age (y)	Tumor stage	Weight (kg)	Weight change[1] (%)	ATP infusion (μg/kg/min)
1	m	31	4	71.6	-20.4	67
2	m	76	3B	91.1	-11.7	42
3	m	85	3B	54.9	-10.2	75
4	f	52	4	64.2	-9.0	75
5	f	57	4	56.6	-22.5	40
6	m	75	3B	70.8	-1.7	67
7	m	49	3B	77.1	-1.0	75
8	m	71	3B	71.8	-4.2	75
9	m	68	4	65.1	-0.3	37
Mean		63		69.2	-9.0	61
SE		6		3.7	2.7	6

[1] Prior to MRS

^{31}P MR spectroscopy of the liver

Subjects were studied after an overnight fast. MR Spectroscopy studies were performed with a whole-body MR system equipped with a Helicon magnet operating at 2 T (Vision Magnetom, Siemens AG, Erlangen, Germany). A 16 cm diameter transmit/receive $^{1}H/^{31}P$ surface coil was used for MRI localization, shimming and ^{31}P MR spectroscopy. Elastic bands were used for positioning the coil lateral to the liver in the mid-axillary plane. Field homogeneity achieved in shimming resulted in water peak line widths which were usually less than 40Hz (≈0.5 ppm). After obtaining an image of the region of interest, a one-dimensional chemical shift imaging (1D-CSI) sequence was applied on a transverse slice of 4 cm centered on the surface coil and the liver (1×4 phase-encoded matrix, field of view 40×40 cm^2), yielding volumes of 40×10×4 cm^3 [195]. Spectra were collected with a 640 μs Hanning-sinc shaped radio frequency pulse resulting in a flip angle of 135° in the center of the coil, where a methylene diphosphonate reference sample was positioned, and 60° (weighted average) in the liver volume. Spectra with repetition time of 15 s (10 acquisitions) were obtained in each patient examination. Time domain data

were Fourier transformed after Gaussian multiplication (center: 0 ms, width 30 ms) and phase corrected. Quantification of spectral peak areas was performed using Numaris-3 software package (Siemens AG, Erlangen, Germany) including polynomial baseline correction followed by frequency domain curve fitting [194]. Metabolite concentrations were calculated from peak areas and expressed relative to total MR-detectable phosphate as previously described [51].

Statistics

Results are presented as means ± standard error of the mean (SE). Changes of liver ATP concentrations and P_i/ATP ratios during ATP infusion were tested for significance using Student's paired t-test. Between-group differences were compared using the Student's t-test for independent groups. Pearson's correlation coefficients were calculated to investigate possible relationships between parameters. P-values of <0.05 indicated statistical significance.

Results and discussion

Baseline ATP levels in the liver of lung cancer patients were significantly lower than in healthy subjects (P<0.05, **Table 6.2**). When patients were stratified for presence of weight loss, it was shown that ATP levels in patients with ≥5% weight loss were as much as 34% lower than in healthy subjects (P<0.05, **Table 6.2**). In contrast, ATP concentrations in patients with <5% weight loss were not significantly different from healthy controls. During ATP infusion, liver ATP levels in lung cancer patients increased by 48 ± 15% (P<0.05) reaching levels similar to those in healthy subjects (**Table 6.2**). In lung cancer patients with >5% weight loss, ATP increased by 64 ± 9% (P<0.01). P_i/ATP ratios during ATP infusion decreased to levels close to those in healthy subjects, although none of these changes were statistically significant. No significant correlations were observed between ATP-dose and the change in liver ATP or phosphorylation status (r=0.08 and r=0.28, respectively).

To our knowledge, no earlier studies have addressed the effect of ATP infusion on ATP concentrations in the liver of human subjects. In tumor-bearing mice, single intraperitoneal injections of ATP increased total liver ATP pools from 3.2 to 8.3 mM [168]. Following this expansion of liver ATP pools, erythrocyte ATP levels increased from 0.6 to 2.4 mM [168]. In patients in the present study we measured whole blood ATP concentrations of 0.71 ± 0.02 mM at baseline. After 22-24 hours of ATP infusion, blood ATP levels increased to plateau levels of 53 ± 3 to 69 ± 2% above baseline, depending on the ATP-dose given.

Table 6.2

Liver energy and phosphorylation status as observed by ^{31}P MRS in healthy subjects and advanced lung cancer patients before and during infusion of adenosine triphosphate (37-75 μg/kg/min).[1]

	Healthy	Lung cancer		
		Total	<5% weight loss	≥5% weight loss
	(n=10)	(n=9)	(n=4)	(n=5)
ATP[2]				
Baseline	11.9 ± 0.9	8.8 ± 0.7*	10.3 ± 1.3	7.9 ± 0.7*
During ATP infusion	-	12.2 ± 0.9†	11.5 ± 1.5	12.8 ± 1.0‡
P_i/ATP				
Baseline	0.73 ± 0.16	0.94 ± 0.15	0.77 ± 0.21	1.07 ± 0.21
During ATP infusion	-	0.68 ± 0.11	0.68 ± 0.11	0.68 ± 0.19

[1] Mean ± standard error of the mean (SE)

[2] Expressed as percentage of total MR-detectable phosphate

Significance of difference from healthy subjects: *P<0.05; significance of difference from baseline: †P<0.05, ‡P<0.01.

It could be argued that the observed increase in liver ATP levels in the present study might be caused by contamination of MR spectra by ATP in blood circulating in the liver voxel, due to elevated erythrocyte ATP concentrations and/or vasodilation. Although ATP concentrations in humans are 3-4 times higher in liver [15,99] than in erythrocytes [54,209,222], and ATP infusions were reported to even further increase liver ATP levels in mice [168], no data are available on the contribution of ATP from blood to the ^{31}P MR signal of liver ATP in humans *in vivo*. Therefore, an estimation of ATP contamination from blood was made using in this study measured whole blood ATP concentrations of 0.7 mM, and assuming a liver ATP concentration of 2.51 mM [99], and a liver blood volume of 0.25 ml/g wet weight [81]. Results showed a relative contribution of blood ATP to total liver ATP in the MRS liver voxel of ~9%. During ATP infusion, whole blood ATP concentrations increased to 1.1 mM which would give a ≤ 5% increase in total MRS-observed ATP concentrations in the liver voxel. This is one order of magnitude less than the 50-60% increase in liver ATP levels.

Yet another effect of ATP is vasodilation [2,176] mediated by purinergic receptors P1 and P2 which are located on endothelial cells of blood vessel walls [17,112]. Vasodilation in the liver could affect the ^{31}P MRS measurement by increasing blood volume within one liver voxel. Indeed, increased liver ATP levels were observed by ^{31}P MRS in rat liver after dopamine administration, probably due to increased hepatic blood flow [159].

Based on reported maximal changes in hepatic blood volume in humans between -20% [76] and +20%[9] depending on the stimulus used, increased blood volume in the liver could account for an increase in liver ATP levels by 8%. Again, this is one order of magnitude short of the observed effect. If liver ATP values are corrected for the highest possible blood contribution from combined vasodilation and raised erythrocyte ATP levels (18%), the increase in patients with >5% weight loss still remains highly significant ($P<0.01$). We therefore conclude that the increase in total liver ATP as measured by ^{31}P MRS during ATP infusion does reflect a significant rise in ATP levels in liver tissue and is not caused by altered ATP contamination from blood.

In conclusion, this study shows that i.v. ATP infusion is able to restore depleted liver ATP pools in patients with advanced lung cancer to levels similar to or above those in healthy subjects. This effect is most marked in weight-losing lung cancer patients. The increase in liver ATP pools could have beneficial effects on the nutritional status of weight-losing lung cancer patients.

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7

Effects of adenosine triphosphate infusion on glucose turnover and gluconeogenesis in patients with advanced lung cancer

Submitted for publication

Hendrik J Agteresch,[1] Susanne Leij-Halfwerk,[1] J Willem O van den Berg,[1] Christien H Hordijk-Luijk,[1] JH Paul Wilson,[1] Pieter C Dagnelie[1,2]

[1] Department of Internal Medicine II, Erasmus University Medical Center Rotterdam, Rotterdam
[2] Department of Epidemiology, University Maastricht, Maastricht, The Netherlands

Abstract

Cancer cachexia is associated with elevated lipolysis, proteolysis, and gluconeogenesis. Recently, adenosine 5'-triphosphate (ATP) infusion was found to significantly inhibit loss of body weight, fat mass and fat-free mass in advanced lung cancer patients. The present study was aimed at exploring the effects of ATP on whole body glucose turnover, alanine turnover, and gluconeogenesis from alanine. Twelve patients with advanced non-small cell lung cancer were studied one week before and at 22-24 hours of continuous ATP infusion. After an overnight fast, turnover rates of glucose and alanine, and gluconeogenesis from alanine were determined using primed-constant infusions of [6,6-$^{2}H_{2}$]-glucose and [3-^{13}C]-alanine. High-dose ATP infusion (75 μg/kg/min) induced an increase in glucose turnover from 0.44 ± 0.13 to 0.62 ± 0.07 mmol/kg/h (P=0.059), and in gluconeogenesis from alanine from 0.30 ± 0.16 to 0.37 ± 0.13 mmol/kg/h (P=0.056). At lower ATP doses (37-50 μg/kg/min) these effects were not detected. The increase of glucose turnover during ATP infusion compared to baseline showed a significant correlation with the ATP dose (r=0.58, P<0.05). No change in alanine turnover was observed at any ATP dose. The percentage glucose derived from alanine showed an increase from 59 ± 26% to 75 ± 17% at high-dose ATP (P<0.05) which was not detected at lower ATP dose. Results of this study indicate an increase in whole-body glucose turnover rate and gluconeogenesis from alanine during high-dose ATP infusion (75 μg/kg/min) in advanced lung cancer patients, with a significant dose-response relationship for ATP dose and glucose turnover.

Introduction

Cachexia is a common phenomenon in lung cancer patients, and contributes significantly to morbidity and mortality [43,56,198]. Cancer cachexia is associated with metabolic alterations including elevated lipolysis [61,188], protein breakdown [132,154,171], and increased glucose turnover [89]. In patients with advanced cancer increased glucose production has been shown from lactate [185], glycerol [133], and alanine [85,219].

It has been argued that the liver plays an important role in the metabolic alterations contributing to the development of weight loss in cancer [20,115]. In liver [49,181,211] and skeletal muscle [181] of tumour bearing rats, significantly reduced adenosine 5'-triphosphate (ATP) levels have been demonstrated which were associated with increased gluconeogenesis [211] and increased gluconeogenic enzyme activity [156]. In mice bearing CT26 colon tumours, daily intraperitoneal injections of ATP, AMP or adenosine for 10 consecutive days significantly inhibited host weight loss [168]. This inhibition was

associated with elevated hepatic ATP pools [167,168].

In a randomised clinical trial in patients with advanced non-small cell lung cancer we recently demonstrated beneficial effects of ATP infusions on body weight, skeletal muscle mass, muscle strength, and body cell mass [1]. The present study was aimed at exploring effects of ATP infusion on whole-body glucose turnover, alanine turnover and gluconeogenesis from alanine. Based on the beneficial clinical outcomes we expected that ATP would have an inhibitory effect on these processes. Alanine was selected as a substrate because this amino acid is the key protein-derived precursor of glucose utilized by the liver [201], and a component of muscle protein degradation.

Subjects and methods

Subjects

Patients with histologically or cytologically proven non-small cell lung cancer, stage IIIB or IV without curative options, and a Karnofsky index of 60% or more were eligible for the study. Patients with cognitive dysfunction or liver, renal, respiratory, or heart failure, and patients undergoing surgery, concurrent chemotherapy, or radiotherapy involving all lesions were excluded. The study was approved by the Medical Ethical Committee of the Erasmus University Medical Center Rotterdam. All participants signed informed consent.

Study design

In the clinical trial 28 patients were randomised to receive a maximum of 10 ATP courses of 30 hours each: 7 courses at 2-week intervals, followed by 3 ATP courses at 4-week intervals. ATP infusions (6.1 mg ATP-$Na_2.3H_2O$ in 1 ml saline 0.9%)(Merck, Darmstadt, Germany) were sterilised by filtration and started with an initial dose of 20 µg/kg/min and were increased by increments of 10 µg/kg/min every 30 minutes until a maximum dose of 75 µg/kg/min, or until the maximally tolerated dose had been reached. If any side effects occurred, the dose was reduced to the last given dose or further until side effects disappeared, usually within minutes after lowering the ATP dose. Thereafter, ATP was infused at a continuous rate. The most frequently occurring side effects were chest discomfort and the need to take a deep breath.

In 12 out of the 28 ATP-allocated patients, glucose and alanine turnover and gluconeogenesis from alanine were studied one week before (baseline) and during a ATP course (22-24 hours after starting ATP infusion). Seven patients received low-dose infusions of 37-50 µg/kg/min ATP, and five high-dose infusions of 75 µg/kg/min ATP.

