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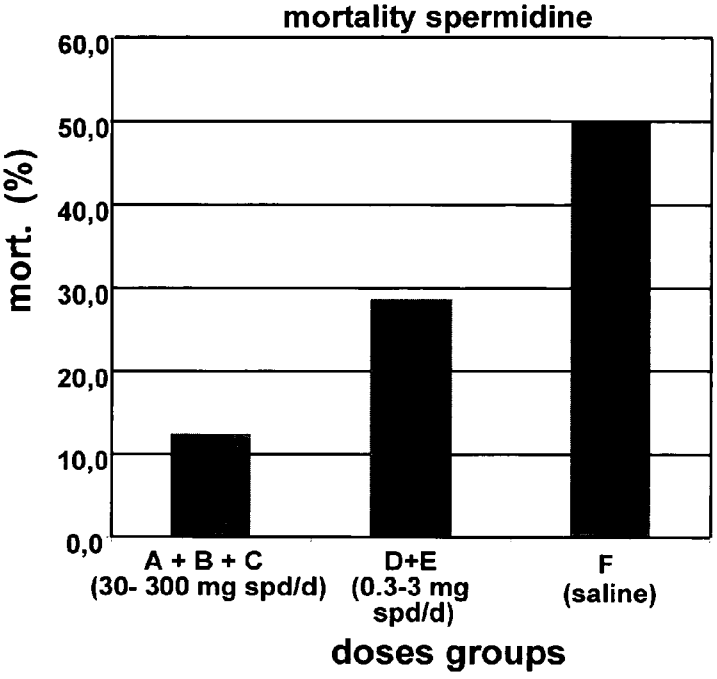

Figure 11

(57) Abstract: The present invention relates to the use of a polyamine or a salt, solvate, or derivative thereof, such as spermine or spermidine for the treatment or prevention of a life threatening condition, such as multiple organ failure, in a critically ill patient with a non-infectuous disorder.

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METHODS AND PREPARATIONS FOR PROTECTING CRITICALLY ILL PATIENTS

Field of the invention

The invention relates to life saving medicaments for critically ill patients and novel methods of treating a clinically ill patient. The invention relates to methods and preparations to increase the survivability of critically ill patients and to reduce or prevent the risk of mortality of the critically ill, mortality due to multiple organ failure and muscle weakness. The invention further relates to methods and preparations for protecting the critically ill, who are subjected to parenteral nutrition, against multiple organ failure or muscle weakness caused by parenteral nutrient delivery, particularly unbalanced or reflect (relative) nutrient overload.

Background of the invention

Thanks to major advances in intensive care medicine, critically ill patients nowadays often survive acute conditions that were previously lethal. Despite much effort, however, the mortality of patients who survive this initial phase and enter a chronic phase of critical illness, remains high worldwide. In these patients, mortality is often due to non-resolving multiple organ failure and muscle weakness. Treatments that have been introduced to improve the weakness, such as hyperalimentation, growth hormone, or androgens, failed because these interventions unexpectedly increased the risk of organ failure and death.

Critically ill or injured patients, particularly the prolonged critically ill, have nutritional needs that are often not, or insufficiently, met by enteral formulas. Severe injury or trauma, including surgery, is associated with loss of the body's nutrient stores due both to the injury itself and the resulting catabolic response. For optimal recovery, critically ill patients need proper nutritional intake. Lack of it can result in malnutrition-associated complications, including prolonged negative nitrogen balance, depletion of somatic and visceral protein levels, immune incompetence, increased risk of infection, and other complications associated with morbidity and mortality. Hormonally mediated hypermetabolism, catabolism, elevated basal metabolic rate and nitrogen excretion, altered fluid and electrolyte balance, synthesis of acute phase proteins, inflammation, and immunosuppression are often observed after severe injury, major surgery, or critical illness. Both anabolic and catabolic processes are accelerated following severe trauma, although catabolism predominates. This response allows muscle breakdown to

occur in order to provide amino acids for synthesis of proteins involved in immunological response and tissue repair. Disuse atrophy contributes to the muscle wasting and negative nitrogen balance frequently observed in the trauma patient and the critically ill patient.

A primary objective of nutritional support for the injured or ill person is to replace or maintain the body's normal level of nutrients by providing adequate energy substrates, protein, and other nutrients essential for tissue repair and recovery. The nutritional support of trauma and surgery patients has been extensively investigated in the prior art. Recently, it has been suggested and documented that the nutritional support to trauma and surgery patients may also have detrimental effects (Vanhorebeek et *al.* (2005) Lancet 365, 53-59).

U.S. 5,576,350 relates to a method for the prophylaxis of shock in a patient induced by endotoxin or bacteremia is disclosed. The method involves administering a therapeutically effective amount of a chemical composition dissolved in a pharmaceutically compatible solvent, such as a phosphate buffered saline, to the patient. The preferred chemical composition is spermidine, which binds to bacterial lipopolysaccharides.

Rasanen *et al.* (US 20040180968) and Hyvonen *et al.* (2006) Am. J. Pathol. 168, 115-122, and Doctoral Dissertation of December 2007, 2007, University of Kuopio, Finland disclose the use of spermine in the prevention of pancreatitis and the use of polyamines, modified at one or more of their NH2 or NH groups in the treatment of pancreatitis.

There is a need in the art to provide critically ill patients with appropriate treatments and adequate nutrition.

Summary of the invention

The present invention demonstrates the beneficial effects of polyamines to improve the condition of critically ill patients who suffer from multiple organ dysfunction. These polyamines allow to ameliorate the condition of critically ill patients.

The above objective is accomplished by polyamine compounds, pharmaceutical compositions, methods and uses of a polyamine compound to manufacture a medicament according to the present invention.

One aspect of the present invention relates to the use of a polyamine or a salt, solvate, or derivative thereof for the treatment or prevention of a life threatening condition in a critically ill patient with a non-infectuous disorder.

In particular embodiments of uses according to the present invention, the polyamine is a metabolisable polyamine, more particularly, the polyamine is a substrate for the enzyme Spermine/Spermidine Acetyltranferase (SSAT), more particularly the polyamine is not modified (particularly not methylated at) one or more of the NH_2 or NH groups.

In particular embodiments of uses according to the present invention, the polyamine is selected from the group consisting of putrescine (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, L-arginyl-3,4-spermidine and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride, more particularly spermine or spermidine.

In particular embodiments of uses according to the present invention, the life threatening condition is selected from the group consisting of lactic acidosis, muscle weakening, hyperglycemia, multiple organ failure and failed or disturbed homeostasis.

In particular embodiments of uses according to the present invention, the critically ill patient is a patient receiving enteral or parenteral nutrition, wherein the polyamine is e.g. administered together with an enteral or parenteral nutritient composition.

In particular embodiments of uses according to the present invention, the disorder of the critically ill patient is selected from the group consting of severe or multiple trauma, high risk or extensive surgery, cerebral trauma or bleeding, respiratory insufficiency, abdominal peritonitis, acute kidney injury, acute liver injury, severe burns and critical illness polyneuropathy.

An other aspect of the present invention relates to the use of a polyamine, or a salt, solvate, or derivative thereof for manufacture of a medicament for the treatment or prevention of a life threatening condition in a critically ill patient with a non-infectuous disorder.

In particular embodiments of uses according to the present invention, the critically ill patient is a patient receiving enteral or parenteral nutrition, wherein e.g. the polyamine is administered together with an enteral or parenteral nutritient composition.

Another aspect of the invention relates to nutrient solution suitable for parenteral administration, said nutrient solution comprising a saccharide, characterised in that said solution further comprises a polyamine or a salt, solvate, or derivative thereof.

In particular embodiments, the solution is suitable for intravenous administration.

Another aspect of the present invention relates to the use of a saccharide and a polyamine or a salt, solvate, or derivative thereof as a medicament.

Another aspect of the present invention relates to the se of a saccharide and a polyamine or a salt, solvate, or derivative thereof for the treatment or prevention of a life threatening condition in a critically ill patient with a non-infectuous disorder.

Another aspect of the present invention relates to the use of a saccharide and a polyamine or a salt, solvate, or derivative for the manufacture of a medicament for the treatment or prevention of a life threatening condition in a critically ill patient with a non infectuous disorder.

In particular embodiments the medicament is a solution suitable for intravenous administration.

Another aspect of the present invention relates to a method of treating a life threatening condition in a critically ill patient with a non infectuous disorder comprising the step of adminstering to said patient of a polyamine, or a salt, solvate, or derivative thereof.

Another aspect of the present invention relates to a pharmaceutical composition comprising a nutrient solution comprising a polyamine or a salt, solvate, or derivative thereof as described above for the treatment or prevention of a life threatening condition in a critically ill patient with a non-infectuous disorder, administering said a polyamine or a salt, solvate, or derivative thereof in one or more doses between 50 µg and 10 gram per day, depending on the body weight, e.g. between 10 µg/kg body weight/day to about 100 mg/kg/day.

It has been surprisingly found that multiple organ dysfuntion in living organisms can be treated by polyamine compounds of the groups consisting of putrescine, spermine, spermidine, cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride or derivatives thereof or combinations thereof. Such multiple organ dysfunction, common in the critical care setting, can be caused or aggravated by unbalanced parenteral nutrient delivery or a parenterially delivered relative or absolute nutrient overload.

It was found that a treatment by polyamine compounds of the groups consisting of putrescine, spermine, spermidine, cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride or derivatives thereof or

combinations thereof can increase the survivability of critically ill patients and can reduce or prevent the risk of mortality of the critically ill which is due to multiple organ failure that does not resolve or heal and to muscle weakness. Such treatment or preparation protects the critically ill, who are subjected to parenteral nutrition, against mortality or morbidity of multiple organ failure or of muscle weakness, in particular when such multiple organ failure or of muscle weakness is caused or aggravated by parenteral nutrient delivery, which may be unbalanced in this context, or parenterally delivered relative or absolute nutrient overload.

The compounds used in the present invention ameliorate the condition of critically ill patients, provide a treatment to treat or to prevent multiple organ dysfunction in the critically ill, provide a treatment to prevent mitochondrial dysfunction induced by inadequate or unbalanced parenteral nutrition to the critically ill and increase the survivability or reduce mortality in such critically ill patients.

The present invention demonstrates that multiple organ dysfunction can be treated in cells, tissues and living organisms by polyamine compounds more in particular polyamines wherein the NH_2 or NH group are not modified, such that they remain a substrate for acetylating enzymes. Examples hereof are putrescine, spermine, spermidine, cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride or derivatives thereof or combinations thereof.

A particular embodiment relates to a polyamine compound of the group consisting of putrescine (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, or a derivative thereof or a pharmaceutically acceptable salt, solvate or isomer thereof, such as cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride or combinations thereof, for use in a treatment of treating or preventing multiple organ dysfunction in a critically ill patient.

In a preferred embodiment, the polyamine compound of present invention is spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

It is an advantage of present invention that the polyamine compound can be used in a treatment of multiple organ dysfunction wherein the polyamine compound is administered parenterally or enterally to the critically ill patient.

Another aspect of the invention relates to a pharmaceutical composition comprising a pharmacologically acceptable amount of a polyamine compound of the group consisting of putrescine (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, or a derivative thereof or a pharmaceutically acceptable salt, solvate or isomer thereof, such as cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride or combinations thereof, for use in a treatment of treating or preventing multiple organ dysfunction in a critically ill patient.