The subjects were studied in the morning after an overnight fast. A cannula (0.8x25

mm) was placed in the left cubital vein for the infusion of stable isotope tracers. In the contralateral cubital vein, an identical cannula was positioned for blood sampling. To study gluconeogenesis, a solution was prepared containing [6,6-$^2H_2$]-D-glucose, 98 atom%, and [3-^{13}C]-L-alanine, 99 atom% (Mass Trace, Woburn, USA), in water and this was sterilised by autoclaving in glass vials. A priming dose of 30 µmol/kg [6,6-$^2H_2$]-D-glucose was administered followed by a continuous infusion of 10 µmol/kg/h [6,6-$^2H_2$]-D-glucose for 90 minutes. Simultaneously, a priming dose of 80 µmol/kg [3-^{13}C]-L-alanine was given followed by a continuous infusion of 40 µmol/kg/h [3-^{13}C]-L-alanine during 90 minutes. Both tracer solutions were infused using calibrated syringe pumps (Perfusor® fm, Braun, Germany). Venous blood samples were drawn immediately before the isotope infusions were started, and at 10 min intervals from 30 to 90 min., i.e. after steady state conditions during the tracer infusions had been achieved.

Analytical methods

Blood samples were collected in tubes containing lithium heparin (Becton Dickinson Vacutainer®, Meylan Cedex, France) and immediately stored on ice. After centrifugation (10 min., 1200 *g*, 4°C), the plasma was collected and stored at -20°C until analysed. An aliquot of the infusate was analysed to document the actual concentrations of the tracers in each study. Blood glucose concentrations were determined enzymatically with glucose-oxidase and peroxidase (Boehringer Mannheim, Mannheim, Germany). Plasma alanine was measured enzymatically as described by Williamson [223]. Isotopic enrichments were determined using the following procedures. Plasma was deproteinized by adding 0.3 M barium hydroxide (Sigma Diagnostics, St.Louis) and 0.3 M zinc sulphate (Merck, Darmstadt, Germany). After centrifugation (8 min,15000 *g*, 4°C) the supernatant was applied to an ion exchange column (mixed bed: AG50W-X8 and AG1-X8, 200-400 mesh, 0.2 g each; BioRad, California). Glucose and alanine were eluted from the column using water and 4 M ammonium hydroxide (Merck, Darmstadt, Germany), respectively, and dried under nitrogen. A glucose derivative (aldonitril penta acetate) was prepared according to Varma et al [216]. An alanine t-butyldimethylsilyl derivative was prepared as described by Chaves Das Neves et al [32].

Isotopic enrichments were measured by injecting 1 µl samples with a split ratio of 50:1 on a fused silica capillary column of 25 m x 0.22 mm, coated with 0.11 µm HT5 (SGE, Victoria, Australia). The relative isotopic enrichments of deuterated glucose and carbon-13 alanine were determined using a Carlo Erba GC8000 gas chromatograph coupled to a Fisons MD800 mass spectrometer (GC-MS) (Intersience B.V., Breda, The Netherlands) in electron impact ionisation mode. In general, the variation coefficient in enrichment was 0.2 mole% for both [6,6-$^2H_2$]-glucose and [3-^{13}C]-alanine measurement,

and no concentration effect was observed at this mole% enrichment level. Ions were selectively monitored at mass per unit charge (m/z) 187 for natural glucose and 189 for the deuterated molecule. The isotopic enrichment of [3-^{13}C]-alanine was determined at the m/z ratios 260 and 261 for carbon-12 and carbon-13 alanine, respectively [137].

Total enrichment of carbon-13 glucose was measured separately (aldonitril penta-acetate derivation) using a gas chromatograph combustion isotope ratio mass spectrometer (GC-IRMS) (Optima, Micromass UK, Middlewich, Cheshire, Great Britain). The [^{13}C]-glucose enrichment in atom% excess (APE) was monitored after combustion to CO_2 at mass 44 for carbon-12 and 45 for carbon-13, respectively.

Calculations

Whole body rates of appearance (R_a) of glucose and alanine were calculated during steady state following a one-compartment model, using the equation:

$$R_a = F_i \times ((IE_i / IE_{ecf}) - 1), \quad (1)$$

where F_i is the isotope infusion rate (mmol/kg/h), IE the isotopic enrichment of the infusate (mole% excess), and IE_{ecf} the isotopic enrichment of the extracellular fluid (mole% excess)[224]. The percentage glucose produced from alanine equals:

$$(IE\ ^{13}C\text{-glucose}_{plasma} / (IE\ ^{13}C\text{-alanine}_{plasma} \times 0.33)) \times 100\% \quad (2)$$

The correction factor in formula (2) is applied in order to correct for the number of carbons in both glucose and alanine. Gluconeogenesis from alanine (mmol/kg/h) is then obtained as:

$$\%\ \text{glucose from alanine} \times R_a\ ([^2H_2]\text{-glucose}). \quad (3)$$

Finally, the percentage of alanine converted into glucose was calculated by dividing the rate of gluconeogenesis from alanine by the rate of appearance of alanine [40].

Statistical analysis

Results are presented as means ± standard deviation (SD). Changes in turnover between baseline and ATP infusion were tested for significance by Student's paired t-test. The correlation between variables was analysed using Spearman's rank correlation coefficient. Results were considered to be statistically significant with a *P* value <0.05.

Results

Study population

Twelve lung cancer patients (9 males, 3 females) with a mean age of 64 ± 13 yrs and weight of 72.7 ± 13.0 kg participated in the study. Mean weight loss at baseline was 7.1 ± 9.0 kg. Clinical details of the patients studied are shown in **Table 7.1.** Patients had

already an average of 2.3 (range 0-7) previous ATP courses.

Table 7.1
Clinical details of patients with non-small cell lung cancer.

Patient	Sex	Age (y)	Stage	Weight change at baseline (%)	Weight at rando (kg)	Weight (kg)	Previous ATP courses	ATP dose (μg/kg/min)
1	M	68	4	-1.4	65.5	65.1	1	37
2	F	57	4	-23.5	57.5	56.6	1	40
3	M	66	4	3.0	68.4	69.0	1	40
4	M	76	3B	-10.7	89.6	91.1	4	42
5	M	54	3B	-22.6	74.5	72.0	4	50
6	M	41	3B	2.3	87.9	98.2	7	50
7	M	77	3B	3.6	81.6	82.9	1	50
8	M	71	3B	-4.3	71.0	71.8	1	75
9	M	48	3B	-4.8	79.7	77.1	5	75
10	M	85	3B	-8.5	53.9	54.9	2	75
11	F	53	4	-7.7	69.0	69.2	0	75
12	F	52	4	-10.8	65.0	64.2	1	75

Glucose and alanine metabolism

Baseline plasma glucose and alanine concentrations were 4.9 ± 0.8 and 0.36 ± 0.02 mmol/l, respectively, and did not change during ATP infusion. When data were analysed for all subjects combined, turnover rates of glucose and alanine, and gluconeogenesis from alanine during ATP infusion did not differ significantly from baseline. However, as shown in **Table 7.2**, stratification for ATP dose revealed remarkable differences according to ATP dose. In patients with low-dose ATP infusion (37-50 μg/kg/min), no change was detected in glucose turnover or gluconeogenesis from alanine. In patients with high-dose ATP infusion (75 μg/kg/min) glucose turnover increased by 50 ± 50% (P=0.059), and gluconeogenesis from alanine by 40 ± 35% (P=0.056). Mean baseline glucose turnover rates and gluconeogenesis from alanine in patients receiving the high-dose ATP infusions tended to be lower than in the low-dose ATP group for glucose turnover (P=0.07; Student's t -test). The change of glucose turnover from baseline was significantly correlated with the ATP dose (r=0.58, P<0.05). No such dose-effect relation

was demonstrated for alanine turnover or gluconeogenesis from alanine.

The percentage glucose derived from alanine increased significantly during high-dose ATP infusion (baseline, 59 ± 26%; ATP, 75 ± 17%; $P<0.05$), but not at low-dose ATP (baseline, 67 ± 29%; ATP, 68 ± 33%; n.s.). No difference in the percentage of alanine converted into glucose was observed between baseline and during ATP infusion at either high-dose ATP (baseline, 63 ± 18%; ATP, 61 ± 21%; n.s.) or low-dose ATP (baseline, 59 ± 33%; ATP, 60 ± 34%; n.s.).

Table 7.2

Glucose turnover and gluconeogenesis from alanine in twelve lung cancer patients before and during ATP infusion.[1]

	Low-dose ATP (37-50 μg/kg/min) (n=5)	High-dose ATP (75 μg/kg/min) (n=7)
Glucose turnover		
baseline (mmol/kg/h)	0.58 ± 0.10	0.44 ± 0.13
during ATP infusion (mmol/kg/h)	0.56 ± 0.13	0.62 ± 0.07
change (mmol/kg/h)[2]	-0.02 ± 0.12	0.18 ± 0.15
change (%)[2]	-2.8 ± 19.0	50.1 ± 49.6
P value of change	0.67	0.059
Gluconeogenesis from alanine		
baseline (mmol/kg/h)	0.34 ± 0.17	0.30 ± 0.16
during ATP infusion (mmol/kg/h)	0.35 ± 0.24	0.37 ± 0.12
change (mmol/kg/h)[2]	0.01 ± 0.14	0.07 ± 0.06
change (%)[2]	1.9 ± 34.3	39.3 ± 35.1
P value of change	0.87	0.056

[1] Scores expressed as mean ± SD

[2] Change from baseline

Discussion

The aim of the present pilot study was to explore effects of intravenous ATP infusion on whole-body glucose turnover, alanine turnover, and gluconeogenesis from alanine, as possible pathways contributing to the reported beneficial effects of ATP on body weight and body composition in advanced lung cancer patients. The effect of ATP on gluconeogenesis from the amino acid alanine was studied because ATP was shown to inhibit loss of skeletal muscle mass and muscle strength [1]. Turnover measurements were performed at 22-24 hours after starting ATP infusion, i.e. after ATP had reached plateau levels in erythrocytes [1]. All but one patients were studied during one of the subsequent ATP infusions. During high-dose ATP infusion (75 μg/kg/min) whole-body glucose turnover increased by approximately 40%, whereas no change whatsoever was shown at low-dose ATP infusion (37-50 μg/kg/min). The ATP-induced increase of gluconeogenesis from alanine explained only part of the increase in total hepatic glucose production. This would suggest that ATP may also stimulate gluconeogenesis from other substrates and glycogenolysis. In vitro studies show that ATP administration stimulated gluconeogenesis from lactate [63,179], pyruvate [29,179], and glutamine [63,117,179,197]. Studies in isolated hepatocytes [31,47,114] and perfused rat liver [27,123] showed that ATP also stimulated glycogenolysis by activating glycogen phosphorylase. It is conceivable that high-dose ATP infusion evoked stimulation of glycogenolysis in our patients since turnover measurements were performed after an overnight fast of 10-12 hours. Glycogen stores in healthy subjects were reported to be depleted only after 36 hours of fasting [152]; no data on glycogen stores in cancer patients are available.

The mechanisms responsible for increased glucose turnover and gluconeogenesis during high-dose ATP infusion remain to be elucidated. Potential mechanisms include receptor-stimulating and catecholamine hormone-stimulating effects of ATP. Studies in isolated hepatocytes showed that extracellular ATP induced phosphatidylinositol hydrolysis, intracellular Ca^{2+}-mobilization, and extracellular Ca^{2+}-influx by stimulation of surface purinergic P2 receptors [31,158] which are involved in the control of gluconeogenesis [8] and glycogenolysis [113]. Furthermore, ATP was shown to act as a cotransmitter of the catecholamine noradrenaline in the nervous system, and was suggested to modulate the release of other neurotransmitters [80]. Noradrenaline is an activator of gluconeogenesis [53,179] and glycogenolysis [53].

Notably, baseline glucose turnover rates in the present study tended to be lower in patients receiving high-dose ATP infusions than in those receiving low-dose ATP. Since patients in the high-dose group had already undergone an average of two ATP courses before the present turnover measurements, the possibility cannot be excluded that the previous ATP infusions had induced a reduction in whole body glucose turnover on a

longer term, which would be consistent with the observed attenuation of weight loss in our patients. In contrast, baseline gluconeogenesis from alanine did not differ significantly between low- and high-dose ATP groups. This would be consistent with the finding in several experimental in vivo studies that the ATP degradation product adenosine inhibited gluconeogenesis from lactate [120,121,130], pyruvate [121,130] and glutamine [121,130], but not from alanine [120,121]. In the present human study, direct effects of ATP on glucose turnover and gluconeogenesis cannot be separated from potential effects of adenosine.

In conclusion, our study demonstrates that ATP infusion causes a dose-dependent increase in whole-body glucose turnover in advanced lung cancer patients. During high-dose ATP infusion (75 μg/kg/min) the increase in glucose turnover was partly accounted for by increased gluconeogenesis from alanine. Despite the direct stimulating effects of ATP on glucose production and gluconeogenesis from alanine, inhibitory effects at longer term cannot be excluded. Further studies will have to show whether differences between direct and long-term effects of ATP infusions on glucose turnover do exist.

Acknowledgment

We are grateful to JDL Wattimena for performing mass spectrometry analyses.

8

Observations on gluconeogenesis and liver metabolites in breast cancer patients

Introduction

Weight loss is a common phenomenon in cancer patients and it contributes to the high morbidity and mortality in this disease. The reported incidence of weight loss varies widely between different tumour types, ranging from 36% in patients with breast cancer to 61% in patients with lung cancer and 83% in patients with pancreatic cancer [56]. Though reduced food intake may be a contributing factor in the etiology of weight loss, it cannot solely explain the occurrence of weight loss. Alterations in host metabolism have been reported, such as elevated glucose turnover, protein catabolism, and increased gluconeogenesis from lactate or protein-derived amino acids. Since these processes put a demand on host reserves, it has been suggested that this elevated metabolism contributes to the weight loss in these patients.

We previously reported elevated rates of glucose turnover and gluconeogenesis from alanine in lung cancer patients, which were dependent on the degree of weight loss in these patients. Moreover, alterations in liver metabolism, such as elevated alanine uptake and high accumulation of gluconeogenic intermediates, were already found before weight loss had occurred. Patients with breast cancer also lose weight, though to a lesser extent than lung cancer patients. It is possible that the difference in the incidence of weight loss between these tumour types is caused by differences in host metabolism. However, information on substrate metabolism in breast cancer patients is limited.

The objective of the present study was to investigate endogenous glucose production in breast cancer patients using stable isotope tracers and ^{31}P magnetic resonance spectroscopy (MRS). In addition, hepatic ATP levels were monitored.

Subjects and methods

Subjects

The study was approved by the Medical Ethical Committee of the Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands. Patients with breast cancer attending the outpatient department of the University Hospital Rotterdam, The Netherlands, were recruited. Patients who were in remission or apparently cured were excluded. Additional exclusion criteria were: liver metastases (as checked for by CT/ultrasound), metabolic disease, corticosteroid treatment, elective surgery <3 months, chemo- or radiotherapy <4 weeks prior to study, hormonal therapy, except for Nolvadex/Tamoxifen of LHRH-replacements, alcohol consumption of more than 100 g/week (=10 glasses), pregnancy, extreme anorexia or artificial weight reduction by dieting. Healthy female subjects without weight loss were included as a control group. All

participants signed an informed consent form.

Experimental design

All subjects kept a dietary record during 7 days and refrained from alcoholic drinks for three days prior to the MRS measurements. Data on pre-illness stable weight, current weight, and weight loss over the previous 6 months were taken from hospital records supplemented with oral information form patients. The subjects were studied between 7:30 a.m. and 1:00 p.m. after an overnight fast (12-14 h). Body weight was measured to the nearest 0.1 kg on an electrical weighing scale (Seca 707, Hamburg, Germany), height was measured to the nearest 0.1 cm, and thickness of four skinfolds (triceps, biceps, subscapular, supra iliac) were measured to the nearest 0.2 mm using a standard skinfold calliper (Holtain Ltd., London, Great Britain). A cannula (0.8×25 mm) was placed in the left cubital vein for the infusion of stable isotope tracers. In the contralateral cubital vein, an identical cannula was introduced for blood sampling. To study gluconeogenesis, a solution was prepared containing [6,6-$^{2}H_{2}$]-D-glucose, 98 atom% and [3-^{13}C]-L-alanine, 99 atom% (Mass Trace, Woburn, USA) in water and this was sterilised by autoclaving in glass vials. A solution of (unlabeled) L-alanine in water (100 g per litre, Bufa B.V., Uitgeest, The Netherlands) was prepared, sterilised by autoclaving in glass bottles, and warmed to ~30°C.

The study consisted of two phases. During the first phase (baseline), a priming dose of 30 µmol/kg [6,6-$^{2}H_{2}$]-D-glucose was administered followed by a continuous infusion of 10 µmol/kg/h [6,6-$^{2}H_{2}$]-D-glucose for 90 minutes. Simultaneously, a priming dose of 80 µmol/kg [3-^{13}C]-L-alanine was given followed by a continuous infusion of 40 µmol/kg/h [3-^{13}C]-L-alanine during 90 minutes. Both tracer solutions were infused using calibrated syringe pumps (Perfusor® fm, Braun, Germany). During the second phase, a priming dose of 1.4 - 2.8 mmol/kg unlabeled L-alanine was administered in 5-8 min and followed by a continuous infusion of 2.8 mmol/kg/h L-alanine for 90 min in order to reobtain a steady state; simultaneously the isotope tracer infusion of deuterated glucose was continued for calculation of glucose turnover.

Venous blood samples were collected as follows: Phase 1) one sample immediately before the isotope tracer infusions were started (for determination of background enrichments) and at 10 min intervals after steady state conditions during the tracer infusions had been reached. Based on observations in our lab and by others [102] steady state was usually obtained between 60-90 min of the tracer infusion; Phase 2) at 15 min intervals during continuous L-alanine infusion. Phosphorus MR spectra of the liver were obtained at baseline and at 3 min intervals during L-alanine infusion.

Biochemistry and turnover measurements

Blood samples were collected in tubes containing lithium heparin (Becton Dickinson Vacutainer®, Meylan Cedex, France) and immediately stored on ice. After centrifugation (10 min, 1200 *g*, 4°C), the plasma was collected and stored at -20°C until analysed. Isotopic enrichment of deuterium-glucose and ^{13}C-alanine (mole% excess, MPE), and ^{13}C-glucose (atom% excess, APE) in plasma was determined as described previously [126].

Whole body rate of appearance (R_a) of glucose and gluconeogenesis from alanine were calculated during steady state assuming an one-compartment model as described by Wolfe [102,224] and were expressed as mmol/kg/h. It was assumed that the underestimation of the gluconeogenic rate due to dilution at oxaloacetate and TCA-cycle [224] would be similar in lung cancer patients and healthy subjects.

^{31}P MR spectroscopy of the liver

Spectroscopy studies were performed with a whole-body MR system equipped with a Helicon magnet operating at 2.0 Tesla (Vision Magneton, Siemens AG, Erlangen, Germany). A 16 cm diameter transmit/receive ^{1}H/^{31}P surface coil was used for MRI localisation, shimming and ^{31}P MR spectroscopy. Elastic bands were used for positioning the coil lateral to the liver in the mid-axillary plane. Field homogeneity achieved in shimming resulted in water peak line widths which were usually less than 40 Hz (≈0.5 ppm). After obtaining an image of the region of interest, an one-dimensional chemical shift imaging (1D-CSI) sequence was applied on a transverse slice of 4 cm centered on the surface coil and the liver (1×4 phase-encoded matrix, field of view 40×40 cm^2), yielding volumes of 40×10×4 cm^3 [195]. Five spectra were collected with a 640 µs Hanning-sinc shaped radio frequency pulse resulting in a flip angle of 135° in the centre of the coil, and 60° (weighted average) in the liver volume with a repetition time (TR) of 1 s (40 acquisitions).

Time domain data were Fourier transformed after Gaussian multiplication (center: 0 ms, width 30 ms) and phase corrected. Quantification of spectral peak areas was performed using Numaris-3 software package (Siemens AG, Erlangen, Germany) including polynomial baseline correction followed by frequency domain curve fitting [194]. Metabolite concentrations were calculated from peak areas and expressed relative to total MR-detectable phosphate as described previously [51]. In each experiment the average of five subsequent ^{31}P MR spectra was used for calculations.

Statistics

Results are presented as means ± standard error of the mean (SEM). In each experiment the average of five subsequent MR-spectra was used as baseline value for calculations.

Mid-time points of MRS data acquisition at 15 min intervals during L-alanine infusion (7.5, 22.5, 37.5 min, etc.) were used for graphical representation, with values being expressed relative to the baseline value of healthy control subjects (=100%). As a measure of overall spectral response, integrals of time-response curves (area under the curve, AUC) of peak areas over 0-90 min intervals during L-alanine infusion were calculated and expressed relative to the baseline values. Between-group differences in baseline values and response to alanine infusion were analysed using Student's t-test for unpaired groups. Changes from baseline values were analysed using Student's paired t-test. Pearson's correlation coefficients were calculated for interrelationship between various variables. Differences were considered statistically significant at P values < 0.05.

Results

Eleven patients with breast cancer and 12 age-matched healthy control subjects, all females, were eligible for the study. Their anthropometric and clinical characteristics are listed in **Table 8.1.** No differences in age and anthropometry were observed. One of the breast cancer patients had lost 7% of her pre-illness stable weight. Albumin levels in breast cancer patients were significantly lower (43 ± 1 g/l) compared to healthy subjects (47 ± 1 g/l, P<0.01), whereas no differences in prealbumin and liver function tests were observed. No differences in total energy, protein, fat, and carbohydrate intake were observed between the groups (data not shown).

Table 8.1
Characteristics of study population.

	Control (n = 12)	Breast cancer (n = 11)
Age (y)	52 ± 3	53 ± 4
Weight (kg)	76.1 ± 2.6	67.7 ± 3.5
Weight change (kg)	-	0 ± 1
Weight change (%)	-	0 ± 1
% Ideal body weight	128 ± 5	128 ± 7
Body mass index (kg/m^2)	26.3 ± 1.1	26.6 ± 1.4
Sum of skinfolds (mm)[d]	75 ± 5	77 ± 10

[1] Mean ± SEM.
Significant difference from healthy control subjects (Students unpaired t-test): * P<0.05.

Turnover measurements

Plasma glucose and alanine concentrations, isotopic enrichment of glucose and alanine, glucose and alanine turnover, and gluconeogenesis from alanine were not significantly different between breast cancer patients and healthy control subjects (**Table 8.2**).

During L-alanine infusion, plasma glucose levels decreased although this was only significant in healthy control subjects. Isotopic enrichment of glucose slightly decreased in both groups, but this was only significantly in breast cancer patients.

Table 8.2

Isotopic enrichments and turnover rates in breast cancer patients and healthy, age-matched female subjects at baseline and during L-alanine infusion.[1]

	Control (n=11)		Breast cancer (n=8)	
	Baseline	During L-alanine	Baseline	During L-alanine
Glucose (mmol/L)	5.6 ± 0.2	5.4 ± 0.2 *	5.1 ± 0.2	4.9 ± 0.3
Alanine (mmol/L)	0.35 ± 0.07	8.0 ± 0.9 **	0.35 ± 0.07	6.4 ± 1.0 **
$^{2}H_2$-glucose (MPE) [2]	1.87 ± 0.15	1.73 ± 0.24	1.84 ± 0.18	1.63 ± 0.19 *
^{13}C-alanine (MPE)	7.75 ± 0.80	-	9.06 ± 0.60	-
^{13}C-glucose (APE) [3]	1.37 ± 0.17	-	1.77 ± 0.17	-
Glucose turnover (mmol/kg/h)	0.55 ± 0.04	0.62 ± 0.06	0.55 ± 0.06	0.65 ± 0.08
Alanine turnover (mmol/kg/h)	0.43 ± 0.03	-	0.38 ± 0.03	-
Gluconeogenesis from alanine (mmol/kg/h)	0.32 ± 0.04	-	0.32 ± 0.04	-

[1] Mean ± SEM
[2] Mole% excess
[3] Atom% excess
Significantly different from baseline value (Paired t-test): *P<0.05, * P<0.01.

^{31}P MR spectroscopy

Baseline values of phosphomonesters (PME) were not significantly different between breast cancer patients and healthy subjects (6.2 ± 0.9 *vs.* 8.8 ± 1.1%, respectively, P=0.09). No differences in baseline levels of PDE, P_i and ATP were observed between the groups.