In a preferred embodiment, the pharmaceutical composition of present invention invention comprises a polyamine compound, which is a pharmacologically acceptable amount of spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

It is an advantage of the pharmaceutical composition that the pharmaceutical composition can be provided as an aqueous liquid composition. Moreover, it is advantageous that the pharmaceutical composition can be administered parenterally or enterally to the critically ill patient. In a preferred embodiment, the critically ill patient further receives total parenteral nutrition.

It is an advantage of the pharmaceutical composition that the pharmaceutical composition can be provided to normalize the plasma spermidine level in the critically ill patient.

In particular embodiments dried polyamine comprising compositions are reconstituted with water to the pharmaceutical composition of present invention.

A further aspect of the invention relates to a method to treat or to prevent multiple organ dysfunction in a critically ill patient by administering to the critically ill patient a pharmaceutical composition comprising a pharmacologically acceptable amount of a polyamine compound of the group consisting of putrescine (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, or a derivative thereof or a pharmaceutically acceptable salt, solvate or isomer thereof such as cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride, or combinations thereof, for use in a treatment of treating or preventing multiple organ dysfunction in a critically ill patient.

In a preferred embodiment, the method to treat or to prevent multiple organ dysfunction in a critically ill patient comprises the step of administering to the critically ill patient a

pharmaceutical composition comprises a polyamine compound which is a pharmacologically acceptable amount of spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

It is an advantage that the method of present invention can normalize the plasma spermidine level in the critically ill patient.

A further aspect of the invention relates to the use of a polyamine compound of the group consisting of putrescine (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, or a derivative thereof or a pharmaceutically acceptable salt, solvate or isomer thereof, such as cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride or combinations thereof, to manufacture a medicament to treat or prevent multiple organ dysfunction in a critically ill patient.

In a preferred embodiment, the use of the polyamine compound of present invention is the use of spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

It is an advantage of present invention that the polyamine compound is used to manufacture a medicament to treat or prevent multiple organ dysfunction wherein the polyamine compound is administered parenterally or enterally to the critically ill patient.

It is an advantage of embodiments of present invention that polyamine compounds and pharmaceutical compositions administered to critically ill patients suffering from multiple organ failure have a decreased length of time spent on ventilator.

In preferred embodiment, the method to treat or to prevent multiple organ dysfunction in a critically ill patient comprises the step of administering to the critically ill patient a pharmaceutical composition comprises a polyamine compound which is a pharmacologically acceptable amount of spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

It is an advantage that the method of present invention can normalize the plasma spermidine level in the critically ill patient.

A further aspect of the present invention relates to the use of a polyamine compound of the group consisting of putrescine, (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride or a derivative thereof or a pharmaceutically acceptable salt, solvate or isomer thereof, or

combinations thereof, to prevent mitochondrial dysfunction induced by inadequate or unbalanced parenteral nutrition delivered to a critically ill patients

Another aspect of the present invention relates to a method to treat or to prevent mitochondrial dysfunction in a critically ill patient comprises the step of administering to the critically ill patient a pharmaceutical composition comprises a polyamine compound which is a pharmacologically acceptable amount of spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof. It is an advantage that such method can normalize the plasma spermidine level in the critically ill patient.

The invention relates further to a pharmaceutical composition comprising a pharmacologically effective amount of a polyamine as described herein as a pharmaceuticaly suitable carrier. In aqueous liquid composition the polyamine concentration can range from 0,05 % to about 4 %, or from about 0,5 % to about 2 % or from about 1.0 % to about 1.5 % of said aqueous liquid composition.

Such a pharmaceutical composition can further comprise a blood glucose regulator and or comprising nutrients.

The methods and compositions of the present invention are for normalising the plasma spermidine level in said critically ill patient, or to augment the plasma spermidine level in said critically ill patient to a level that is 1 to 2,5 times, 4 or even 5 times the plasma spermidine level of a healthy person with a similar body weight as said critically ill patient, for example to augment the plasma spermidine level in said critically ill patient to a level that is about twice the plasma spermidine level of a healthy person with a similar body weight as said critically ill patient or for example to augment the plasma spermidine level in said critically ill patient to a level that is restoring the plasma spermidine level to that of a healthy person with a similar body weight as said critically ill patient.

In particular embodiments the treatment is a treatment to augment the plasma spermidine level in said critically ill patient to a level in the range of 50 to 6000 nmol/l plasma, to augment the plasma spermidine level in said critically ill patient to a level in the weight range of 100 to 6000 nmol/l plasma, to augment the plasma spermidine level in said critically ill patient by administering daily said polyamine compound in the weight range of 0,05-1, 1 – 200, 5 – 150, 10 – 120 mg, or 40 – 80 mg per kg body weight.

Typically between 50µg to 10g of a polyamine compound per daily serving in one or more portion is administered to a human critically ill patient.

Polyamines as described in the present invention can be administered parenterally or

enterally to a critically ill patient, or by a bolus injection or by an intravenous bolus injection to said critically ill patient.

Polyamines as described in the present invention are suitable for inter alia;

- treatment of multiple organ dysfunction in a critically ill patient with failed or disturbed homeostasis receiving parenteral nutrition.

-protection a critically ill patient against multiple organ dysfunction by inducing adipocytes dedifferentiation. treatment of the development of lactic acidosis (lowering of blood pH and an increase in lactate),

- treatment of preventing parenteral nutrition induced development of lactic acidosis,

-treatment of treating or preventing muscle weaking in a critically ill patient.

- decreasing or preventing parenteral nutrition aggravated morbidity or mortality in the critical ill patient ,

- preventing body system collapse,

- treatment of preventing morbidity or mortality in a critical ill patient,

- treatment of preventing development of lactic acidosis in a critically ill patients.

The compositions, methods and uses of the present invention provide several advantages such as:

- the condition of critically ill animals or critically ill humans is improved.

- excessive catabolism in a critically ill patient is prevented or treated.

- morbidity or mortality due to excessive catabolism, e.g. in a critically ill patient, is reduced.

- the polyamine compound can be administered intravenously.

- multiple organ dysfunction syndrome in a critically ill patient can be reversed, treated or cured.

- the polyamine compound can be provided in an economically viable way.

- the polyamine compound can be administered in a pharmacologically acceptable way.

Particular and preferred aspects of the invention are set out in the accompanying independent and dependent claims. Features from the dependent claims may be combined with features of the independent claims and with features of other dependent claims as appropriate and not merely as explicitly set out in the claims.

The above and other characteristics, features and advantages of the present invention will become apparent from the following detailed description, taken in conjunction with the accompanying figures, which illustrate, by way of example, the principles of the invention. This description is given for the sake of example only, without limiting the

scope of the invention. The reference figures quoted below refer to the attached figures.

Brief description of the drawings

Figure 1: Flow chart of the treatment of critically ill rabbits according to embodiments of present invention.

Figure 2: Adjustment of glucose and insulin infusions in burn-injured rabbits of group 1 and group 2 according to embodiments of present invention.

Figure 3: Flow chart of the treatment of critically ill rabbits according to embodiments of present invention.

Figure 4: Adjustment glucose and insulin infusions in burn-injured rabbits of groups 1-6 according to an embodiment of present invention.

Figure 5: Graphs showing glucose uptake and glycolysis in healthy versus critically ill patients. The data represent (A) gene expression levels (mRNA A.U.) and (B) protein translation levels (protein A.U.) of GLUT1, GLUT3, GLUT4, as well as (C) glucose levels (µmol/g tissue) of subcutaneous (Subc. A.T.) and omental (Omental A.T.) adipose tissue biopsies, and (D) serum glucose levels (mg/dl) of 61 prolonged critical ill patients (taken minutes after death) and of 20 non-critically ill patients (taken during abdominal surgery).

Figure 6: Graphs showing lipogenesis in healthy versus critically ill patients. The data represent: (A) ACC protein translation levels (A.U.), (B) FAS activity (% cpm of control), and (C) SCD gene expression levels (mRNA A.U.) of subcutaneous (Subc. A.T.) and omental (Omental A.T.) adipose tissue biopsies, as well as (D) serum insulin levels (mIU/l) and (E) serum triglyceride levels (mg/dl) of 61 prolonged critical ill patients (taken minutes after death) and of 20 non-critically ill patients (taken during abdominal surgery).

Figure 7: Graphs showing adipose tissue morphology in healthy versus critically ill patients. The data represent: (A) median cell area (µm2) and (B) perilipin gene expression levels (mRNA A.U.) of subcutaneous (Subc. A.T.) and omental (Omental A.T.) adipose tissue biopsies of 61 prolonged critical ill patients (taken minutes after death) and of 20 non-critically ill patients (taken during abdominal surgery).

Figure 8: Picture illustrating Cd68 (macrophage) coloring in healthy versus critically ill patients.

Figure 9: (A) Cell line chart showing cell mean in critically ill rabbits (sperm d7.2) and healthy control rabbits (sperm baseline.2) at day 7, data representing 8 animals per group. (B) Box plot showing spermidine levels in plasma of critically ill rabbits (sperm d7.2) and healthy control rabbits (sperm baseline.2) at day 7, data representing 8 animals per group.

Figure 10: (A) Cell line chart showing cell mean in critically ill rabbits (sperm d7.2) and healthy control rabbits (sperm baseline.2) at day 7, data representing 7 animals per group. (B) Box plot showing spermidine levels in plasma of critically ill rabbits (sperm d7.2) and healthy control rabbits (sperm baseline.2) at day 7, data representing 7 animals per group.

Figure 11 and 12 show a decrease in mortality of parenterally fed hyperglycemic critically ill animals receiving spermidine

Figure 13 and 14 show a correlation between lactate levels in surviving and non-surviving animals.

Figure 15 shows that spermidine adminstration decrease the levels of creatinine, a marker of renal failure.

Figure 16 shows that spermidine adminstration decrease the levels of ureum, a marker of renal failure.

Figure 17 shows that spermidine adminstration decrease the levels of AST (ASpartate Transaminase), a marker of liver failure.

Figure 18 shows the effect of spermidine on the expression of autophagy markers and markers of mitochondrial acitivity.

Figure 19 shows that spermidine leads to hypoacetylation of histone H3 and strongly induces autophagy in flies and mammalian cell culture. **(A)** Relative acetylation (normalized to respective controls, dashed line) of indicated histone H3 lysine residues determined by quantification of immunoblot analysis using site specific antibodies. Data represent means of two independent analyses. For calculation details and representative blots (Balasundaram *et al. Proc Natl Acad Sci U S A* **88**, 5872-5876 (1991)). **(B)** Relative acetylation of indicated histone H3 lysine residues of *Δspe1* cells (open bars) compared to wild type cells (closed bars) chronologically aged to day 3 (Lys18 and Lys56) or day 12 (Lys9+14). Data represent means of two independent analyses. **(C)** Relative inhibition of histone acetyltransferase activity (HAT-activity) by spermidine determined by an in vitro HAT-activity assay of yeast nuclear extracts of wild type cells. Data

represent means ± SEM of three independent experiments. *p = 0,024. **(D)** Chronological aging of wild type and *Δiki3Δsas3* with (open symbols) or without (closed symbols) addition of 4 mM spermidine. Data represent means ± SEM (n = 4). **(E)** Relative acetylation of histone H3 lysine 9+14 residues determined by quantification of immunoblot analysis performed at day 20 of the experiment shown in **(E)**. Data represent means ± SEM (n = 3). *p < 0,05, ***p < 0,001. **(H)** LysoTracker Red staining of vacuoles indicative of autophagy in oesophagus-tissue from flies fed with 1 mM spermidine for two days compared to controls (without spermidine). Nuclei were visualized by Hoechst staining. Representative pictures are show. Scale bars represent 10 µm. **(I)** Fluorescence microscopy of Hoechst-counterstained HeLa cells transiently transfected with LC3-GFP subjected to 100 µM spermidine for 6 h. Representative pictures are shown.