In **Figure 8.1** changes in hepatic metabolite concentrations during L-alanine infusion

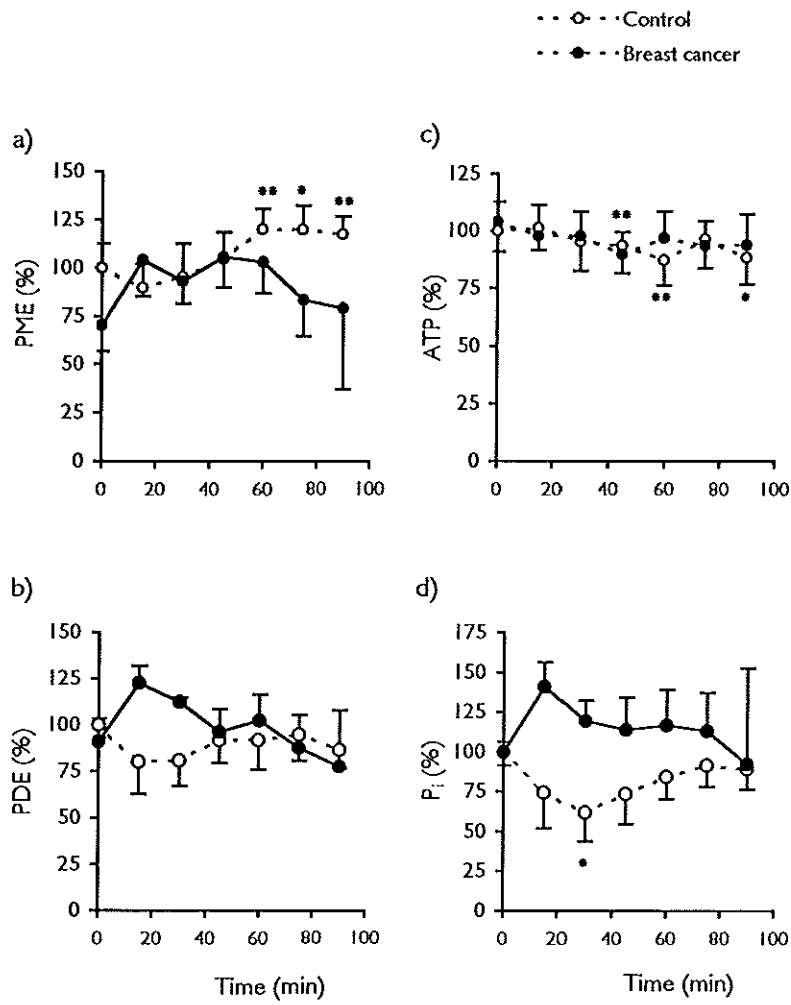

Figure 8.1

Metabolite concentrations in the liver of healthy control subjects (--o--; n = 7) and weight-stable breast cancer patients (–●–; n = 6) during a primed-constant infusion of 1.4-2.8 mmol/kg + 2.8 mmol/kg/h L-alanine: a) phosphomonoesters (PME), b) phosphodiesters (PDE), c) ATP, d) inorganic phosphate (P_i). Curves and error bars represent means ± SE. Baseline values are means from 5 spectra acquired in each subject before L-alanine infusion. Values are expressed as percentage of mean baseline value of healthy subjects (=100%). Times during L-alanine infusion are mid-time points of ^{31}P MRS data collection referenced to the start of the L-alanine infusion (0=baseline). Significance of changes during L-alanine infusion compared to baseline (Student's paired t-test): $*P<0.05$, $**P<0.01$.

are shown. PME levels increased gradually in healthy subjects and reached statistical significance at 60 min ($P<0.01$) (**Figure 8.1**). In breast cancer patients, PME showed a sharp rise during the first 20 min, but was only significantly different from baseline at 60 min of L-alanine infusion ($P<0.05$) after which PME decreased. PDE levels as such were not significantly different from baseline at any time point in either of the two groups.

ATP levels showed a slow but steady decrease in both groups which became statistically significant from baseline values after 40 min of alanine infusion in healthy subjects ($P<0.01$), but never in breast cancer patients. Between 60-80 min of alanine infusion, partial recovery of ATP was observed in healthy control subjects. P_i levels in healthy subjects decreased sharply during the first 30 min ($P<0.05$), but recovered to baseline values between 30 and 90 min of alanine infusion. In contrast, P_i levels in breast cancer patients showed a sharp initial rise (NS), but slowly decreased to baseline values during alanine infusion.

The overall increase PME levels in healthy control subjects was 41 ± 12% ($P=0.02$), whereas in breast cancer patients the increase was 67 ± 34% ($P=0.09$). ATP levels significantly declined in healthy subjects (-13 ± 4%, $P=0.01$), whereas in breast cancer patients no change was observed (-4 ± 6, $P=0.39$).

Correlations

Gluconeogenesis from alanine was inversely correlated with percentage of ideal body weight and body mass index (r=-0.67 and r=-0.68, respectively, $P<0.05$) in healthy subjects, but not in patients with breast cancer (r=-0.27 and r=-35, respectively, $P>0.40$). In breast cancer patients, both hepatic PME and PDE levels were correlated with percentage of ideal body weight (r=0.67 and 0.93, respectively, $P<0.05$) and body mass index (r=0.68 and 0.94, respectively, $P<0.05$), whereas β-ATP was inversely correlated with body mass index and percentage of ideal body weight (r=-0.89 and r=-0.88, respectively, $P<0.01$). In healthy subjects, only PDE was correlated with percentage of ideal body weight (r=0.63 and 0.63, respectively, $P<0.05$). Liver PME levels in breast cancer patients were inversely correlated with glucose turnover (r= -0.90, $P<0.05$) and gluconeogenesis from alanine (r=-0.90, $P<0.05$), whereas in healthy subjects no such relation was observed (r=-0.37 and r=-0.22, respectively, $P>0.33$).

Discussion

Alterations in hepatic gluconeogenesis have been previously reported in patients with lung cancer by ^{31}P MRS with L-alanine infusion [124]. In the present study, hepatic gluconeogenesis was investigated in patients with breast cancer using the same techni-

que. Simultaneously, glucose turnover before and during L-alanine infusion was measured using stable isotope tracers. Alanine turnover and gluconeogenesis from alanine were measured at baseline only.

Glucose and alanine turnover, and gluconeogenesis in breast cancer patients were not different from those in healthy control subjects, confirming previous data in weight-stable lung cancer patients [126]. This suggests that whole-body glucose and alanine metabolism are not altered in cancer patients without weight loss. Since weight loss occurs less frequently in patients with breast cancer [56] only one weight-losing breast cancer patient could be included in the study, so that the relation between turnover measurements and weight loss could not be determined. Changes in glucose turnover during L-alanine infusion were minimal, as could be expected in view of the autoregulatory mechanisms which controls hepatic glucose output [57,108,225].

PME levels in the liver of breast cancer patients and healthy control subjects were similar at baseline. During L-alanine infusion, PME increased in both groups confirming studies performed in healthy animals [30] and humans [50,124,177]. This rise in PME was attributed to increased concentrations of 3-phosphoglycerate (3PG) [30,50]. However, the overall rise in PME was not significantly different in breast cancer patients compared to healthy subjects. This finding is in contrast with observations in weight-stable lung cancer patients where a rapid and significant increase in PME was observed [124]. Moreover, hepatic PME levels at baseline in breast cancer patients in the present study were inversely correlated with glucose production, whereas in lung cancer patients the opposite was found [125]. It may be speculated that due to a high gluconeogenic rate during L-alanine infusion, less gluconeogenic intermediates accumulate within the liver of these patients resulting in lower PME levels in breast cancer patients. In healthy control subjects, no correlations between PME and glucose turnover or gluconeogenesis from alanine were observed, confirming previous reports [125].

Baseline ATP levels in the liver of breast cancer patients in the present study were not different from healthy subjects. During L-alanine infusion, ATP levels in healthy subjects decreased significantly, confirming previous observations [124]. This could be that elevated gluconeogenesis from alanine put an increased demand on hepatic ATP supply. In contrast, in breast cancer patients L-alanine did not reduce liver ATP levels. It may be hypothesised that breast cancer patients have larger energy reserves or enhanced ATP regeneration in the liver than healthy subjects. As no ATP depletion exists in breast cancer patients, gluconeogenic flow is not inhibited resulting in lower PME levels in these patients.

It should be noted that most of the patients were on stable therapy (Tamoxifen). Since the exact influence of Tamoxifen on hepatic glucose mechanism is not clear, an influence of therapy cannot be excluded. In addition, several breast cancer patients

showed side-effects (nausea, sweating) during L-alanine infusion, which may be due to decreased capacity for the L-alanine dose.

In summary, weight-stable breast cancer patients do not show the alterations in hepatic glucose and energy metabolism observed in lung cancer patients, indicating that alterations in host-metabolism may be tumour-specific.

9

Elevated liver phosphomonoester levels predict weight loss in cancer

Susanne Leij-Halfwerk,[1] Pieter C Dagnelie[1,2]

[1] Department of Internal Medicine II, Erasmus University Medical Center Rotterdam, Rotterdam
[2] Department of Epidemiology, University Maastricht, Maastricht, The Netherlands

Abstract

Raised hepatic phosphomonoester (PME) levels have been observed by ^{31}P magnetic resonance spectroscopy (MRS) in cancer patients with weight loss and have been attributed to increased gluconeogenic intermediates. The aim of the present study was to assess the prognostic relevance of elevated PME levels for the development of weight loss in cancer.

Advanced cancer patients diagnosed with lung (n=31), breast (n=20), or cancer of other origin (n=5), without liver metastases, were studied after an overnight fast. Body weight, body mass index, upper arm circumference, and triceps skinfold thickness were measured on the day of the MRS and after three months. Dietary intake was calculated using a seven-day food record. Localised hepatic ^{31}P MR spectra were obtained using a repetition time of 20 s.

Hepatic PME levels in the cancer patients, expressed relative to total MR-detectable phosphate, were 10.3 ± 0.6% (mean ± SEM). Cancer patients lost 0.6 ± 0.5 kg of their weight within three months after MRS. After adjustment for energy intake and weight loss prior to MRS, an increase of 5% in PME was prognostic for a 1.8 ± 0.6 kg decrease in body weight and a 0.6 ± 0.2 kg/m^2 reduction in body mass index within three months after MRS (P<0.05).

Elevated hepatic PME levels and PME/P_i ratios in cancer patients predict the development of weight loss. Further exploration of the use of ^{31}P MRS as a non-invasive tool for early identification of cancer patients at risk for weight loss is warranted.

Introduction

Weight loss frequently occurs in patients with cancer and contributes to the high morbidity and mortality in this disease [56,93,169,198]. Several mechanisms have been suggested to play a role in the development of weight loss in cancer, including anorexia [82], increased energy expenditure [196], and alterations in host metabolism [79]. Increased rates of glucose turnover [116,133] and gluconeogenesis [25,126,219] have been observed in weight-losing cancer patients, and may be related to altered liver metabolism.

Recently, ^{31}P magnetic resonance spectroscopy (^{31}P MRS) of the liver has been applied as a non-invasive tool to study liver metabolism in cancer patients. Elevated phosphomonoester (PME) levels were observed in the tumour-free liver of weight-losing patients with various tumour types [51] and lung cancer [126].

Although weight loss and altered liver metabolism were related in these cancer

patients, it remains to be established whether changes in liver metabolite concentrations are able to predict the development of weight loss in cancer. Therefore, the aim of the present study was to assess the prognostic relevance of PME for weight loss in cancer.

Subjects and methods

Subjects

The study was approved by the Medical Ethical Committee of the Erasmus University Medical Center Rotterdam, The Netherlands. All participants signed informed consent. Cancer patients attending the outpatient department of the University Hospital Rotterdam, The Netherlands, were recruited. Patients who were in remission or apparently cured were excluded. Additional exclusion criteria were: liver metastases (as checked for by CT or ultrasound), metabolic disease, corticosteroid treatment, elective surgery <3 months, chemo- or radiotherapy <4 weeks prior to study, alcohol consumption of more than 100 g/week (=10 glasses), pregnancy, extreme anorexia or artificial weight reduction by dieting.

Dietary intake

Food intake was recorded using a standard food diary during seven days preceding the experiment. The subjects received oral and written information from a trained nutritionist (PD,SL) about the procedures for filling in the diary and registering all consumed drinks and foods using household measures. Subjects were asked to maintain their usual dietary habits and none reported any significant changes in their diet. Subjects abstained from alcohol during 5 days prior to the experiment. All medication taken was noted.

Household measures were converted into weights using a standard food conversion table (Department of Human Nutrition, Wageningen, The Netherlands) and a nutrition software program (Komeet version 2.0, B-Ware, Arnhem, The Netherlands). Average daily intake of energy, fat, carbohydrate and protein was calculated.

Study design

Data on pre-illness stable weight, current weight, and weight loss over the previous 6 months were taken from hospital records supplemented with oral information from patients. On the day of the MRS measurements, body weight was measured to the nearest 0.1 kg on an electrical weighing scale (Seca 707, Hamburg, Germany), height was measured to the nearest 0.1 cm, and body mass index was calculated. Upper arm circumference was measured to the nearest 0.1 cm and thickness of the triceps skinfold was measured to the nearest 0.2 mm using a standard skinfold caliper (Holtain Ltd.,

London, Great Britain). The anthropometric measurements were repeated at three months following MRS.

^{31}P MR spectroscopy of the liver

Hepatic 31-phosphorus MR spectra were obtained in the morning after an overnight fast between 8:00 and 12:00 a.m. Spectroscopy studies were performed with a whole-body MR system equipped with a Helicon magnet operating at 2 Tesla (Siemens Magnetom SP4000, upgraded to Vision, Siemens AG, Erlangen, Germany). A 16 cm diameter transmit/receive ^{1}H/^{31}P surface coil was used for MRI localization, shimming, and ^{31}P MR spectroscopy. Elastic bands were used for positioning the coil lateral to the liver in the mid-axillary plane. Field homogeneity achieved in shimming resulted in water peak line widths which were usually less than 40 Hz (≈0.5 ppm). After obtaining an image of the region of interest, an one-dimensional chemical shift imaging (1D-CSI) sequence was applied on a transverse slice of 4 cm centered on the surface coil and the liver (1×4 phase-encoded matrix, field of view 40×40 cm^2), yielding volumes of 40×10×4 cm^3 [195]. Five spectra were collected with a 640 μs Hanning-sinc shaped radio frequency pulse resulting in a flip angle of 135° in the center of the coil, and 60° (weighted average) in the liver volume with a repetition time of 15 to 20 s.

Time domain data were Fourier transformed after Gaussian multiplication (center: 0 ms, width 30 ms) and phase corrected. Quantification of spectral peak areas was performed using Numaris-3 software package (Siemens AG, Erlangen, Germany) including polynomial baseline correction followed by frequency domain curve fitting [194]. Metabolite concentrations were calculated from peak areas and expressed relative to total MR-detectable phosphate as described previously [51].

Statistics

Results are presented as means ± standard error of the mean (SEM). Differences between group means were compared using analysis of variance. The predictive value of PME for weight loss was examined by multivariate regression analysis with adjustment for energy intake and weight loss prior to MRS. Differences were considered statistically significant at P values < 0.05.

Table 9.1

Characteristics of the cancer patients.[1]

	Mean ± SEM	Range
Tumour type (L/B/O)[2]	31 / 20 / 5	-
Age (y)	60.6 ± 1.6	38 - 81
Weight (kg)	67.5 ± 1.6	45 - 96
Previous weight change (% / 6 mo)	-5 ± 1	-8 - 24
Body mass index (kg/m^2)	24.1 ± 0.6	16.3 - 33.3
Arm circumference (cm)	29 ± 1	20 - 37
Triceps skinfold (mm)	16.4 ± 1.3	4.4 - 39.4

[1] Mean ± SEM.

[2] L: lung; B: breast; O: other; number of patients.

Results

Fifty-six cancer patients, twenty-five males and thirty-one females, were included in the study (**Table 9.1**). Forty-four patients were eligible for follow-up. Main tumour types represented were non-small cell lung cancer (n=31) and breast cancer (n=20). All patients had normal liver function tests. Energy intake was 1944 ± 67 kcal/day and did not differ between tumour types. Though previous weight change varied considerably among individual patients, no systematic differences between patients with different tumour types were observed (P=0.17). Albumin and prealbumin concentrations were 42 ± 1 g/l (mean ± SEM) and 0.23 ± 0.01 g/l, respectively.

Hepatic PME levels in cancer patients were 10.3 ± 0.6% (range 3-18%), and PME/P_i ratios were 1.31 ± 0.09 (range 0.39-3.59), and did not differ significantly between tumour types. During the three months following MRS, body weight decreased by 0.6 ± 0.5 kg, body mass index by 0.2 ± 0.2 kg/m^2, arm circumference by 0.5 ± 0.2 cm, and triceps skinfold by 1.1 ± 0.6 mm.

After adjustment for energy intake and weight loss prior to MRS, a rise in PME levels of 5% significantly predicted a decrease of 1.8 kg body weight and 0.6 kg/m^2 in body mass index during the following three months (**Table 9.2**, both P<0.05). In contrast, decreases in arm circumference and triceps skinfold thickness were not predicted by changes in PME. Plasma albumin or prealbumin did not predict weight loss in this population of cancer patients. Tumour type was no confounding factor in the analysis.

Table 9.2

Predictive value of a 5% rise in PME/P for subsequent changes in anthropometric measures.

	PME/P	P Value
Weight (kg)	-1.8 ± 0.6[2]	0.010
Body mass index (kg/m^2)	-0.6 ± 0.2	0.015
Arm circumference (cm)	-0.6 ± 0.3	0.081
Triceps skinfold (mm)	-1.6 ± 0.9	0.071

[1] Multivariate analysis adjusted for energy intake and presence of weight loss prior to MRS.
[2] Change per three months after MRS (mean ± SEM).

Discussion

Weight loss in cancer is known to be a prognostic factor for reduced survival in cancer [43,93,198]. Although attempts have been made to elucidate underlying mechanisms and to reverse the weight loss in cancer, little attention have been given to prognostic factors for the development of weight loss itself.

The present study demonstrates that elevated PME levels in the non-metastasised liver of cancer patients significantly predict the development of weight loss within three months following MRS. This effect was not caused by confounding by energy intake, nor by weight loss prior to the MRS examination, as was shown by multivariate analysis including these variables. In addition, no confounding effect of tumour type was observed.

Phosphorus MRS has been applied in the non-invasive study of nutritional conditions and metabolic changes [7]. However, possible applications of ^{31}P MRS as a prognostic tool for various disease states have only rarely been investigated. In patients with hepatic lymphoma, the degree of liver infiltration was reflected by increased hepatic PME levels [58]. In response to chemotherapy a decrease in PME levels was observed. The authors suggested that ^{31}P MRS might provide a useful tool in the diagnosis of liver infiltration and response to chemotherapy [58]. In a study in patients with primary biliary cirrhosis, the prognostic value of ^{31}P MRS was assessed in patients waiting for orthotopic liver transplantation [103]. Elevated PME/P_i ratios in the patients with primary biliary cirrhosis were positively correlated with standard prognostic indices based on plasma parameters indicating disease severity [103]. The metabolic background for the observed hepatic changes was not investigated in that study. When we analysed the prognostic value of hepatic PME/P_i ratios in cancer patients in the present study, it was significant for weight loss only. It should be noted, however, that in this study only cancer patients without

infiltrated livers were studied, as was confirmed by CT/ultrasound and liver function tests.

In a ^{31}P MRS study in weight-losing cancer patients of various tumour types, without hepatic malignancies, increased hepatic PME levels were observed [51]. This was suggested to be due to elevated concentrations of gluconeogenic intermediates within the liver [51]. Dynamic liver ^{31}P MRS studies in rats showed that increased liver PME levels coincided with increased concentrations of 3-phosphoglycerate, a gluconeogenic intermediate [30,50]. Indeed, liver PME levels in lung cancer patients were reported to significantly correlate with rates of glucose turnover and gluconeogenesis [125].

Several authors have suggested that elevated gluconeogenesis observed in cancer could be a contributing factor in the development of weight loss [189,204]. Gluconeogenesis is an energy-consuming process. The present study confirms that high concentrations of gluconeogenic intermediates in the liver of cancer patients are indeed prognostic for the development of weight loss. Early detection of cancer patients at risk for weight loss would facilitate early treatment, thus preventing or reversing the process of weight loss, and might therefore contribute to improved survival. We conclude that ^{31}P MRS may be useful clinically as a non-invasive prognostic tool for predicting weight loss in cancer patients. Further investigation in a larger cancer patient population is warranted.

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10

General discussion

Weight loss is frequently observed in patients with cancer and contributes to the high morbidity and mortality in this disease. Decreased food intake cannot fully explain the origin of weight loss, especially so in patients with lung cancer, suggesting that alterations in host metabolism must play a role. A typical feature of the weight loss in cancer is that not only fat mass, but also muscle protein is degraded. In lung cancer, elevated protein turnover and glucose production have been reported and it was suggested that elevated gluconeogenesis from muscle-derived amino acids, especially alanine, may contribute to the weight loss observed. It has remained unclear, however, whether gluconeogenesis from alanine is elevated in lung cancer patients and whether this is related with weight loss. Also, it is not known whether changes in hepatic glucose metabolism are associated with alterations in energy (ATP) metabolism in the liver of lung cancer patients.

Therefore, the present research project was aimed at obtaining quantitative information on gluconeogenesis from alanine and liver energy metabolism in relation with weight loss in lung cancer, using turnover measurements with stable isotope tracers and hepatic ^{31}P magnetic resonance spectroscopy (MRS).

Glucose turnover

Gluconeogenesis from alanine was significantly elevated in lung cancer patient with weight loss compared to weight-stable lung cancer patients and healthy control subjects (Chapter 2), and correlated with the degree of weight loss. These data were obtained under steady state conditions using primed-constant infusions of [6,6-$^{2}H_2$]-glucose and [3-^{13}C]-alanine assuming an one-compartment model.

Total glucose production, as measured by deuterated glucose in the present study, did not change during L-alanine infusion in weight-losing lung cancer patients and healthy control subjects (Chapter 4), confirming studies in healthy subjects and dogs [57,108,140,225] in which gluconeogenesis from alanine increased during L-alanine infusion, whereas the amount of alanine released from the gut decreased [57]. It was also suggested that decreased glycogenolysis [108,150], or increased amounts of alanine shunted to glycogen [225] or protein [36], might play a role in the constant rate of endogenous glucose production. In contrast, in weight-stable lung cancer patients in the present study, total glucose production increased by 8% during L-alanine infusion. This would suggest that glucose production from other substrates was not inhibited to the same extent by alanine infusion as in healthy subjects. It is unlikely that increased glycogenolysis played a role in the elevated glucose production during L-alanine infusion, since decreased glycogen stores have been observed in patients with colorectal cancer

[97]. One explanation for the observation that glucose production during L-alanine infusion did not increase in weight-losing lung cancer patients, is that gluconeogenesis was already maximally induced at baseline, although other explanations remain possible.

To obtain an indication of stimulation of protein synthesis by L-alanine infusion, total amino acid concentrations in plasma of two weight-losing lung cancer patients and two healthy subjects were measured. It was shown that during L-alanine infusion, apart from the marked elevation in alanine levels, concentrations of glutamine, glycine, proline, serine, and threonine increased in both cancer patients and healthy subjects.

The contribution of alanine to the elevated endogenous glucose production observed in weight-losing lung cancer patients was estimated based on our turnover data. The percentage glucose derived from alanine was not elevated in weight-losing lung cancer patients as compared to both weight-stable lung cancer patients or healthy control subjects (Chapter 2), which implies a similar relative contribution of alanine to total glucose production in the three groups. When comparing the data in absolute terms, however, total glucose turnover was 23 mmol/kg/h higher in weight-losing lung cancer patients than in healthy subjects, whereas gluconeogenesis from alanine in these patients was only 18 mmol/kg/h higher. This suggests that, although elevated gluconeogenesis from alanine explains ≈70% of the enhanced glucose production in weight-losing lung cancer patients, glucose production from other substrates, such as lactate, may have been elevated as well.

Information on gluconeogenesis from lactate could not be obtained in the present study, because this would have requested a separate labelled tracer (for example [3-^{14}C]-lactate). Nevertheless, we were able to measure ^{13}C-enrichment in lactate in most of the subjects which may provide an indication of gluconeogenesis from lactate. Results showed that lactate enrichment was 1.91 ± 0.30 MPE in weight-losing lung cancer patients, 2.49 ± 0.11 MPE in weight-stable lung cancer patients and 2.95 ± 0.16 MPE in healthy subjects, and did not differ significantly between the groups. Values of enrichment were lower for lactate than for alanine (CaWL: 7.22 ± 0.60, CaWS: 8.09 ± 0.64, C: 9.19 ± 1.06 MPE). This was unexpected since alanine rapidly equilibrates with pyruvate-lactate which leads to similar ^{13}C enrichment in these substrate at complete equilibration in the body pools [109,111]. Apparently this was not the case in the present study. However, we may conclude that in our study the proportion of gluconeogenesis from lactate to total glucose production is likely to have been similar in weight-losing lung cancer patients compared to weight-stable patients and healthy subjects, as there were no differences in ^{13}C-lactate enrichment between the three groups. Nevertheless, we should be aware of possibility that Cori cycling may have contributed to the gluconeogenesis from alanine as measured by the present technique.

Mechanisms of elevated gluconeogenesis from alanine

Elevated gluconeogenesis from alanine as observed in weight-losing lung cancer patients could be driven by high precursor availability [57,70] from muscle protein breakdown [66,94], or by elevated hepatic alanine uptake and/or increased gluconeogenic enzyme activity.

Alanine availability

It has been suggested that enhanced muscle protein breakdown and higher alanine release reported in weight-losing cancer patients as compared to healthy control subjects [24,95] provides increasing amounts alanine for endogenous glucose production.

In weight-losing lung cancer patients in the present study, alanine flux was 0.17 mmol/kg/h higher than in healthy subjects (Chapter 2). Although this extra amount of alanine could be solely released from muscle protein breakdown, this is not very likely since alanine accounts for only 7-10% of the amino acids in muscle [69]. Enhanced muscle alanine production [40] or glucose-alanine cycling within weight-losing lung cancer patients is more likely, in combination with part of the alanine being produced from glutamine in the gut [14,151] (**Figure 1.1**). The origin of the enhanced alanine production by muscle in weight-losing lung cancer patients is not clear. However, it has been reported in healthy subjects that elevated amounts of circulating lactate [40] and/or glucose [173,187] stimulate alanine production in muscle, which explains increased release of alanine by muscle in weight-losing lung cancer patients.