Figure 20 shows autophagy induced by spermidine in flies and in mammalian cell culture. **(A)** Quantification of autophagic vesicles per nucleus in LysoTracker Red stained muscle tissue of female flies fed with 1 mM spermidine or with 10% glucose (starved) for 48 hours compared to normal food (controls). Data represent means ± SEM of at least 20 flies for each group. **p < 0,01. **(B)** HeLa cells transiently transfected with LC3-GFP were subjected to spermidine (100 µM) for 6 h and the percentage of adherent cells exhibiting a clear LC3-GFP relocalization into cytoplasmic vacuoles was determined using micrographs of Hoechst 33342-counterstained cells. Data represents means ± S.D. of three independent experiments. **(C, D)** Immunoblot analyses of accumulating LC3-II protein as well as p53 and GAPDH protein levels evaluated in dose (0,1 – 100 µM) and time (1 – 6 h) responses in HeLa cells subjected to spermidine.

Figure 21 shows that application of spermidine extends life span of yeast, flies, and human immune cells and inhibits oxidative stress in aging mice. **(A)** Survival determined by clonogenicity during chronological aging of wild type yeast (BY4741) with (○) and without (■) addition of 4 mM spermidine at day 1. Data represent means ± SEM (n = 5).

(B) Survival determined by AnnexinV / 7-AAD staining (unstained cells were considered as viable) of isolated human immune cells (PBMC) cultured for 6 and 12 days with PHA in the absence (black bar) or presence (white bars) of various spermidine concentrations (as indicated). Data

represent means ± SEM of 3 independent experiments (each done on PBMC from different donors). *p < 0,05 and **p < 0,01. **(C and D)** Survival determined by the number of living individuals of *Drosophila melanogaster* during aging of (C) female flies under normal conditions and (D) male flies under stressful conditions (addition of preservatives propionic acid and phosphoric acid) with and without (■) supplementation of food with various concentration of spermidine (as indicated). Representative aging experiments of at least 50 flies per sample are shown. **(E)** Replicative life span analysis of BY4741 wild type yeast after separation into old (fraction V) and young (fraction II) cells by elutriation centrifugation. The remaining life span with or without 1 mM spermidine on 2 % glucose full media is shown. **(F)** Free thiol group concentration (indicative of oxidative stress level) in blood serum of aging mice with or without (control) supplementation of drinking water with 0,3 and 3 mM spermidine for 200 days. Data represent means ± SEM (n = 3). **p < 0,01. **(G)** Intracellular spermidine of mouse liver cells, obtained from the same mice used for RSH measurements presented in (F). Data represent means ± SEM (n = 3).

Figure 22 shows that spermidine treatment of yeast results in strong resistance against heat shock and peroxide treatment. Survival of pre-aged wild type cells stressed for 4 h with hydrogen peroxide (3 mM H_2O_2) or heat shock (42 °C) compared to unstressed cells. Cells were chronologically aged until day 24 with or without addition of 4 mM spermidine. Data represent means ± SEM (n = 4). *p < 0,05 and ***p < 0,001.

Figure 23 shows that spermidine inhibits necrotic cell death of PBMC. Quantification (FACS analysis) of phosphatidylserine externalization (FITC channel) and loss of membrane integrity indicative of necrosis (PerCP channel) using AnnexinV/7-ADD costaining of 12 day old human PBMC's. Unstained cells were considered as viable. Dot Plots with 30,000 cells evaluated of a representative experiment are shown. Numbers indicate the percentage of cells located in the respective gate.

Figure 24 shows that histone H3 acetylation is regulated by intracellular polyamines in part mediated through Iki3p and Sas3p. **(A)** Immunoblot of whole cell extracts of wild type cells chronologically aged to designated time points with (+) or without (-) spermidine application. Blots were probed with antibodies against total histone H3 or H3 acetylation sites at the indicated

lysine residues. **(B)** Relative acetylation of histone H3 lysine 9+14 of *Δspe1* cells compared to wild type cells chronologically aged to day 5 with (open bars) or without (closed bars) adjustment of pH_{ex} to 6. Data represent means ± SEM of three independent experiments. **p < 0,01. **(C)** Quantification (FACS analysis) of phosphatidylserine externalization and loss of membrane integrity using AnnexinV/PI co-staining performed at day 20 of the chronological aging experiment shown in (Fig. 20D). For each staining 30,000 cells were evaluated. ***p < 0,001. **(D)** Immunoblot of whole cell extracts of wild type and *Δiki3Δsas3* cells with (+) or without (-) spermidine application obtained at day 20 of the aging experiment shown in (Fig. 20D). Blots were probed with antibodies against total histone H3 or H3 acetylation sites at lysine 9+14 (Lys9+14).

Figure 25 is a graphic display showing that polyamine depletion shortens yeast chronological life span evoking markers of oxidative stress and necrosis. **(A)** Intracellular spermidine of five-day-old Δ*spe1* cells compared to wild type. Data represent means ± SEM (n = 3). ***p < 0,001. **(B)** Chronological aging of wild type (■) and polyamine depleted *Δspe1* (Δ) yeast cells. Data represent mean ± SEM (n = 6). Cells were tested for cell death markers at day 3 (C-E). **(C)** Fluorescence microscopy of DHE stained wild type and *Δspe1* cells indicating ROS accumulation. Scale bars represent 10 µm. **(D)** Quantification (fluorescence reader) of ROS accumulation using DHE staining of wild type and *Δspe1* cells. Data represent means ± SEM (n =4). ***p < 0,001. **(E)** Quantification (FACS analysis) of phosphatidylserine externalization and loss of membrane integrity using Annexin V/PI costaining and of DNA-fragmentation using TUNEL staining of chronologically aging wild type and Δ*spe1* cells at day 3. Data represent means ± SEM (n = 3). **p < 0,01.

Figure 26 shows life span extension upon external alkalinization strictly depends on endogenous polyamines. **(A)** Chronological aging of wild type (closed symbols) and *Δspe1* (open symbols) with (▲) and without (■) adjustment of extracellular pH to 6. Data represent means ± SEM (n = 3). **(B)** Intracellular pH determined by staining with the pH-dependent fluorescent dye SNARF-4F of wild type and *Δspe1* cells with and without adjustment of extracellular pH to 6 during chronological aging. Data represent means ± SEM (n = 3). *p < 0,05, **p < 0,01, and ***p < 0,001.

Figure 27 shows that spermidine application suppresses necrotic cell death. **(A)** Fluorescence microscopy of DHE staining and Annexin V/PI costaining of wild type cells at day 18 of the chronological aging experiment shown in Fig. 23 B. Scale bars represent 10 µm. **(B and C)** Quantification of DHE staining (**B**) and Annexin V/PI costaining (**C**) by FACS analysis performed at indicated time points of the chronological aging experiment shown in Fig. 23B. Data represent means ± SEM (n = 3). ***p < 0,001. **(D)** Fluorescence microscopy of chronologically aged wild type cells (day 3 and 14) expressing an EGFP-tagged version of the yeast HMGB1 homolog (Nhp6A-EGFP) with or without (control) addition of 4 mM spermidine. Scale bars represent 5 µm. **(E)** Electron microscopy of young log-phase cells (day 0) and of 20 day old wild type cells aged with or without (control) 4 mM spermidine. Representative cells are shown.

Figure 28 shows necrotic disintegration of subcellular structures during aging is inhibited by spermidine. Overview pictures of electron microscopy of 20 day old wild type cells aged with or without (control) treatment of 4 mM spermidine and of healthy young cells. Higher resolution images of representative cells are shown in Figure 28 E.

Figure 29 shows life span extension by spermidine treatment is not due to regrowth of better adapted mutants. (**A**) Budding index of wild type cells at indicated time points during chronological aging with (○) or without (■) application of 4 mM spermidine, similar to the aging experiment as shown in Figure 29A. Data represent means ± SEM (n = 3) with at least 500-1000 cells evaluated for each replicate. **p < 0,01. (**B**) Mutation rate per 10^6 living cells determined by canavanine resistance of wild type cells at indicated time points during chronological aging with (open bars) or without (closed bars) application of 4 mM spermidine, similar to the aging experiment as shown in Fig. 29A. Data represent means ± SEM (n = 5). *p < 0,05.

Figure 30 shows that spermidine application temporarily protects from excessive ROS accumulation and loss of survival in *sod2* mutant cells during aging. **(A)** Survival during chronological aging of wild type (WT) and Δ*sod2* yeast with (open symbols) or without (closed symbols) application of 4 mM spermidine. Data represent means ± SEM (n = 4). **(B)** Quantification (fluorescence reader) of ROS accumulation using DHE staining of cells

obtained from the aging experiment shown in (A). Data represent means ± SEM (n = 4).

Figure 31 shows autophagy induced by spermidine in flies and in mammalian cell culture. **(A)** Quantification of autophagic vesicles per nucleus in LysoTracker Red stained muscle tissue of female flies fed with supplementation of 1 mM spermidine or with 10% glucose (starved) for 48 hours compared to normal food (control). Data represent means ± SEM of at least 20 flies for each group. **p < 0,01. **(B)** HeLa cells transiently transfected with LC3-GFP were subjected to spermidine (100 µM) for 6 h and the percentage of adherent cells exhibiting a clear LC3-GFP relocalization into cytoplasmic vacuoles was determined using micrographs of Hoechst 33342-counterstained cells. Data represents means ± S.D. of three independent experiments. **(C, D)** Immunoblot analyses of accumulating LC3-II protein (indicative of autophagy) as well as p53 and GAPDH protein levels evaluated in dose (0,1 – 100 µM) and time (1 – 6 h) responses in HeLa cells subjected to spermidine.

Figure 32 shows that deletion of the polyamine acetyltransferase *PAA1* shortens chronological life span and enhances oxidative stress. **(A)** Survival during chronological aging of wild type and Δ*paa1* yeast cells. Data represent means ± SEM (n = 4). **(B)** Quantification (fluorescence reader) of ROS accumulation using DHE staining of cells obtained from the aging experiment shown in (A). Relative fluorescence units have been normalized to wild type at day1. Data represent means ± SEM (n = 4). ***p < 0,001.

Figure 33 shows that spermidine treatment causes remodelling of chronologically aging cells into a low metabolic, quiescence-like state. **(A)** Sucrose gradient centrifugation of 22 day old wild type yeast chronologically aged with or without (control) treatment of 4 mM spermidine. A representative photograph is shown, picturing upper, middle, and lower (quiescent) cells. **(B)** Quantification of upper, middle, and lower fraction of cells after sucrose gradient centrifugation representatively shown in (A). Data represent means ± SEM of three independent experiments. **p < 0,01, ***p < 0,001. **(C)** Oxygen consumption of wild type cells treated with or without (control) 4 mM spermidine during chronological aging. Oxygen consumption has been determined using O_2-electrode measurements and

normalized to living cells (see Methods section). Data represent means ± SEM (n = 3). *p< 0,05, ***p < 0,001.

Description of illustrative embodiments

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. The drawings described are only schematic and are non-limiting. In the drawings, the size of some of the elements may be exaggerated and not drawn on scale for illustrative purposes. The dimensions and the relative dimensions do not correspond to actual reductions to practice of the invention.

Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequence, either temporally, spatially, in ranking or in any other manner. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

It is to be noticed that the term "comprising", used in the claims, should not be interpreted as being restricted to the means listed thereafter; it does not exclude other elements or steps.

Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances, of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment, but may. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to one of ordinary skill in the art from this disclosure, in one or more embodiments.

Similarly it should be appreciated that in the description of exemplary embodiments of the invention, various features of the invention are sometimes grouped together in a single embodiment, figure, or description thereof for the purpose of streamlining the disclosure and aiding in the understanding of one or more of the various inventive aspects.

The invention will now be described by a detailed description of several embodiments of the invention. It is clear that other embodiments of the invention can be configured

according to the knowledge of persons skilled in the art without departing from the technical teaching of the invention, the invention being limited only by the terms of the appended claims.

The following terms are provided solely to aid in the understanding of the invention.

The term "pharmaceutically acceptable" is used adjectivally herein to mean that the compounds are appropriate for use in a pharmaceutical product. The term "physiologically acceptable" also means that the compounds are appropriate for use in a pharmaceutical product.

As used herein, the phrase "physiologically acceptable salts" or "pharmaceutically acceptable salts" or "nutraceutically acceptable salts" refers to salts prepared from pharmaceutically acceptable, preferably nontoxic, acids and bases, including inorganic and organic acids and bases, including but not limited to, sulfuric, citric, maleic, acetic, oxalic, hydrochloride, hydro bromide, hydro iodide, nitrate, sulfate, bisulfite, phosphate, acid phosphate, isonicotinate, acetate, lactate, salicylate, citrate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucaronate, saccharate, fornate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate and pamoate (i.e. 1,1'-methylene-bis-(2-hydroxy-3-naphthoate)) salts. Pharmaceutically acceptable salts include those formed with free amino groups such as, but not limited to, those derived from hydrochloric, phosphoric, acetic, oxalic, and tartaric acids. Pharmaceutically acceptable salts also include those formed with free carboxyl groups such as, but not limited to, those derived from sodium, potassium, ammonium, sodium lithium, calcium, magnesium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, and procaine.

As used herein, the term "carrier" refers to a diluent, adjuvant, excipient, or vehicle. Such carriers can be sterile liquids, such as saline solutions in water, or oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. A saline solution is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions.

As used herein, the tern "mineral" refers to a substance, preferably a natural substance that contains calcium, magnesium or phosphorus. Illustrative nutrients and minerals include beef bone, fish bone, calcium phosphate, egg shells, sea shells, oyster shells,

calcium carbonate, calcium chloride, calcium lactate, calcium gluconate and calcium citrate.

The term "treatment" refers to any process, action, application, therapy, or the like, wherein a mammal, including a human being, is subject to medical aid with the object of improving the mammal's condition, directly or indirectly.

In its broadest sense, the term a "**critically ill patient**" (herein designated CIP) refers to a patient who is experiencing an acute life-threatening episode or who is diagnosed to be in imminent danger of such an episode. A critically ill patient is medically unstable, and when not treated, likely to die.

The term critically ill patient refers to a patient who has sustained or is at risk of sustaining acutely life-threatening single or multiple organ system failure due to disease or injury, a patient who is being operated and where complications supervene, and a patient who has been operated in a vital organ within the last week or has been subject to major surgery within the last week.

In a more restricted sense, the term a "critically ill patient", as used herein refers to a patient who has sustained or are at risk of sustaining acutely life-threatening single or multiple organ system failure due to disease or injury, or a patient who is being operated and where complications supervene.

In an even more restricted sense, the term a "critically ill patient", as used herein refers to a patient who has sustained or are at risk of sustaining acutely life-threatening single or multiple organ system failure due to disease or injury. Similarly, these definitions apply to similar expressions such as "critical illness in a patient" and a "patient is critically ill". A critically ill patient is also a patient in need of cardiac surgery, cerebral surgery, thoracic surgery, abdominal surgery, vascular surgery, or transplantation, or a patient suffering from neurological diseases, cerebral trauma, respiratory insufficiency, abdominal peritonitis, multiple trauma, severe burns, or critical illness polyneuropathy.

The term "**critical illness**" as used herein refers to the condition of a "critically ill patient".

The term "**Intensive Care Unit**" (herein designated ICU), as used herein refers to the part of a hospital where critically ill patients are treated. Of course, this might vary from country to country and even from hospital to hospital and the part of the hospital may not necessary, officially, bear the name "Intensive Care Unit" or a translation or derivation thereof. Of course, the term "Intensive Care Unit" also covers a nursing

home, a clinic, for example, a private clinic, or the like if the same or similar activities are performed there. The term “ICU patient” refers to a “critically ill patient”.

The term “**multiple organ dysfunction**” or “**multiple organ dysfunction syndrome**” or “**MODS**” refers to a condition resulting from infection, injury (accident, surgery), hypoperfusion or hypermetabolism.

The “multiple organ failure” of which critically ill patients die, is considered a descriptive clinical syndrome, defined by a dysfunction or failure of at least two vital organ systems. The vital organ systems that are uniformly and most specifically affected are the liver, the kidneys, the lungs, as well as the cardiovascular system, the nervous sytem and the hematological system.

MODS comprises but is not limited to acute respiratory distress syndrome, heart failure, liver failure, renal failure, respiratory insufficiency, intensive care, shock and systemic inflammatory response syndrome. MODS is characterized by a progressive deterioration and subsequent failure of the body’s physiological system. The primary cause triggers an uncontrolled inflammatory response. In operative and non-operative patients sepsis is the most common cause. Sepsis may result in septic shock. In the absence of infection a sepsis-like disorder is termed systemic inflammatory response syndrome (SIRS). Both SIRS and sepsis could ultimately progress to MODS. However, in one-third of the patients no primary focus can be found. MODS is well established as the final stage of a continuum ranging from SIRS to sepsis to severe sepsis to MODS.

The terminology **"enterally administering"** encompasses oral administration (including oral gavage administration) as well as rectal administration, oral administration being most preferred. Unless indicated otherwise, the dosages mentioned in this application refer to the amounts delivered during a single serving or single administration event. If the present composition is ingested from a glass or a container, the amount delivered during a single serving or single administration will typically be equal to the content of the glass or container.

The term **"parenterally administering"** refers to delivery of substances given by routes other than the digestive tract, and covers adminstration routes such as intravenous,,intraarterial, intramuscular, intracerebroventricular, intraosseous intradermal, intrathecal, and intraperitoneal adminstration and intravesical infusion and intracavernosal injection.

Typically **"parenteral administration"** refers to intravenous adminstration. A particular form of parenteral adminstration refers to the delivery by intravenous adminstration of

nutrition (**"parenteral nutrition"**). Parenteral nutrition is called **"total parenteral nutrition"** when no food is given by other routes.

"Parenteral nutrition" is a isotonic or hypertonic aqueous solution (or solid compositions to be dissolved, or liquid concentrates to be diluted to obtain an isotonic or hypertonic solution) comprising a saccharide such as glucose and further comprising one or more of lipids, amino acids, and vitamins.

The present invention discloses the surprising finding that polyamines have a beneficial effect on combating life-treating conditions in critically ill patients.

Whereas the use of polyamines to treat infectious disorders can be explained by the fact that polyamines bind to lipopolysacharides of bacteria, it was unexpected that polyamines have a therapeutic activity on life threatening conditions caused by non-infectious disorders.

The invention further discloses the surprising finding that polyamines have a beneficial effect even when a patient is already is at far-developed stage of a disorder in that the patient is a critically ill patient in a life threatening condition.

Hyvonen *et al.* and Rasanen *et al.* (cited above) discuss the use of polyamines in the prevention an treatment of pancreatitis. However Hynonen emphasizes the fact that after induction of the pancreatitis the treatment can be started when symptoms occur, but does not suggest or encourages to perform a treatment when the diseases is further developed into life-threatening conditions. As indicated in Hyvonen, pancreatitis can result into multiple organ failure due to systemic factors, leading to complications associated with high mortality. However the experiments performed by Vynonen in mice models of pancreatitis do not show or suggest the treatment of these animals in further developed stages of pancreatitis, let alone suggest the treatment of multiple organ failure in other conditions apart from pancreatitis.

The invention further discloses the surprising finding that life threatening conditions can be alleviated or treated by metabolisable polyamines, i.e polyamines with primary (NH2) or secondary (NH) amines. The prior art of Vynonen and Rasanen explain that a depletion in spermine and spermidine is caused by an enhanced activity of the acetylating enzyme SSAT (Spermine/Spermidine Actyl Transferase) leading to the degradation of the polyamine. The prior teaches that in order to treat pancreatitis, polyamines should be modified (e.g. methylated) at at least one amine position to avoid acetylation of the enzymes. The findings of the present invention show that unmodified polyamines, which are substrates for the degradation via acetylation, are active indeed. Accordingly the teaching of the present invention allows to use commercially available,

natural compounds occurring in the human body, which are metabolized, whereas the modified compounds of Hyvonen and Hassane are foreign to the body and may accumulate in the body without being degraded.

The invention further discloses the surprising finding that the treatment with polyamines according to embodiments of the present invention can be combined with the administration of high nutrient compositions such as glucose comprising solutions for enteral or parenteral (e.g. intravenous) administration.

Vanhorebeek *et al.* (2005) Lancet 365, 53-59 discuss the detrimental effects of glucose in critically ill patients. The experiments performed in the present invention on mammalian cells and model organisms such as yeast and *Drosophila* show that the addition of polyamines has a beneficial effect on critically ill patients who are at risk of nutrient overload or starvation. The present invention discloses that the removal of damaged cell organelles by autophagy acts as a cell protective mechanism and that this autophagy process functions better under calorie restricted conditions. At the one hand, starvation in critically ill patients is not an option as this results in muscle degradation and severe weakness. But also prolonged hypocaloric feeding can result in impaired outcome (Villet *et al.* (2005) Clin Nutr 24, 502-509.

On the other hand damaged organelles, which are even more abundantly present in humans and in animals who are in critically ill conditions (such as caused by trauma or complex injuries) need to be removed to preserve the cellular function in vital organs and systems. This removal, which is stimulated by autophagy which is considered to be an important defense process in critical, is inhibited by when the calorie restricted conditions are compensated by nutrient overload and hyperglycemia.

Although parenteral feeding has shown to reduce muscle wasting during critical illness, it is a powerful suppressor of autophagy in vital organs, and thus we hypothesized that autophagy is an important defence process in critical illness. Indeed, damaged organelles, which are even more abundantly present in patients and in animals who have undergone critical illness (induced by trauma or complex injuries) and who receive parenteral nutrition and hence are exposed to additional risks such as nutrient overload and hyperglycemia, need to be removed properly to preserve the cellular function in vital organs and systems. As such, feeding-induced suppression of autophagy could counteract any benefit obtained by feeding. Such a mechanism may explain why we found that starved animals have a better functioning of liver mitochondria, because damaged mitochondria are better removed by starvation-induced autophagy.

Without being bound by theory, the present experiments on mammalian cells, yeast and flies show that polyamines such as spermidine offer protection against cellular damage, which can be at least partially explained by reactivating autophagy, leading to, but not restricted to e.g. a better functioning mitochondria (increased clearance of damaged mitochondria) and subsequent protection of vital organ systems. It has been found that these beneficial effect of polyamines abrogate vital organ dysfunction and lethality induced by parenteral feeding in critically ill animals, even in the presence of pronounced hyperglycemia. Accordingly the present invention that the suppression of autophagy caused by parental feeding is compensated by the administration of polyamines such as spermidine.