Hepatic alanine uptake

Hepatic alanine uptake and glucose production from alanine are known to be closely related with alanine levels in plasma [57,70,108]. In the present study, alanine uptake by the liver was estimated by calculating alanine clearance[1]. Weight-losing lung cancer patients had an alanine clearance of 1.65 ± 0.15 l/kg/h which was significantly higher than the rates observed in weight-stable lung cancer patients and healthy subjects (1.16 ± 0.12 and 1.19 ± 0.08, respectively; $P<0.01$). The elevated alanine clearance was also reflected by significant lower plasma alanine concentrations during steady state of L-alanine infusion (1.4-2.8 mmol/kg followed by a continuous load of 2.8 mmol/kg/h) in weight-losing lung cancer patients (5.0 mM), compared to weight-stable lung cancer patients (6.3 mM) and healthy subjects (8.1 mM)(Chapter 4). Measurements in 18 lung cancer patients and 6 healthy subjects showed that of the total L-alanine dose infused, a

[1] Alanine clearance (l/kg/h) was calculated as alanine turnover (mmol/kg/h) divided by plasma alanine concentration (mmol/l).

maximum of 3% was excreted in urine, indicating that >97% of the alanine infused had been taken up and metabolised by body tissues. Most of this alanine has been taken up by the liver, whereas splanchnic and peripheral alanine uptake may have been unchanged, as reported in healthy dogs after an L-alanine load [57]. Thus, the attenuated increase in plasma alanine levels during constant L-alanine infusion in weight-losing lung cancer patients is likely to be caused by increased hepatic alanine uptake in these patients. In weight-stable lung cancer patients, plasma alanine concentrations during L-alanine infusion were also significantly lower than in healthy subjects indicating an enhanced hepatic alanine uptake in weight-stable patients as well.

Gluconeogenic enzyme activities

Various animal models suggested increased gluconeogenic enzyme activities in the liver in the cancer-bearing state [83,128,144]. In our study, we did not measure gluconeogenic enzymes directly. However, in addition to the turnover studies which provide information on the whole-body level, we used ^{31}P magnetic resonance spectroscopy (MRS) to investigate gluconeogenesis non-invasively at the actual site of metabolism: the liver. ^{31}P-MRS with L-alanine infusion has been applied to study gluconeogenesis in healthy rats [30] and humans [50,177], showing increasing concentrations of hepatic gluconeogenic intermediates, especially 3-phosphoglycerate and phosphoenolpyruvate. This was reflected in the present study by increased levels of phosphomonoesters (PME) and phosphodiesters (PDE), respectively.

In the present study, high hepatic PME levels in weight-losing lung cancer patients were observed even without stimulation of the gluconeogenic pathway (Chapter 3). Simultaneous turnover measurements revealed that the degree of elevation of PME in lung cancer patients was positively correlated with the rate of gluconeogenesis from alanine, confirming that elevated PME levels in the liver of weight-losing lung cancer patients were due to elevated concentrations of gluconeogenic intermediates. Subsequent L-alanine infusion in these patients (Chapter 4) did not further increase hepatic PME levels, suggesting that hepatic gluconeogenesis from alanine was already maximally induced. Weight-stable lung cancer patients expressed normal liver PME levels at baseline, but showed an enhanced increment of PME levels during L-alanine infusion compared to healthy control subjects (Chapter 4). The elevated PME levels indicate enhanced hepatic accumulation of 3-phosphoglycerate, which is consistent with facilitated induction of gluconeogenesis. Moreover, increasing concentrations of hepatic PDE levels following L-alanine administration were observed in lung cancer patients both with and without weight loss, in contrast to healthy subjects implicating higher accumulation of phosphoenolpyruvate in the liver of these patients. This observation would be consistent with an elevated activity of phosphoenolpyruvate carboxykinase (PEPCK, converting

oxaloacetate to phosphoenolpyruvate) and possibly pyruvate carboxylase (PC, converting pyruvate to oxaloacetate) as observed in the liver of tumour-bearing rats [83,128] (see also **Scheme 3.1**).

Notably, elevated PME in the liver of lung and breast cancer patients were prognostic for the development of weight loss. This is consistent with a causal relationship between elevated gluconeogenesis and weight loss, although other explanations cannot be excluded.

Hormones

Although enhanced gluconeogenesis from alanine observed in weight-losing lung cancer patients in the present study could be hormonally regulated, results showed that serum insulin and glucagon concentrations were not different between lung cancer patients with/without weight loss and healthy control subjects, nor were they correlated with glucose turnover or gluconeogenesis from alanine (Chapter 2). During L-alanine infusion, both insulin and glucagon levels increased significantly in all groups (Chapter 4). Remarkably, the insulin:glucagon ratio during L-alanine infusion was lower in weight-losing lung cancer patients (0.17 ± 0.03) than in weight-stable lung cancer patients (0.44 ± 0.09) and healthy subjects (0.91 ± 0.42) which implies a more gluconeogenic state in lung cancer patients, especially weight-losing patients, than in healthy subjects.

Concentrations of rT_3 in weight-losing lung cancer patients were significantly increased compared to weight-stable lung cancer patients and healthy control subjects, and were correlated with glucose turnover. High rT_3 levels are non-specific for the cancer-bearing state but occur in other diseases as well, indicating energy depletion of the liver [65,90]. Indeed, in the liver of weight-losing lung cancer patients in the present study decreased energy status was observed as discussed in the next paragraph.

Liver energy status

Liver ATP levels in weight-losing lung cancer patients were lower than in weight-stable lung cancer patients and healthy control subjects (Chapter 5 and 6), confirming data from tumour-bearing animals [6,20,77,211]. Infusion of L-alanine as a gluconeogenic substrate induced a drop in liver ATP levels in both weight-losing and weight-stable lung cancer patients and healthy control subjects (Chapter 5). This reduction in ATP could be due to increased ATP utilisation for alanine uptake into the hepatocyte [72,163,212], for gluconeogenesis [111], and/or for urea production [151]. Remarkably, at 75-90 min of L-alanine infusion, hepatic ATP levels in weight-stable lung cancer patients and healthy control subjects were partly restored, whereas ATP levels in weight-losing lung cancer

patients continued to decrease. The enhanced decrease in ATP as well as the depleted ATP stores at baseline in weight-losing lung cancer patients, indicate elevated ATP utilisation for increased gluconeogenesis and/or decreased efficiency of ATP-regeneration [226].

MRS data from the present study were analysed for a possible relation between hepatic ATP levels and gluconeogenesis. Unexpectedly, no correlations between hepatic PME levels and ATP levels were observed. In contrast, a strong inverse correlation between hepatic ATP and PDE levels was observed in lung cancer patients (Pearson's r=-0.81, $P<0.001$), breast cancer patients (r=-0.98, $P<0.01$), as well as in healthy control subjects (r=-0.73, $P<0.01$). Furthermore, increases in PDE levels during L-alanine infusion were larger when ATP levels at baseline were higher ($P<0.001$). As previously described, PDE contains contributions from phosphoenolpyruvate. Alanine has an inhibitory effect on pyruvate kinase, converting phosphoenolpyruvate to pyruvate, and a stimulatory effect on PEPCK [59]. Thus, the presence of increased alanine concentrations within the hepatocyte would stimulate the conversion of pyruvate towards phosphoenolpyruvate and further to glucose. Four out of the six energy-rich phosphate bonds necessary for gluconeogenesis are utilised by the gluconeogenic enzymes PC and PEPCK [151] which are the most energy-consuming steps in gluconeogenesis [111]. In addition, PC and PEPCK activities are stimulated by a high ATP content. Although we cannot exclude the utilisation of ATP for other energy-consuming processes, our finding that PDE and ATP are closely related would be consistent with enhanced ATP consumption for elevated gluconeogenesis.

We further tested the hypothesis that ATP infusion would be able to reverse the ^{31}P MRS-observed depletion of ATP pools in the liver of weight-losing and weight-stable lung cancer patients. Indeed, intravenous infusion of ATP caused a significant 39% rise in hepatic ATP levels which was independent of the ATP dose given (Chapter 6). Simultaneously, a dose-dependent rise in total glucose production rates and gluconeogenesis from alanine was observed (Chapter 7). It could be hypothesised that the restoration of hepatic ATP levels by ATP infusion provided energy necessary for the elevated gluconeogenesis in weight-losing lung cancer patients, as previously suggested by the close relation between PDE and ATP. However, this relation does not seem straightforward since changes in liver ATP content during ATP infusion and glucose turnover or gluconeogenesis from alanine were not correlated. Other mechanisms by which ATP infusion could stimulate glucose production may be through binding on purinergic membrane receptors, or by acting as cotransmitter of noradrenaline in the nervous system thereby indirectly stimulating glucose production [31,80,158]. ATP was also reported to inhibit binding of insulin to its receptor [210], which may stimulate glucose production as well.

Though the precise way of action of ATP has not been clarified, we speculate that ATP may act predominantly on the liver rather than on muscle, since ATP infusion did not have any effect on alanine flux in lung cancer patients in our study. The stimulatory action of ATP on glucose production may be acute, since glucose turnover and gluconeogenesis from alanine appeared lower in lung cancer patients one week after they had received an ATP course. A breakdown product of ATP, adenosine, has been reported to inhibit gluconeogenesis [121,130].

Conclusions

From the results of the studies described in this thesis, the following conclusions can be drawn:

1. Hepatic gluconeogenesis from alanine is elevated, whereas liver energy status is decreased in lung cancer patients with weight loss. These alterations are correlated with the degree of weight loss. Nevertheless, even in patients without weight loss alterations in hepatic gluconeogenesis are observed. There is evidence for elevated hepatic alanine uptake and gluconeogenic enzyme activity in the early steps of the gluconeogenesis occurring even in the absence of weight loss.
2. Depleted ATP levels in the liver can be restored by intravenous infusion of ATP in lung cancer patients, especially weight-losing patients. High-dose ATP infusion also stimulates glucose turnover and gluconeogenesis from alanine during the infusion, but may lead to a reduction in gluconeogenesis after the ATP infusion has been stopped.
3. Elevated hepatic PME levels predict the development of weight loss in cancer patients in the next three months. This would be consistent with the hypothesis that elevated gluconeogenesis contributes to weight loss in these patients.

Future perspectives

Though the present study has clarified some of the metabolic changes involved in weight loss in lung cancer, underlying mechanisms remain to be investigated. As elevated hepatic gluconeogenesis is prognostic for developing weight loss in cancer, further research on aberrations in the gluconeogenic pathway may help develop new therapeutic strategies.

The following issues would appear to be especially relevant. First, it would be important to analyse the underlying mechanisms of the elevated glucose production in weight-losing lung cancer patients, which appeared to occur from both alanine as well as

lactate. Though in the present study evidence for alterations in liver metabolism in lung cancer was observed, its relation with other organs, especially in skeletal muscle, needs to be clarified. There may exist a regulating mechanism between altered liver and muscle metabolism involving alanine, glucose and lactate. Selective labelling of lactate, alanine and glucose would permit to investigate metabolism of these substrates simultaneously [40,110,150]. In addition, the forearm balance technique could be applied in order to investigate the fractional extraction of lactate, alanine, and glucose by muscle [40]. Total glucose production by gluconeogenesis could be assessed simultaneously using $^{2}H_{2}O$ as described by Landau et al [119].

In addition, measurement of concentrations of individual gluconeogenic intermediates and activities of gluconeogenic enzymes within the liver would be of interest. Although chemical analysis of liver biopsies is possible [12], the use of non-invasive techniques is to be preferred in patients. We have shown that ^{31}P MRS can be a valuable tool for this especially if combined with turnover measurements. Additional information could be obtained by ^{13}C MRS of the liver. This method allows not only the determination of concentrations of gluconeogenic substrates, but also the flow through the various steps of gluconeogenesis or cycling [178,200]. Another advantage of this technique is that glycogen formation can be measured simultaneously [166,184,213]. This is of particular interest since glycogen depletion could be another driving force in the enhanced glucose production in cancer [97,127]. Moreover, ^{13}C and ^{31}P MRS could be used simultaneously for the investigating glucose metabolism and energy metabolism [193]. The approach of combining ^{31}P and ^{13}C MRS may be used to obtain information on intermediates of glucose and energy metabolism in liver and skeletal muscle in cancer patients with or without weight loss. This is of particular interest when investigating muscular fatigue or loss of muscle strength in these patients. Finally, the effect of intravenous ATP on liver and muscle ATP content could be measured in combination by ^{31}P MRS.

The studies described in the present thesis clearly point out that alterations in host liver metabolism exist in lung cancer patients which are prognostic for the development of weight loss. Unravelling the underlying mechanisms of altered host metabolism is the starting point for successful development of therapies for weight loss in lung cancer.

Summary

Weight loss is a common phenomenon in lung cancer patients and is associated with increased morbidity and mortality (**Chapter 1**). A typical feature of weight loss in cancer is that not only fat tissue but also muscle protein is lost. Decreases in food intake cannot fully account for the weight loss, suggesting alterations in host metabolism.

Elevated muscle protein degradation and endogenous glucose production have been observed in cancer. Muscle-derived amino acids, especially alanine, are used for gluconeogenesis within the liver. It has remained unclear, however, whether gluconeogenesis from alanine is elevated in lung cancer patients and whether it is related with weight loss. Also, it is not known whether changes in hepatic glucose metabolism are associated with alterations in energy (ATP) metabolism in the liver of lung cancer patients.

The present research project was aimed at obtaining quantitative information on gluconeogenesis from alanine and on liver energy metabolism in relation with weight loss in lung cancer. The following specific study questions were formulated:

1. Are liver gluconeogenesis increased and energy status decreased in lung cancer patients, and are these alterations related with weight loss?
2. Can depleted liver energy stores in advanced lung cancer patients be restored by ATP infusion, and does this affect glucose metabolism?
3. Do alterations in hepatic gluconeogenesis predict subsequent weight loss in cancer patients?