The present invention discloses an animal model of prolonged critical illness that mimicks the human condition. Indeed, these critically ill animals undergo the same metabolic, immunological and endocrine disturbances and development of organ failure and muscle wasting as the human counterpart. In this animal model, parenteral feeding has an effect on the overall outcome of the animals. Compared to starvation, a small dose of parenteral feeding in critically ill animals decreased muscle catabolism and did not induce significant lethality. A higher dose of parenteral feeding however holds risk of death, which thus reflects a trade-off for improved muscle preservation. As soon as hyperglycemia is allowed to develop, a higher lethality precludes any benefit from parenteral feeding. Indeed, parenteral feeding has also disadvantages, one of which is development of hyperglycemia, which, if left untreated, leads to increased mortality, multiple organ failure and muscle breakdown. Even brief cellular hyperglycemia and nutrient overload exerts direct toxic cellular effects in the setting of critical illness, leading to these disastrous effects. Prevention of hyperglycemia in the critically ill, however, is difficult to achieve, specifically since there is a risk of hypoglycemia, which could counteract any benefit.

The present invention illustrates that such lethal effects of parental feeding in critically ill animals can be abrogated by administration of polyamines such as spermidine.

The present invention describes polyamines, compositions comprising polyamines and and their use in the treatment of life threatening conditions in critically ill patients.

Polyamines are generally described as basic, water soluble, low molecular weight aliphatic molecules with two (diamines) or more amine groups.

Particular amines in the context of the present invention are diamines represented by the general formula NH_2-$(CH2)_{2-10}$-NH_2, which are unsubstituted at the carbon atoms or wherein one or more carbon atoms are optionally substituted with a methylgroup, an NH or oxygen. The diamine group of polyamines comprises ethylene diamine, 1,3 diaminopropane, 1,4 diaminobutane (putrescine), 1,5 diaminopentane (cadaverine), 1,6-diamino-hexane, 1,7-diamino-heptane and 1,8-diamino-octane. Particular diamines in the context of the present invention are 1,4 diaminobutane (putrescine) and 1,5 diaminopentane (cadaverine), more particularly are 1,4 diaminobutane (putrescine) Other particular polyamines have a general structure NH_2-$((CH_2)_m$-$NH)_n$ -H, wherein m and n are each independently integers from 2 to 6. These polyamines are typically unsubstituted at the carbon atoms. Optionally one or more carbon atoms are substituted with a methylgroup, and/or NH and/ or oxygen group.

In particular embodiments m is 3, 4 or 5, more particularly 4. In particular embodiments n is 3 or 4. Particular polyamines are spermine $H_2N((CH_2)_4$-$NH)_3$-H (m is 4 and n is 3) and spermidine $NH_2((CH_2)_4$-$NH)_2H$ (m is 4 and n is 2).

More particular embodiments in the context of the present invention are polyamines wich are metabolisable, i.e. in that the polyamines are a substrate for the acetylating enzyme SSAT. According to this embodiment, polyamines which are methylated at one or more NH_2 or NH groups are disclaimed.

Alternatively, according to an alternative embodiment polyamines can be acetylated at one or more of NH_2 or NH groups.

The present invention relates in particular embodiments to a polyamine compound of the group consisting of putrescine (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, or a derivative thereof or a pharmaceutically acceptable salt, solvate or isomer thereof, such as or combinations thereof, for use in a treatment of treating or preventing multiple organ dysfunction in a critically ill patient.

In one embodiment, the polyamine compound of present invention is spermidine or a spermidine analog of the group consisting of spermidine phosphate hexahydrate, spermidine phosphate hexahydrate and L-arginyl-3,4-spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

In a particular embodiment, the polyamine compound of present invention is a polyamine compound of the group consisting of putrescine, spermine, and spermidine.

In a preferred embodiment, the polyamine compound of present invention is spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

In embodiments of the present invention, the polyamine compound is used in a treatment of treating or preventing multiple organ dysfunction in a critically ill patient wherein the multiple organ dysfunction is not caused or associated with sepsis.

The polyamine compound can be used in a treatment of multiple organ dysfunction wherein the polyamine compound is administered parenterally or enterally to the critically ill patient, or is administered by a bolus injection, e.g. an intravenous bolus injection.

In a preferred embodiment, the polyamine compound of present invention is used in a treatment of multiple organ dysfunction wherein the critically ill patient further receives total parenteral nutrition.

In another preferred embodiment, the polyamine compound of present invention is used in a treatment of multiple organ dysfunction in a critically ill patient receiving parenteral nutrition.

In yet another preferred embodiment, the polyamine compound of present invention is used in a treatment of multiple organ dysfunction in a critically ill patient with failed or disturbed homeostasis receiving parenteral nutrition.

In a particular embodiment, the polyamine compound of present invention is used in a treatment to protect a critically ill patient against multiple organ dysfunction by inducing adipocytes dedifferentiation.

In a particular embodiment, the polyamine compound of present invention is used in a treatment to protect a critically ill patient against multiple organ dysfunction by inducing dedifferentiation of adipocytes, e.g. inducing dedifferentiation of adipocytes into new into adipogenic, chondrogenic and osteogenic lineages, which results in reduced size of adipocytes and increased adipose mass.

Mature, lipid-containing adipocytes possess the ability to undergo symmetrical or asymmetrical cell division by a process called dedifferentiation of adipocytes. Such dedifferentiated adipocytes can function as seed cells and are capable of further differentiating into adipogenic, chondrogenic and osteogenic lineages. Differentiation of adipocytes has been observed in critical ill patients. This process of adipocyte dedifferentiation and the formation of new adipocytes from the seed/precursor cells can be further be enhanced by a treatment with a polyamine compound of the group consisting of putrescine (1, 4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-

heptane, 1,8-diamino-octane, spermine, or a derivative thereof or a pharmaceutically acceptable salt, solvate or isomer thereof, such as spermidine, cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride or combinations thereof. Such induced dedifferentiation of adipocytes and further differentiation of the seed cells turn adipose tissue into a functional 'waist bin' for toxic metabolites such as glucose during critical illness and is protective against multiple organ dysfunction in a critically ill patient.

A preferred embodiment of present invention is spermidine or a spermidine analogue of the group consisting of spermidine phosphate hexahydrate, spermidine phosphate hexahydrate and L-arginyl-3,4-spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof for use in a treatment to protect a critically ill patient against toxic metabolites by enhance dedifferentiation of adipocytes and of absorption of toxic metabolites in the adipose tissue protection of a critically ill patient.

A preferred embodiment of present invention is spermidine or a spermidine analogue of the group consisting of spermidine phosphate hexahydrate, spermidine phosphate hexahydrate and L-arginyl-3,4-spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof for use in a treatment to protect a critically ill patient against multiple organ dysfunction by enhance dedifferentiation of adipocytes and of absorption of toxic metabolites in the adipose tissue protection of a critically ill patient.

A polyamine compound of the group consisting of putrescine (1, 4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, or a derivative thereof or a pharmaceutically acceptable salt, solvate or isomer thereof, such as cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride or combinations thereof, for use in a treatment of to induce or enhance the dedifferentiation of adipocytes and absorption of toxic metabolites into the protection of the critically ill patent against toxic metabolites.

The present invention also relates to a pharmaceutical composition comprising a pharmacologically acceptable amount of a polyamine compound of the group consisting of putrescine, 1,4-diamino-butane, 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, or a derivative thereof or a

pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof, such as cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride for use in a treatment of treating or preventing multiple organ dysfunction in a critically ill patient.

In one embodiment, the pharmaceutical composition of present invention comprises a pharmacologically acceptable amount of a polyamine compound of the group consisting of cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride or a derivative thereof or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

In a particular embodiment, the pharmaceutical composition of present invention comprises a polyamine compound which is spermidine or a spermidine analog of the group consisting of spermidine phosphate hexahydrate, spermidine phosphate hexahydrate and L-arginyl-3,4-spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

In a preferred embodiment, the pharmaceutical composition of present invention invention comprises a polyamine compound, which is a pharmacologically acceptable amount of spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

In one embodiment, the pharmaceutical composition of present invention comprises the polyamine compound of present invention in the range of about 0,05 % to about 4 % of the aqueous liquid composition.

In one embodiment, the pharmaceutical composition of present invention comprises the polyamine compound in the range of about 0,5 % to about 2 % of the aqueous liquid composition.

In one embodiment, the pharmaceutical composition of present invention comprises the polyamine compound in the range of about 1.0 % to about 1.5 % of the aqueous liquid composition.

In another embodiment, the pharmaceutical composition of present invention further comprises a pharmaceutically acceptable carrier or a blood glucose regulator or nutrients, e.g. essential nutrients.

The pharmaceutical composition can be provided as an aqueous liquid composition. Moreover, the pharmaceutical composition can be administered parenterally or enterally to the critically ill patient, or is administered by a bolus injection, e.g. an

intravenous bolus injection. In a preferred embodiment, the critically ill patient further receives total parenteral nutrition.

The pharmaceutical composition can be provided to normalize the plasma spermidine level in the critically ill patient, or to augment the plasma spermidine level in the critically ill patient to a level that is 1 to 2,5, 4 or even 5 times the plasma spermidine level of a healthy person with a similar body weight as the critically ill patient. In an embodiment, the pharmaceutical composition can be provided to augment the plasma spermidine level in the critically ill patient to a level that is twice the plasma spermidine level of a healthy person with a similar body weight as the critically ill patient. Also, the pharmaceutical composition of present invention is for use in a treatment to augment the plasma spermidine level in the critically ill patient to a level in the weight range of 0,5 – 4 µg per kg body weight.

In one embodiment, the pharmaceutical composition of present invention is for use in a treatment to augment the plasma spermidine level in the critically ill patient to a level that is restoring the plasma spermidine level to that of a healthy person with a similar body weight as the critically ill patient.

In another embodiment, the pharmaceutical composition of present invention is for use in a treatment to augment the plasma spermidine level in the critically ill patient to a level in the range of 50 to 3500 nmol/l plasma.

In another embodiment, the pharmaceutical composition of present invention is for use in a treatment to augment the plasma spermidine level in the critically ill patient to a level in the range of 100 to 6000 nmol/l plasma.

In another embodiment, the pharmaceutical composition of present invention is for use in a treatment to augment the plasma spermidine level in the critically ill patient by administering daily the polyamine compound in the weight range of 0,01 µg per kg to 100 mg per kg body weight.

In a preferred embodiment, the pharmaceutical composition of present invention is for use in a treatment of treating or preventing multiple organ dysfunction in a critically ill patient that is not caused or associated with sepsis.

In a preferred embodiment, the pharmaceutical composition of present invention is for use in a treatment to protect a critically ill patient against multiple organ dysfunction by inducing dedifferentiation of adipocytes, e.g. inducing dedifferentiation of adipocytes into new into adipogenic, chondrogenic and osteogenic lineages, which results in reduced size of adipocytes and increased adipose mass.

In a particular embodiment, the pharmaceutical composition of present invention is for use in a treatment to protect a critically ill patient against toxic metabolites by enhancing dedifferentiation of adipocytes and of absorption of toxic metabolites in the adipose tissue protection of a critically ill patient.

In a particular embodiment, the pharmaceutical composition of present invention is for use in a treatment to induce or enhance the dedifferentiation of adipocytes and absorption of toxic metabolites into the protection of the critically ill patent against toxic metabolites.

The present invention also relates to a composition that can be reconstituted with water to the pharmaceutical composition of present invention.

The present invention also relates to a method to treat or to prevent multiple organ dysfunction in a critically ill patient by administering to the critically ill patient a pharmaceutical composition comprising a pharmacologically acceptable amount of a polyamine compound of the group consisting of putrescine (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, or a derivative thereof or a pharmaceutically acceptable salt, solvate or isomer thereof, such as cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride or combinations thereof, for use in a treatment of treating or preventing multiple organ dysfunction in a critically ill patient.

In one embodiment, the method to treat or to prevent multiple organ dysfunction in a critically ill patient comprises the step of administering to the critically ill patient a pharmaceutical composition comprises a pharmacologically acceptable amount of a polyamine compound of the group consisting of cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride or a derivative thereof or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

In a particular embodiment, the method to treat or to prevent multiple organ dysfunction in a critically ill patient comprises the step of administering to the critically ill patient a pharmaceutical composition comprises a polyamine compound which is spermidine or a spermidine analog of the group consisting of spermidine phosphate hexahydrate, spermidine phosphate hexahydrate and L-arginyl-3,4-spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof

In a preferred embodiment, the method to treat or to prevent multiple organ dysfunction in a critically ill patient comprises the step of administering to the critically ill patient a pharmaceutical composition comprises a polyamine compound which is a pharmacologically acceptable amount of spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

In another preferred embodiment, the method to treat or to prevent multiple organ dysfunction in a critically ill patient comprises the step of administering to the critically ill patient a pharmaceutical composition that further comprises a pharmaceutically acceptable carrier and/or a blood glucose regulator and/or nutrients, e.g. essential nutrients. The pharmaceutical composition used in the method of present invention can be an aqueous liquid composition. The pharmaceutical composition can comprise the polyamine compound of present invention in the range of about 0,05, 0,1, 0,2 or 0,5 % to about 1, 2, 3 or 4 % of the aqueous liquid composition. The pharmaceutical composition can comprise the polyamine compound of present invention in the range of about 0,5 % to about 2 % of the aqueous liquid composition.

The pharmaceutical composition can comprise the polyamine compound of present invention in the range of about 1.0 % to about 1.5 % of the aqueous liquid composition.

The method of present invention can normalize the plasma spermidine level in the critically ill patient. The method of present invention can augment the plasma spermidine level in the critically ill patient to a level that is twice the plasma spermidine level of a healthy person with a similar body weight as the critically ill patient. The method of present invention can augment the plasma spermidine level in the critically ill patient to a level in the range of 50, -6000 nmol/l plasma, or can augment the plasma spermidine level in the critically ill patient by administering daily the polyamine compound in the weight range of 0,01, 0,05, 0,1, 0,2 or 0,5 to 1, 10, 20, 30 or 100 mg per kg body weight. Multiple organ dysfunction can thus be treated or prevented in a critically ill patient, for instance multiple organ dysfunction that is not caused or associated with sepsis.

The polyamine compound can be administered parenterally or enterally to the critically ill patient, or is administered by a bolus injection, e.g. an intravenous bolus injection. The critically ill patient can further receive total parenteral nutrition.

The pharmaceutical composition can be provided as an aqueous liquid composition. Moreover, it is advantageous of the method of present invention that the pharmaceutical composition can be administered parenterally or enterally to the

critically ill patient. In a preferred embodiment, the critically ill patient further receives total parenteral nutrition.

The pharmaceutical composition can be provided to normalize the plasma spermidine level in the critically ill patient.

The pharmaceutical composition can be provided to augment the plasma spermidine level in the critically ill patient to a level that is 1 to 2,5, 4 or even 5 times the plasma spermidine level of a healthy person with a similar body weight as the critically ill patient. In an embodiment, the pharmaceutical composition can be provided to augment the plasma spermidine level in the critically ill patient to a level that is twice the plasma spermidine level of a healthy person with a similar body weight as the critically ill patient.

In one embodiment, the method of present invention can augment the plasma spermidine level in the critically ill patient to a level in the range of 50 to 6000 nmol/l plasma.

In one embodiment, the method of present invention can augment the plasma spermidine level in the critically ill patient to a level that is restoring the plasma spermidine level to that of a healthy person with a similar body weight as the critically ill patient.

In one embodiment, the method of present invention can augment the plasma spermidine level in the critically ill patient to a level in the range of 50 to 6000 nmol/l plasma.

In one embodiment, the method of present invention can augment the plasma spermidine level in the critically ill patient to a level in the range of 100 to 6000 nmol/l plasma.

In one embodiment, the method of present invention can augment the plasma spermidine level in the critically ill patient by administering daily the polyamine compound in the weight range of 0,01 to 100 mg per kg body weight.

In one embodiment, the method of present invention can treat or prevent multiple organ dysfunction in a critically ill patient that is not caused or associated with sepsis.

In one embodiment, the method of present invention can protect a critically ill patient against multiple organ dysfunction by inducing dedifferentiation of adipocytes, e.g. inducing dedifferentiation of adipocytes into new into adipogenic, chondrogenic and osteogenic lineages, which results in reduced size of adipocytes and increased adipose mass.

In one embodiment, the method of present invention can protect a critically ill patient against toxic metabolites by enhancing dedifferentiation of adipocytes and of absorption of toxic metabolites in the adipose tissue protection of a critically ill patient.

In one embodiment, the method of present invention can induce or enhance the dedifferentiation of adipocytes and absorption of toxic metabolites into the protection of the critically ill patent against toxic metabolites.

In one embodiment, the method is used to treat a patient who has been diagnosed as having a paradoxal muscle waste syndrome.

In a particular embodiment, the method is used to treat an animal under a starvation condition, or a critically ill animal, or a critically ill patient. In a particular embodiment, the animal is a fasting animal such as a fasting mammal, more in particular a fasting human.

In a particular embodiment, the method is used to prevent or treat excessive catabolism in a critically ill patient or to reduce morbidity or mortality due to excessive catabolism in a critically ill patient. The treatment can particularly be applied to prevent loss of lean body mass due to critical illness or to prevent mortality due to significant loss of lean body mass in a critically ill patient. Moreover, the treatment is particularly used to induce adipocyte differentiation by a direct action of the polyamine compound on adipocytes for prevention of contradictory adipose mass increase in a critically ill patient.

In another particular embodiment of the invention, the method is used to improve the nitrogen balance in a critically ill patient. The treatment can particularly be applied to increase lean body mass in a critically ill patient. Moreover, the treatment is particularly used to decrease length of time spent on ventilator in a critically ill patient.

In a particular embodiment, the method is used to induce a positive nitrogen balance and lean body mass in an animal in need thereof.

In a particular embodiment, the method is used to induce adipocyte differentiation for reducing the size of adipocytes and to prevent adipose mass increase in an animal in need thereof.

In another particular embodiment of the invention, the method is used to treat a lipid disorder or a dyslipidemia.

The present invention further relates to the use of a polyamine compound of the group consisting of putrescine, (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, or a derivative thereof or a

pharmaceutically acceptable salt, solvate or isomer thereof, such as cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride or combinations thereof, to manufacture a medicament to treat or prevent multiple organ dysfunction in a critically ill patient.

In one embodiment, the use of the polyamine compound of present invention is the use of spermidine or a spermidine analog of the group consisting of spermidine phosphate hexahydrate, spermidine phosphate hexahydrate and L-arginyl-3,4-spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

In a particular embodiment, the use of the polyamine compound of present invention is the use of a polyamine compound of the group consisting of putrescine, spermine, and spermidine.

In a preferred embodiment, the use of the polyamine compound of present invention is the use of spermidine or a pharmaceutically acceptable salt, solvate or isomer thereof, or combinations thereof.

In embodiments of the present invention, the polyamine compound is used to manufacture a medicament to treat or prevent multiple organ dysfunction in a critically ill patient wherein the multiple organ dysfunction is not caused or associated with sepsis.

The polyamine compound is used to manufacture a medicament to treat or prevent multiple organ dysfunction wherein the polyamine compound is administered parenterally or enterally to the critically ill patient, or is administered by a bolus injection, e.g. an intravenous bolus injection.

In a preferred embodiment, the polyamine compound of present invention is used to manufacture a medicament to treat or prevent multiple organ dysfunction wherein the critically ill patient further receives total parenteral nutrition.

In another preferred embodiment, the polyamine compound of present invention is used to manufacture a medicament to treat or prevent multiple organ dysfunction in a critically ill patient receiving parenteral nutrition.

In yet another preferred embodiment, the polyamine compound of present invention is used to manufacture a medicament to treat or prevent multiple organ dysfunction in a critically ill patient with failed or disturbed homeostasis receiving parenteral nutrition.

In a particular embodiment, the polyamine compound of present invention is used to manufacture a medicament to treat or prevent multiple organ dysfunction by inducing adipocytes dedifferentiation.

In a particular embodiment, the polyamine compound of present invention is used to manufacture a medicament to induce dedifferentiation of adipocytes.

In a particular embodiment, the polyamine compound of present invention is used to manufacture a medicament to induce dedifferentiation of adipocytes into new into adipogenic, chondrogenic and osteogenic lineages.

In a particular embodiment, the polyamine compound of present invention is used to manufacture a medicament to induce dedifferentiation of adipocytes resulting in reduced size of adipocytes and increased adipose mass.

In a particular embodiment, the polyamine compound of present invention is used to manufacture a medicament to protect a critically ill patient against toxic metabolites by enhancing dedifferentiation of adipocytes and of absorption of toxic metabolites in the adipose tissue protection of a critically ill patient.

In a particular embodiment, the polyamine compound of present invention is used to manufacture a medicament to induce or enhance the dedifferentiation of adipocytes and absorption of toxic metabolites into the protection of the critically ill patent against toxic metabolites.

Examples of trauma that can lead to MOD that can be treated prophylactically with the present method include surgery and major injuries such as burns, lesions and haemorrhage. The present method is particularly suitable for preventing MOD resulting from surgery, particularly prescheduled surgery. In case of, for instance, prescheduled surgery it is possible to administer the present liquid composition prior to the occurrence of the trauma Administration of the liquid composition prior to the occurrence of the trauma offers the important advantages that the composition can be administered simply by asking the patient to drink it and that the effect will be manifest when the actual trauma occurs.

Usually and preferably, the treatment of a critical ill patent necessitates prolonged minute-to-minute therapy and/or observation, usually and preferably in an intensive care unit (ICU) or a special hospital unit, for example, a post operative ward or the like which is capable of providing a high level of intensive therapy in terms of quality and immediacy.

According to particular embodiments of the present invention, the critical ill patient is selected from the group consisting of a patient in need of cardiac surgery, a patient in need of thoracic surgery, a patient in need of abdominal surgery, a patient in need of vascular surgery, a patient in need of transplantation, a patient suffering from neurological diseases, a patient suffering from cerebral trauma, a patient suffering from

respiratory insufficiency, a patient suffering from abdominal peritonitis, a patient suffering from multiple trauma, a patient suffering from severe burns, a patient suffering from CIPNP and a patient being mechanically ventilated.

In a further preferred embodiment of the present invention, the critical ill patient is a patient suffering from multiple organ dysfunction syndrome (MODS). Patients with life threatening illness are cared for in hospitals in the intensive care unit ("ICU"). These patients may be seriously injured from automobile accidents, etc., have had major surgery, have suffered a heart attack, or may be under treatment for cancer, or other major disease. While medical care for these primary conditions is sophisticated and usually effective, a significant number of patients in the ICU will not die of their primary disease. Rather, a significant number of patients in the ICU die from a secondary complication known commonly as "multiple organ failure". The medical terms for the general terms "sepsis" and "septic shock" are systemic inflammatory response syndrome ("SIRS"), multiple organ dysfunction syndrome ("MODS"), and multiple organ system failure ("MOSF") (collectively "SIRS/MODS/MOSF").