In **Chapter 2**, whole-body turnover rates of glucose and alanine, and gluconeogenesis from alanine were determined in lung cancer patients with weight loss (>5% of pre-illness stable weight) or without weight loss. Primed-constant infusions of [6,6-$^{2}H_{2}$]-glucose and [3-^{13}C]-L-alanine were used. The relations between turnover rates and the degree of weight loss were investigated. Healthy control subjects without weight loss were included as a reference.

Energy intake and plasma concentrations of glucose, alanine, insulin, and glucagon were not different between lung cancer patients and healthy subjects. Whole body glucose and alanine turnover, and gluconeogenesis from alanine were 35%, 39% and 57% higher, respectively, in weight-losing lung cancer patients compared to weight-stable patients and healthy subjects. The degree of weight loss was positively correlated with glucose and alanine turnover, as well as with gluconeogenesis from alanine.

In **Chapter 3** the relation between elevated hepatic phosphomonoesters (PME) and phosphodiesters (PDE), as observed by ^{31}P MRS, and glucose flux and gluconeogenesis from alanine, as determined by turnover measurements, in lung cancer patients is reported.

Patients with advanced lung cancer without liver metastases, with/without weight

loss and healthy control subjects were studied after an overnight fast. Liver PME concentrations were 6% higher in lung cancer patients compared to controls; in particular, patients with weight loss had significantly higher PME levels than weight-stable patients. Levels of PDE did not differ between the groups. PME levels were significantly correlated with glucose turnover and gluconeogenesis from alanine in lung cancer patients.

In **Chapter 4**, changes in levels of gluconeogenic intermediates in the liver of lung cancer patients during continuous L-alanine infusion are reported, using simultaneous ^{31}P MRS and turnover measurements. Lung cancer patients without liver metastases with/without weight loss and healthy control subjects were studied during an intravenous L-alanine bolus of 1.4-2.8 mmol/kg followed by continuous infusion of 2.8 mmol L-alanine/kg/h for 90 minutes.

During L-alanine infusion, plasma alanine concentrations increased 15 to 17-fold in lung cancer patients and 24-fold in healthy subjects. Whole-body glucose turnover slightly increased (8%) in weight-stable lung cancer patients, whereas no significant change in weight-losing lung cancer patients and healthy subjects were observed. Liver PME levels increased by 50% in healthy subjects (area under the curve) and by 87% in weight-stable lung cancer patients after 45-90 min of alanine infusion. In contrast, no significant changes in PME were observed in weight-losing lung cancer patients. Plasma insulin concentrations increased during L-alanine infusion in all groups. Peak plasma insulin levels were lower in weight-losing lung cancer patients than in weight-stable lung cancer patients and healthy subjects. In lung cancer patients, changes in PME and PDE levels during alanine infusion were inversely correlated with their respective baseline levels. No such relation was observed in healthy control subjects. Also, changes in PME during alanine infusion in lung cancer patients were inversely correlated with the degree of weight loss.

In the same protocol, it was investigated whether increasing rates of gluconeogenesis, stimulated by L-alanine infusion, have an effect on bioenergetics in the liver of lung cancer patients with or without weight loss. Results are reported in **Chapter 5**. Baseline ATP levels were reduced in weight-losing lung cancer patients and inversely correlated with the degree of weight loss. P_i/ATP ratios in these patients were increased, indicating reduced liver phosphorylation status. During L-alanine infusion, ATP levels decreased in all groups. In weight-losing lung cancer patients, ATP levels were lower at all time-points between 0-90 min of alanine infusion as compared to both weight-stable lung cancer patients and healthy controls. Between 60-90 min of alanine infusion, partial recovery of ATP was observed in both healthy control subjects and weight-stable lung cancer patients, but not in weight-losing lung cancer patients where ATP continued to decrease. P_i/ATP ratios were significantly elevated after 70-90 min of L-alanine infusion in weight-

losing lung cancer patients compared to weight-stable lung cancer patients and healthy subjects.

Since liver ATP levels in weight-losing lung cancer patients were decreased and infusion of adenosine 5'-triphosphate (ATP) had shown to increase liver ATP levels in mice, two studies were carried out to investigate the effect of ATP infusion on liver ATP and glucose metabolism. The study described in **Chapter 6** was aimed at investigating whether ATP infusion restores liver ATP levels and phosphorylation status in advanced lung cancer patients. Patients with advanced non-small cell lung cancer were studied one week before and at 22-24 hours of continuous ATP infusion (37-75 µg/kg/min) after an overnight fast. During ATP infusion ^{31}P MRS-detected hepatic ATP levels in lung cancer patients increased from 8.8% of total MR-detectable phosphate to 12.2% during ATP infusion, a level similar to that in healthy subjects (11.9%). ATP levels tended to increase more in patients with >5% weight loss than in patients without weight loss. Although P_i/ATP ratios in weight-losing lung cancer patients decreased slightly during ATP infusion, this was not statistically significant.

In the study described in **Chapter 7**, the effects of ATP infusion on whole body glucose and alanine turnover metabolism were investigated one week before and at 22-24 hours of continuous ATP infusion using stable isotope tracers. High-dose ATP infusion (75 µg/kg/min) increased glucose turnover by 41% and gluconeogenesis from alanine by 23%, whereas at lower ATP doses (37-50 µg/kg/min) no effects were detected. The change in glucose turnover during ATP infusion was positively correlated with ATP dose. The percentage glucose derived from alanine increased from 59% to 75% at high-dose ATP. No change in alanine turnover was observed at any ATP dose.

In **Chapter 8** observations on gluconeogenesis and liver metabolism in patients with breast cancer (mean weight loss 0 ± 1%) are described. Glucose and alanine turnover, and gluconeogenesis from alanine in breast cancer patients were not different compared to healthy subjects. Baseline liver metabolites as observed by ^{31}P MRS were similar in both groups. In breast cancer patients, PME levels were inversely correlated with glucose production and gluconeogenesis from alanine. During L-alanine infusion, PME levels increased in breast cancer patients and healthy subjects, but this was only significant in healthy subjects. No changes in PDE, ATP or P_i levels were observed during L-alanine in breast cancer patients.

The study described in **Chapter 9** was conducted to investigate whether high hepatic PME levels in cancer patients predict the development of weight loss. For this analysis, ^{31}P MRS liver data of fifty-six cancer patients with various tumour types, predominantly

lung (n=31) and breast cancer (n=25), were pooled. Body weight, body mass index, upper arm circumference, and triceps skinfold thickness decreased within 3 months post-MRS. After adjustment for energy intake and weight loss prior to MRS, a 5% rise in PME significantly predicted a reduction in body weight of 1.8 ± 0.6 kg and a reduction in body mass index of 0.6 ± 0.2 kg/m^2 at 3 months after MRS.

In conclusion, the present project demonstrates elevated glucose and alanine turnover, and gluconeogenesis from alanine in weight-losing lung cancer patients. Elevated PME levels in the liver of lung cancer patients are correlated with increased glucose flux and gluconeogenesis from alanine, indicating that PME levels reflect contributions from gluconeogenic intermediates. In weight-losing lung cancer patients, maximal levels of gluconeogenic intermediates within the liver have been observed. In weight-stable lung cancer patients gluconeogenesis is more rapidly induced by alanine infusion than in healthy control subjects suggesting that the first steps of the gluconeogenic pathway are enhanced. Altered gluconeogenic enzyme activities, possibly PEPCK, and elevated alanine uptake by the liver may play a role. Weight-stable breast cancer patients do not show the alterations in hepatic glucose metabolism as observed in lung cancer, indicating that alterations in host-metabolism may be tumour-specific. Nevertheless, the observation that elevated hepatic PME levels in cancer patients of different tumour types predict weight loss within the following three months is consistent with the suggestion that elevated gluconeogenesis contributes to weight loss in cancer.

Hepatic energy and phosphorylation status is decreased in lung cancer patients with weight loss. An impaired energy regenerating capacity during stimulated gluconeogenesis exists in these patients. Decreased hepatic ATP levels in advanced lung cancer patients, especially weight-losing patients, can be regenerated by intravenous infusion of ATP which may contribute to improving the nutritional status in these patients. High-dose intravenous ATP infusion also increases glucose turnover and gluconeogenesis from alanine in these patients.

The studies described in the present thesis clearly point out that alterations in host liver metabolism exist in lung cancer patients which are prognostic for the development of weight loss. Unravelling the underlying mechanisms of altered host metabolism will be the starting point for successful development of therapies for weight loss in lung cancer.

Samenvatting

Gewichtsverlies komt veelvuldig voor bij patiënten met longkanker. Een kenmerk van gewichtsverlies bij kanker is dat er niet alleen een afname van het vetweefsel optreedt maar er ook spiereiwit verloren gaat. Bij de gezonde mens wordt spiereiwit tijdens (langdurig) vasten juist gespaard. Deze afname in spiereiwit leidt tot een verslechtering van de lichamelijke conditie en een kortere overlevingsduur bij kankerpatiënten. Het ontstaan van gewichtsverlies bij kankerpatiënten kan niet volledig worden verklaard doordat deze minder eten dan gezonde personen. Er zijn aanwijzingen dat veranderingen in de stofwisseling een belangrijke rol spelen bij het ontstaan van gewichtsverlies.

Bij patiënten met kanker zijn een verhoogde afbraak van spiereiwit en verhoogde gluconeogenese, dit is de vorming van glucose (suiker, koolhydraten) uit niet-koolhydraat substraten in het lichaam, waargenomen. Gluconeogenese is een energieverbruikend proces. Aminozuren afkomstig uit de afbraak van spierweefsel, met name alanine, kunnen worden gebruikt voor gluconeogenese in de lever. Het is echter nog onduidelijk of gluconeogenese uit alanine verhoogd is bij patiënten met longkanker en of een verhoogde gluconeogenese samenhangt met gewichtsverlies. Verder zou het kunnen zijn dat een verhoogde gluconeogenese een effect heeft op de energiehuishouding van de lever. Het is onbekend of verlaging van de energiestatus van de lever samenhangt met gewichtsverlies.

Het doel van het in dit proefschrift beschreven onderzoek was na te gaan of de gluconeogenese uit alanine en de energiehuishouding in de lever afwijkend zijn bij longkankerpatiënten met, respectievelijk zonder gewichtsverlies. De hiervoor gebruikte methoden zijn turnovermetingen met stabiele isotopen en fosfor magnetische resonantie spectroscopie (^{31}P MRS) van de lever. De eerste methode is gebaseerd op het meten van de omzettingssnelheid (turnover) van een bepaald substraat (molecuul) in het bloed door het "merken" van het molecuul met een zwaar atoom. In dit onderzoek werden glucose en alanine gemeten, gemerkt met de atomen deuterium (^{2}H) en koolstof-13 (^{13}C). De tweede methode is gebaseerd op verschillen in magnetische eigenschappen tussen verschillende moleculen die een fosfor atoom bevatten. Met behulp van ^{31}P MRS kunnen relatieve concentraties van deze moleculen in specifieke organen, zoals de lever, gemeten worden.

De onderzoeksvragen van dit proefschrift waren:

1. Is gluconeogenese in de lever verhoogd en de energiereserve van de lever verlaagd bij patiënten met longkanker, en zijn deze veranderingen gerelateerd aan gewichtsverlies?
2. Kan een verlaagde energiestatus van de lever bij longkankerpatiënten hersteld worden door een infuus van ATP en wat is het effect hiervan op de gluconeogenese?
3. Voorspellen veranderingen van de gluconeogenese in de lever van kankerpatiënten

gewichtsverlies?

In **Hoofdstuk 2** wordt een onderzoek beschreven naar de stofwisseling van glucose, alanine, en gluconeogenese uit alanine bij longkankerpatiënten met gewichtsverlies (>5% van het stabiele gewicht voor de ziekte) en zonder gewichtsverlies, gemeten met stabiele isotopen. Ook wordt beschreven of er een relatie bestaat tussen de stofwisseling van de genoemde substraten en de mate van gewichtsverlies. De waarden bij kankerpatiënten werden vergeleken met die van gezonde vrijwilligers zonder gewichtsverlies.

De voedingsinname en plasmaconcentraties van glucose, alanine, insuline en glucagon bleken niet te verschillen tussen longkankerpatiënten en gezonde controlepersonen. De glucose- en alanineturnover en de gluconeogenese uit alanine bij longkankerpatiënten met gewichtsverlies waren respectievelijk 35%, 39% en 57% hoger dan bij patiënten zonder gewichtsverlies en gezonde vrijwilligers. De snelheid van de glucose- en alanineturnover, en gluconeogenese uit alanine was hoger naarmate het gewichtsverlies bij longkankerpatiënten toenam.