Medical illness, trauma, complication of surgery, and, for that matter, any human disease state, if sufficiently injurious to the patient, may elicit SIRS/MODS/MOSF. The systemic inflammatory response within certain physiologic limits is beneficial. As part of the immune system, the systemic inflammatory response promotes the removal of dead tissue, healing of injured tissue, detection and destruction of cancerous cells as they form, and mobilization of host defenses to resist or to combat infection. If the stimulus to the systemic inflammatory response is too potent, such as massive tissue injury or major microbial infection, however, then the systemic inflammatory response may cause symptoms which include fever, increased heart rate, and increased respiratory rate. This symptomatic response constitutes SIRS. If the inflammatory response is excessive, then injury or destruction to vital organ tissue may result in vital organ dysfunction, which is manifested in many ways, including a drop in blood pressure, deterioration in lung function, reduced kidney function, and other vital organ malfunction. This condition is known as MODS. With very severe or life threatening injury or infection, the inflammatory response is extreme and can cause extensive tissue damage with vital organ damage and failure. These patients will usually die promptly without the use of ventilators to maintain lung ventilation, drugs to maintain blood pressure and strengthen the heart, and, in certain circumstances, artificial support for the liver, kidneys, coagulation, brain and other vital systems. This condition is known as MOSF. These support measures partially compensate for damaged and

failed organs, they do not cure the injury or infection or control the extreme inflammatory response which causes vital organ failures.

Because no effective treatments have been developed so far, MODS is associated with high mortality rates. MODS is no longer viewed as a series of isolated failures. On autopsy, the involved organs display similar patterns of tissue damage although they are often remote from the initial injury site or septic source. This complex syndrome, once thought to be solely related to cardiovascular dysfunction and/or isolated organ failure, is now recognized as a systemic disturbance mediated by a sustained inflammatory response to injury, regardless of the initiating factor(s). MODS attests to the complex interaction between organ systems in both their functioning and pathological states.

Several mechanisms have been postulated to be involved in post-ischemia induction of MODS. The gut-liver-lung axis has been associated to play a dominant role in the incidence and severity of this single and multiple organ dysfunction syndrome (S)MODS. More specifically, the intestine is often referred to as the driving force of MODS. The post-ischemic increase in reactive oxygen species can directly or indirectly (by macrophages and lymphocytes) activate neutrophils that subsequently can infiltrate at the site of inflammation causing tissue injury. These neutrophils have recently also been reported to increase paracellular transport in ileum. This damage of the intestinal barrier has often been mentioned to result in increased trans-epithelial bacterial transport and their endotoxins resulting in an inflammatory challenge of the patient, which has been reported to be involved in the incidence of MODS.

In a specific embodiment, the polyamine of present invention is spermine or spermidine.

When administered to a patient, a polyamine compound is preferably administered as a component of a composition that optionally comprises a pharmaceutically acceptable carrier or vehicle. In one embodiment, these compositions are administered orally. In a preferred embodiment, the polyamine compound of present invention is a component of a pharmaceutical composition that is administered intravenously.

A pharmaceutical composition comprising a polyamine compound of present invention can be administered via one or more routes such as, but not limited to, oral, intravenous infusion, subcutaneous injection, intramuscular, topical, depo injection, implantation, time-release mode, and intracavitary. The pharmaceutical composition is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intramuscular,

intraperitoneal, intracapsular, intraspinal, intrasternal, intratumor, intranasal, epidural, intra-arterial, intraocular, intraorbital, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical-particularly to the ears, nose, eyes, or skin), transmucosal (e.g., oral) nasal, rectal, intracerebral, intravaginal, sublingual, submucosal, and transdermal administration.

Administration can be via any route known to be effective by a physician of ordinary skill. Parenteral administration, i.e., not through the alimentary canal, can be performed by subcutaneous, intramuscular, intra-peritoneal, intratumoral, intradermal, intracapsular, intra-adipose, or intravenous injection of a dosage form into the body by means of a sterile syringe, optionally a pen-like syringe, or some other mechanical device such as an infusion pump. A further option is a composition that can be a powder or a liquid for the administration in the form of a nasal or pulmonary spray. As a still further option, the administration can be transdermally, e.g., from a patch. Compositions suitable for oral, buccal, rectal, or vaginal administration can also be provided. In a preferred embodiment, administration of the polyamine compound of present invention is via an intravenous injection, e.g. an intravenous bolus injection or by gradual perfusion over time.

The polyamine compound and the pharmaceutical composition of present invention can also be administered by a small bolus injection followed by a continuous infusion. One protocol for treatment with spermidine or a spermidine analog is as follows: (i) initial bolus injection over a period of 1-2 minutes; (ii) high level infusion for 1 hour; (2) low level maintenance infusion for 2-3 hours.

The whole of the dose of spermidine required to achieve a protective effect could also be administered as one or more bolus injections, e.g. administered with a 50cc syringe at a rate of 2 ml per hour.

The polyamine compound and the pharmaceutical composition of present invention can also be administered by a small bolus injection followed by a continuous infusion. One protocol for treatment with spermidine or a spermidine analog is as follows: (i) initial bolus injection over a period of 1-2 minutes; (ii) high level infusion for 1 hour; (2) low level maintenance infusion for 2-3 hours.

The whole of the dose of spermidine required to achieve a protective effect could also be administered as one or more bolus injections, e.g. administered with a 50cc syringe at a rate of 2 ml per hour.

In one embodiment, a pharmaceutical composition of the invention is delivered by a controlled release system. For example, the pharmaceutical composition can be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump can be used (See e.g., Langer, 1990, Science 249:1527-33; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald *et al.*, 1980, Surgery 88:507; Saudek *et al.*, 1989, N. Engl. J. Med. 321:574). In another embodiment, the compound can be delivered in a vesicle, in particular a liposome (See e.g., Langer, 1990, Science 249:1527-33; Treat *et al.*, 1989, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-65; Lopez-Berestein, ibid., pp. 317-27; International Patent Publication No. WO 91/04014; U.S. 4,704,355). In another embodiment, polymeric materials can be used (See e.g., Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Press: Boca Raton, Fla., 1974; Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley: New York (1984); Ranger and Peppas, 1953, J. Macromol. Sci. Rev. Macromol. Chem. 23:61; Levy *et al.*, 1985, Science 228:190; During *et al.*, 1989, Ann. Neurol. 25:351; Howard *et al.*, 1989, J. Neurosurg. 71:105).

In yet another embodiment, a controlled release system can be placed in proximity of the target. For example, a micropump can deliver controlled doses directly into bone or adipose tissue, thereby requiring only a fraction of the systemic dose (See e.g., Goodson, 1984, in Medical Applications of Controlled Release, vol. 2, pp. 115-138). In another example, a pharmaceutical composition of the invention can be formulated with a hydrogel (See, e.g., U.S. Pat. Nos. 5,702,717; 6,117,949; 6,201,072).

In one embodiment, it may be desirable to administer the pharmaceutical composition of the invention locally, i.e., to the area in need of treatment. Local administration can be achieved, for example, by local infusion during surgery, topical application (e.g., in conjunction with a wound dressing after surgery), injection, catheter, suppository, or implant. An implant can be of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

In certain embodiments, it may be desirable to introduce the polyamine compound into the central nervous system by any suitable route, including intraventricular, intrathecal, and epidural injection. Intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent, or via perfusion in a fluorocarbon or synthetic pulmonary surfactant.

In one embodiment, the invention provides for the treatment of a patient using implanted cells that have been regenerated or stimulated to proliferate in vitro or in vivo prior to reimplantation or transplantation into a recipient. Conditioning of the cells ex vivo can be achieved simply by growing the cells or tissue to be transplanted in a medium that has been supplemented with a growth-promoting amount of the combinations and is otherwise appropriate for culturing of those cells. The cells can, after an appropriate conditioning period, then be implanted either directly into the patient or can be encapsulated using established cell encapsulation technology, and then implanted.

The skilled artisan can appreciate the specific advantages and disadvantages to be considered in choosing a mode of administration. Multiple modes of administration are encompassed by the invention. For example, a polyamine compound of the invention can be administered by subcutaneous injection, whereas another therapeutic agent can be administered by intravenous infusion. Moreover, administration of one or more species of polyamine compounds, with or without other therapeutic agents, can occur simultaneously (i.e., co-administration) or sequentially. In another embodiment, the periods of administration of a polyamine compound, with or without other therapeutic agents can overlap. For example a polyamine compound can be administered for 7 days and another therapeutic agent can be introduced beginning on the fifth day of polyamine compound treatment. Treatment with the other therapeutic agent can continue beyond the 7-day polyamine compound treatment.

A pharmaceutical composition of a polyamine compound can be administered before, during, and/or after the administration of one or more therapeutic agents. In one embodiment, polyamine compound can first be administered to stimulate the expression of insulin, which increases sensitivity to subsequent challenge with a therapeutic agent. In another embodiment, polyamine compound can be administered after administration of a therapeutic agent. In yet another embodiment, there can be a period of overlap between the administration of the polyamine compound and the administration of one or more therapeutic agents.

A pharmaceutical composition of the invention can be administered in the morning, afternoon, evening, or diurnally. In one embodiment, the pharmaceutical composition is administered at particular phases of the circadian rhythm. In a specific embodiment,

the pharmaceutical composition is administered in the morning. In another specific embodiment, the pharmaceutical composition is administered at an artificially induced circadian state.

The present pharmaceutical compositions can take the form of solutions, suspensions, emulsion, tablets, pills, pellets, capsules, capsules containing liquids, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, or any other form suitable for use. In one embodiment, the pharmaceutically acceptable vehicle is a capsule (See e.g., U.S. 5,698,155).

Pharmaceutical compositions adapted for parenteral administration include, but are not limited to, aqueous and non-aqueous sterile injectable solutions or suspensions, which can contain antioxidants, buffers, bacteriostats and solutes. Other components that can be present in such pharmaceutical compositions include water, alcohols, polyols, glycerine and vegetable oils, for example. Compositions adapted for parenteral administration can be presented in unit-dose or multi-dose containers (e.g., sealed ampoules and vials), and can be stored in a freeze-dried (i.e., lyophilized) condition requiring the addition of a sterile liquid carrier (e.g., sterile saline solution for injections) immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules and tablets.

Pharmaceutical compositions adapted for transdermal administration can be provided as discrete patches intended to remain in intimate contact with the epidermis for a prolonged period of time. Pharmaceutical compositions adapted for topical administration can be provided as, for example, ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays, aerosols or oils. A topical ointment or cream is preferably used for topical administration to the skin, mouth, eye or other external tissues. When formulated in an ointment, the active ingredient can be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient can be formulated in a cream with an oil-in-water base or a water-in-oil base.

Pharmaceutical compositions adapted for topical administration to the eye include, for example, eye drops or injectable pharmaceutical compositions. In these pharmaceutical compositions, the active ingredient can be dissolved or suspended in a suitable carrier, which includes, for example, an aqueous solvent with or without carboxymethylcellulose. Pharmaceutical compositions adapted for topical administration in the mouth include, for example, lozenges, pastilles and mouthwashes.

Pharmaceutical compositions adapted for nasal administration can comprise solid carriers such as powders (preferably having a particle size in the range of 20 to 500 microns). Powders can be administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nose from a container of powder held close to the nose. Alternatively, pharmaceutical compositions adopted for nasal administration can comprise liquid carriers such as, for example, nasal sprays or nasal drops. These pharmaceutical compositions can comprise aqueous or oil solutions of a polyamine compound. Compositions for administration by inhalation can be supplied in specially adapted devices including, but not limited to, pressurized aerosols, nebulizers or insufflators, which can be constructed so as to provide predetermined dosages of the polyamine compound.