Gluconeogenese uit alanine vindt voornamelijk plaats in de lever. Met behulp van ^{31}P MRS kunnen verhoogde concentraties van tussenproducten van de gluconeogenese worden gemeten als een stijging van de phosphomonoesters (PME) en phosphodiesters (PDE) in de lever. In het onderzoek beschreven in **Hoofdstuk 3** werd nagegaan of verhoogde PME- en phosphodiester (PDE) concentraties samenhangen met verhoogde glucoseturnover en gluconeogenese uit alanine, zoals gemeten met stabiele isotopen bij longkankerpatiënten en gezonde vrijwilligers. De PME-concentratie in de lever bij longkankerpatiënten bleek 6% hoger te zijn dan bij gezonde controlepersonen. De PME-waarden in patiënten met gewichtsverlies waren significant hoger dan in patiënten zonder gewichtsverlies. PDE-concentraties in de lever waren niet verschillend tussen de groepen. Hogere PME-concentraties in de lever bij longkankerpatiënten gingen gepaard met een hogere glucoseturnover en gluconeogenese uit alanine. Bij gezonde controlepersonen werd dit verband niet gevonden.

In **Hoofdstuk 4** wordt een dynamisch onderzoek naar concentraties van tussenproducten van de gluconeogenese in de lever van longkankerpatiënten beschreven. Hierin werden ^{31}P MRS-metingen van de lever en turnovermetingen verricht tijdens een infuus van L-alanine (bolus van 1,4-2,8 mmol/kg gevolgd door 2,8 mmol/kg/uur gedurende 90 minuten).

Tijdens het infuus van L-alanine stegen de plasma alanineconcentraties met een factor 15-17

bij longkankerpatiënten en met een factor 24 bij gezonde controlepersonen. De glucoseturnover tijdens het L-alanine infuus steeg met 8% bij longkankerpatiënten zonder gewichtsverlies, terwijl geen significante verandering werd waargenomen bij patiënten met gewichtsverlies en gezonde controlepersonen. Na 45-90 minuten alanine infuus waren de PME-concentraties in de lever gestegen met 50% bij gezonde controles en met 87% bij longkankerpatiënten zonder gewichtsverlies. Bij longkankerpatiënten met gewichtsverlies werden geen significante veranderingen in PME-concentraties in de lever waargenomen. De plasma insulineconcentraties namen weliswaar bij alle groepen tijdens het L-alanine infuus toe, maar stegen het minst bij longkankerpatiënten met gewichtsverlies. Hoe hoger de concentraties van PME en PDE waren in de lever van longkankerpatiënten in de uitgangssituatie, des te kleiner waren de veranderingen in deze componenten tijdens het L-alanine infuus. Opnieuw werden deze relaties niet gevonden bij gezonde controlepersonen. Verder waren de veranderingen in PME in de lever bij longkankerpatiënten tijdens het L-alanine infuus kleiner naarmate het percentage gewichtsverlies groter was.

Dezelfde studieopzet werd gebruikt om na te gaan of een verhoogde gluconeogenese tijdens alanine infuus invloed heeft op de energiehuishouding in de lever van longkankerpatiënten met en zonder gewichtsverlies. Dit onderzoek wordt beschreven in **Hoofdstuk 5**. Veranderingen in concentraties van adenosine triphosphate (ATP) en anorganisch fosfaat (P_i) werden gemeten met behulp van ^{31}P MRS.

Basale ATP-concentraties waren verlaagd in de lever bij longkankerpatiënten met gewichtsverlies. De ATP-concentraties waren lager naarmate het gewichtsverlies groter was. De P_i/ATP ratio's in de lever bij longkankerpatiënten met gewichtsverlies waren verhoogd, hetgeen duidt op een verlaagde fosforyleringsstatus (energiestatus) van de lever. Tijdens het L-alanine infuus daalde de concentratie van ATP in de lever bij alle groepen. Gedurende de gehele infuusperiode (0-90 min.) was het ATP-gehalte bij longkankerpatiënten met gewichtsverlies significant lager dan bij longkankerpatiënten zonder gewichtsverlies en gezonde controlepersonen. Opmerkelijk was dat bij de laatste twee groepen na 60-90 minuten een zekere stabilisatie van de ATP niveau's in de lever plaatsvond, maar niet bij longkankerpatiënten met gewichtsverlies. Na 70-90 min infuus van L-alanine waren de P_i/ATP ratio's significant verhoogd bij longkanker-patiënten met gewichtsverlies ten opzichte van gewichtsstabiele longkankerpatiënten en gezonde controlepersonen.

De volgende twee studies werden uitgevoerd om het effect van een infuus van ATP op de energiehuishouding in de lever en de glucosestofwisseling bij patiënten met longkanker te

bestuderen. In het onderzoek beschreven in **Hoofdstuk 6** werd nagegaan of een infuus van ATP het ATP-gehalte en de fosforyleringsstatus in de lever bij longkankerpatiënten kan verbeteren. Dit werd onderzocht met behulp van ^{31}P MRS. Patiënten met longkanker werden tweemaal onderzocht: één week vóór, resp. tijdens 22-24 uur continu ATP infuus (37-75 µg/kg/min). Het ATP-gehalte in de lever bij longkankerpatiënten nam toe van 8,8% in de uitgangssituatie (ten opzichte van totaal MR-fosfaat) tot 12,2% tijdens het ATP-infuus. Deze laatste waarden waren gelijk aan die bij gezonde controlepersonen. Het ATP-gehalte nam meer toe bij patiënten met gewichtsverlies dan bij patiënten zonder gewichtsverlies. De P_i/ATP verhoudingen daalden licht tijdens het ATP-infuus, maar dit was niet statistisch significant.

In **Hoofdstuk 7** wordt het effect van het ATP-infuus op de glucose- en alanineturnover, en op de gluconeogenese uit alanine beschreven. Een hoge ATP-dosis (75 µg/kg/min) bleek bij longkankerpatiënten een stijging in de glucoseturnover van 41% en een stijging in de gluconeogenese uit alanine van 23% te veroorzaken. Lagere doses ATP (37-50 µg/kg/min) hadden dit effect niet. De toename in de glucoseturnover was afhankelijk van de ATP-dosis. Tijdens de hoge ATP-dosis nam het percentage glucose afkomstig van alanine toe van 59% tot 75%. De alanineturnover bij longkankerpatiënten veranderde bij geen van de gegeven ATP-doses.

In **Hoofdstuk 8** worden resultaten beschreven van gluconeogenese en leverstofwisseling bij patiënten met borstkanker (gewichtsverlies: 0 ± 1%). Glucose- en alanineturnover en gluconeogenese uit alanine bij patiënten met borstkanker waren niet verschillend van die bij gezonde controlepersonen. Concentraties van metabolieten in de lever gemeten met ^{31}P MRS waren niet verschillend tussen de groepen. Bij borstkankerpatiënten waren PME niveau's in de lever negatief gecorreleerd met glucoseproductie en gluconeogenese uit alanine. Lever-PME concentraties namen toe tijdens L-alanine infuus bij zowel borstkankerpatiënten als gezonde controlepersonen, maar alleen bij de laatste groep was dit significant. Er werden geen verandering waargenomen in PDE, ATP en P_i concentraties tijdens L-alanine infuus bij borstkankerpatiënten.

In de studie beschreven in **Hoofdstuk 9** werd onderzocht of een hoge concentratie van PME in de lever bij kankerpatiënten het optreden van gewichtsverlies kan voorspellen. Gegevens van ^{31}P MRS-studies van de lever bij 56 kankerpatiënten met diverse tumortypen, voornamelijk long- (n=31) en borstkanker (n=25), werden samengevoegd voor deze analyse. Tijdens 3 maanden na de MRS-meting namen het lichaamsgewicht, de quetelet-index, de

armomtrek, en de triceps huidplooi dikte af. Na correctie voor de energieinname en de quetelet-index ten tijde van de MRS-meting, bleek een stijging in de PME waarde van 5% was voorspellend voor een gewichtsafname van 1,8 ± 0,6 kg en een afname in de quetelet-index van 0,6 ± 0,2 in 3 maanden.

Conclusie

Het onderzoek beschreven in dit proefschrift toont aan dat de glucose- en alaninestofwisseling verhoogd zijn bij longkankerpatiënten met gewichtsverlies. Deze afwijkingen zijn afhankelijk van de mate van gewichtsverlies bij longkankerpatiënten en niet alleen van de aanwezigheid van longkanker als zodanig. De resultaten wijzen erop dat een verhoogde concentratie van PME in de lever van longkankerpatiënten het gevolg is van een verhoogde glucoseturnover en gluconeogenese uit alanine, door een toename van - concentraties van tussenproducten van de gluconeogenese. In de lever van longkankerpatiënten met gewichtsverlies is de gluconeogenese uit alanine maximaal. Bij longkankerpatiënten zonder gewichtsverlies zijn waarschijnlijk de eerste stappen van de gluconeogenese versneld. Mogelijke achterliggende mechanismen zijn een verhoogde activiteit van gluconeogenese-enzymen, met name fosfoenolpyruvaat carboxykinase, en een verhoogde opname van alanine in de lever vanuit het bloed. De bevindingen dat verhoogde PME-waarden in de lever bij kankerpatiënten het gewichtsverlies in de 3 daaropvolgende maanden voorspellen wijst op een mogelijk oorzakelijk verband tussen een verhoogde gluconeogenese en gewichtsverlies bij patiënten met long- en borstkanker.

De ATP- en fosforyleringsstatus van de lever zijn verlaagd bij longkankerpatiënten met gewichtsverlies. Ook bestaat er een verminderde capaciteit voor het herstellen van het energieniveau bij longkankerpatiënten met gewichtsverlies. Een infuus van ATP kan de energiestatus van de lever bij longkankerpatiënten verbeteren, met name in patiënten met gewichtsverlies. Deze veranderingen kunnen een rol spelen in de gunstige effecten van ATP op het lichaamsgewicht zoals gemeten bij muizen. Een hoge dosis ATP veroorzaakt ook een toename in de glucoseturnover en de gluconeogenese uit alanine bij patiënten met longkanker.

Het in dit proefschrift beschreven onderzoek toont aan zien dat er veranderingen bestaan in de leverstofwisseling bij patiënten met longkanker en dat deze veranderingen gewichtsverlies kunnen voorspellen. Het ontrafelen van de oorzaken van deze afwijkende stofwisseling zou een uitgangspunt kunnen zijn voor de ontwikkeling van succesvolle therapieën tegen gewichtsverlies bij kanker.

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Abbreviations

^{13}C	carbon-13
^{2}H	deuterium
^{31}P	phosphorus-31
1D-CSI	one-dimensional chemical shift imaging
3PG	3-phosphoglycerate
ANOVA	analysis of variance
APE	atom percent excess
ATP	adenosine 5'-triphosphate
AUC	area under the curve
BCAA	branched-chain amino acids
C	healthy control subject
CaWL	weight-losing cancer patient
CaWS	weight-stable cancer patient
CT	computed tomography
F_i	isotopic infusion rate
F1,6P	fructose-1,6-bisphosphate
F6P	fructose-6-phosphate
GC	gas chromatograph
G6P	glucose-6-phosphate
GPC	glycerophosphorylcholine
GPE	glycerophosphorylethanolamine
IE	isotopic enrichment
IRMS	isotopic ratio mass spectrometer
MDP	methylene diphosphonate
MS	mass spectrometer
MPE	mole percent excess
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
R_a	rate of appearance
PC	phosphorylcholine
PCr	phosphocreatine
PDE	phosphodiesters
PE	phosphorylethanolamine
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
P_i	inorganic phosphate
PME	phosphomonoesters

r	Pearson's correlation coefficient
rT_3	reverse triiodothyronine
SD	standard deviation
SE(M)	standard error of the mean
T_3	triiodothyronine
T_4	thyroxine
TCA	tricarboxylic acid
TR	repetition time

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Susanne Leij-Halfwerk

Curriculum vitae

Susanne Leij-Halfwerk werd op 21 april 1970 geboren te Leiden. Na het behalen van haar eindexamen V.W.O. aan het Rijnlands Lyceum Oegstgeest in 1988, begon zij met de studie Humane Voeding aan de Landbouwuniversiteit in Wageningen. Binnen de afstudeerrichting "Voeding en Gezondheid" voerde zij een viertal 5-maands afstudeerprojecten uit: 1) afstudeervak voeding op de afdeling maag-, darm-, en leverziekten, van het academisch ziekenhuis Nijmegen, 2) stage voeding bij de Human Nutrition Unit, university of Sydney, Australië, 3) afstudeervak toxicologie bij de vakgroep toxicologie, Landbouwuniversiteit Wageningen, en 4) afstudeervak fysiologie bij het Institut de Physiology, université de Lausanne, Zwitserland. In augustus 1994 studeerde zij af en in december werkte zij als statistisch analist bij IMRO in Oss. Vanaf februari 1995 was zij als assistent in opleiding aangesteld bij de Erasmus Universiteit Rotterdam en werkte op de afdelingen Inwendige Geneeskunde II, Dijkzigt Ziekenhuis, en Radiodiagnostiek, Dr. Daniel den Hoed Kliniek. De resultaten van het promotieonderzoek zijn beschreven in dit proefschrift.

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