Typically, pharmaceutical compositions for injection or intravenous administration are solutions in sterile aqueous buffers. Where necessary, the composition can also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically sealed container such as an ampoule or sachet indicating the quantity of active agent.

Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle, bag, or other acceptable container, containing sterile pharmaceutical grade water, saline, or other acceptable diluents. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

For a patient who cannot orally ingest a nutrient, it is essential to supply all nutrients such as an amino acid, a saccharide and an electrolyte through a vein. This way is called the total parenteral nutrition therapy, (TPN therapy) which can be provided by a TPN solution. Such TPN solutions are particularly suitable for critically ill patients for a therapy in the Intensive Care Unit. As a TPN solution employed in the TPN therapy, there has been known (1) a TPN solution containing a saccharide, an amino acid, a fat and an electrolyte (Japanese Unexamined Patent Publications No. 186822/1989, WO08503002 and EP-A-0 399 341), (2) an emulsion for injection comprising an amino acid and a fat (Japanese Unexamined Patent Publication No. 74637/1986), (3) a TPN solution comprising two separate infusions, one of which contains glucose and an electrolyte and the other of which contains an amino acid (Japanese Unexamined Patent Publications No. 52455/1982 and No. 103823/1986) and the like. In the TPN

therapy, an infusion containing a high concentration of saccharide is usually administered to a patient.

As indicated the high nutritional content of such TPN solutions may lead to hyperglycemia and has been found to have a detrimental effect on the repair processes in critically ill patients by inhibition the autophagy process, which contributes to the removal of damaged organelles.

Accordingly, a further aspect of the present invention relates to a TNP solution combined with a polyamine compound of the present invention. This combined composition is used to improve the condition of a critically ill patient or to reduce or treat multiple organ dysfunction syndrome in a critically ill patient.

Compositions for parenteral nutrition, in particular for intravenous adminstration are isotonic or hypertonic solutions (e.g. prepared by NaCl and/or dextrose or lactated Ringers) further comprising a saccharide such as glucose in a range between 10 and 20 to obtain a high nutritional content, and further comprising lipids and/or amino acids and/or vitamines.

Compositions for parenteral administration comprise further to polyamine compound of the present invention a saccharide such as glucose. Final glucose concentrations in a composition for adminstration are typically in the range from 10 to 20 % (w/v) e.g. 12,5 or 16 %.

Compositions for parenteral administration typically further comprise saturated, mono-unsaturated and essential poly-unsaturated fatty acids such as refined olive oil and/or soybean oil. Final lipid concentrations in a composition for adminstration are typically in the range of 2 to 6 % (w/v) e.g 4 %.

Compositions for parenteral administration typically further comprise one or more amino acids. Final amino acid concentrations are typically in the range from 2 to 6 % (w/v) e.g. 4 %.

Compositions for parenteral administration optionally further comprise trace elements such as one or more of Fe, Zn, Cu, Mn, F, Co, I, Se, Mo, Cr e.g. under the form of respectively the following salts ferrous gluconate, copper gluconate, manganese gluconate,zinc gluconate, sodium fluoride,cobalt II gluconate, sodium iodide,sodium selenite, ammonium molybdate and chromic chloride.

Compositions for parenteral administration optionally further comprise one or more vitamins such as Vitamin A (Retinol), Vitamin D3,Vitamin E (α tocopherol), Vitamin C, Vitamin B1 (thiamine), Vitamin B2 (riboflavin), Vitamin B6 (pyridoxine), Vitamin B12,

Folic Acid, Pantothenic acid, Biotin, and Vitamin PP (niacin), e.g. under the form of Retinol palmitate, Colecalciferol, DL-α-tocopherol, Ascorbic acid, Cocarboxylase tetrahydrate, Riboflavin dihydrated sodium phosphate, Pyridoxine hydrochloride, Cyanocobalamin, Folic acid, Dexpanthenol, D-Biotin and Nicotinamide.

Compositions for parenteral administration prior to adminstration can be isotonic solutions, or more particularly hypertonic solutions e.g. solutions with osmolarity between 1000 and 1500, or between 1200 and 1500 mOsm/liter, eg. 1250 or 1500 mOsm/liter.

Compositions for parenteral administration can be provided as one solution comprisinfg all constituent or as a kit of parts wherein different consituents are provided separately (saccharide, lipids, amino acids) and wherein the polyamine is dissolved in one of the constituent or is provided seperately. One or more of the different constituents may be provided in a dried form, which is redissolved prior to use.

The compositions for parenteral nutrition in accordance with the present invention further comprise a polyamine such as spermine, spermidine or putrescine in a concentration between 0,05, 0,1, 0,2 or 0,5 to 1, 2, 3 or 4 % (w/v).

The compostions for intravenous adminstration are typically packed in plastic bags with spike ports for delivery by intravenous drips.

In a specific embodiment, the present compositions contain spermine or spermidine.

For patients which do not rely on parenteral food pharmaceutical compositions herein described can be provided in the form of oral tablets, capsules, elixirs, syrups and the like.

Compositions for oral administration might require an enteric coating to protect the composition(s) from degradation within the gastrointestinal tract. In another example, the composition(s) can be administered in a liposomal formulation to shield the polyamine compound disclosed herein from degradative enzymes, facilitate the molecule's transport in the circulatory system, and affect delivery of the molecule across cell membranes to intracellular sites.

A polyamine compound intended for oral administration can be coated with or admixed with a material (e.g., glyceryl monostearate or glyceryl distearate) that delays disintegration or affects absorption of the polyamine compound in the gastrointestinal tract. Thus, for example, the sustained release of a polyamine compound can be achieved over many hours and, if necessary, the polyamine compound can be protected from being degraded within the gastrointestinal tract. Taking advantage of

the various pH and enzymatic conditions along the gastrointestinal tract, pharmaceutical compositions for oral administration can be formulated to facilitate release of a polyamine compound at a particular gastrointestinal location.

Selectively permeable membranes surrounding an osmotically active driving compound are also suitable for orally administered compositions. Fluid from the environment surrounding the capsule is imbibed by the driving compound, which swells to displace the polyamine compound through an aperture, can provide an essentially zero order delivery profile instead of the spiked profiles of immediate release formulations. A time delay material such as, but not limited to, glycerol monostearate or glycerol stearate can also be used.

Suitable pharmaceutical carriers also include starch, glucose, lactose, sucrose, gelatin, saline, gum acacia, talc, keratin, urea, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, and ethanol. If desired, the carrier, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. In addition, auxiliary, stabilizing, thickening, lubricating, and coloring agents may be used. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

For oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic, pharmaceutically acceptable, inert carrier such as, but not limited to, lactose, starch, sucrose, glucose, methyl cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, mannitol, and sorbitol. For oral administration in liquid form, the oral drug components can be combined with any oral, non-toxic, pharmaceutically acceptable carrier such as, but not limited to, ethanol, glycerol, and water. Moreover, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include, but are not limited to, starch, gelatin, natural sugars (e.g., glucose, beta-lactose), corn sweeteners, natural and synthetic gums (e.g., acacia, tragacanth, sodium alginate), carboxymethylcellulose, polyethylene glycol, and waxes. Lubricants useful for an orally administered drug, include, but are not limited to, sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, and sodium chloride. Disintegrators include, but are not limited to, starch, methyl cellulose, agar, bentonite, and xanthan gum.

Pharmaceutical compositions adapted for oral administration can be provided, for example, as capsules or tablets; as powders or granules; as solutions, syrups or

suspensions (in aqueous or non-aqueous liquids); as edible foams or whips; or as emulsions. For oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic, pharmaceutically acceptable, inert carrier such as, but not limited to, lactose, starch, sucrose, glucose, methyl cellulose, magnesium stearate, dicalcium phosphate, magnesium carbonate, stearic acid or salts thereof, calcium sulfate, mannitol, and sorbitol. For oral administration in the form of a soft gelatin capsule, the active drug component can be combined with an oral, non-toxic, pharmaceutically acceptable, inert carrier such as, but not limited to, vegetable oils, waxes, fats, semi-solid, and liquid polyols. For oral administration in liquid form, the oral drug components can be combined with any oral, non-toxic, pharmaceutically acceptable carrier such as, but not limited to, ethanol, glycerol, polyols, and water. Moreover, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include, but are not limited to, starch, gelatin, natural sugars (e.g. glucose, beta-lactose), corn sweeteners, natural and synthetic gums (e.g., acacia, tragacanth, sodium alginate), carboxymethylcellulose, polyethylene glycol, and waxes. Lubricants useful for an orally administered drug, include, but are not limited to, sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, and sodium chloride. Disintegrators include, but are not limited to, starch, methyl cellulose, agar, bentonite, and xanthan gum.

Orally administered compositions may contain one or more agents, for example, sweetening agents such as, but not limited to, fructose, aspartame and saccharin. Orally administered compositions may also contain flavoring agents such as, but not limited to, peppermint, oil of wintergreen, and cherry. Orally administered compositions may also contain coloring agents and/or preserving agents.

The polyamine compounds of present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines. A variety of cationic lipids can be used in accordance with the invention including, but not limited to, N-(1(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTMA") and diolesylphosphotidylethanolamine ("DOPE"). Such compositions suit the mode of administration.

The polyamine compounds of present invention can also be delivered by the use of monoclonal antibodies as individual carriers to which the compounds can be coupled.

The compounds can also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinylpyrrolidone, pyran copolymer, polyhydroxypropylmethacrylamide-phenol, polyhydroxyethylaspartamide-phenol, or polyethyleneoxide-polylysine substituted with palmitoyl residues. Furthermore, the polyamine compounds can be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyglycolic acid, copolymers of polylactic and polyglycolic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacrylates and cross linked or amphipathic block copolymers of hydrogels.

Pharmaceutical compositions adapted for rectal administration can be provided as suppositories or enemas. Pharmaceutical compositions adapted for vaginal administration can be provided, for example, as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

Suppositories generally contain active ingredients in the range of 0,5% to 10% by weight. Oral formulations preferably contain 10% to 95% active ingredient by weight. In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intratumoral injection, implantation, subcutaneous injection, or intravenous administration to humans.

Conveniently, the blood spermidine level is kept within the ranges mentioned in connection with the present invention for as long a period of time as the patient is critically ill. Hence, as a general rule, the blood spermidine level is kept within the ranges mentioned in connection with the present invention as long as the patient is critically ill. Consequently, the blood spermidine level is usually kept within the ranges mentioned in connection with the present invention for a period of time of more than about 8 hours, preferably more than about 24 hours, even more preferred more than about 2 days, especially more than about 4 days, and even more than about 7 days. In certain cases, it may even be preferred that the blood spermidine level is kept within the ranges mentioned in connection with the present invention after the patient (previously) considered as being critically ill has been transferred from the Intensive Care Unit to another part of the hospital or even after the patient has left the hospital.

A critically ill patient, optionally entering an ICU, may be fed continuously, on admission with mainly intravenous glucose (for example, about 200 g to about 300 g per 24 hours) and from the next day onward with a standardised feeding schedule aiming for a caloric content up to between about 10 and about 40, preferably between about 20 and about 30, non-protein Calories/kg/24 hours and a balanced composition (for example,

between about 0,05 and about 0,4, preferably between about 0,13 and about 0,26, g nitrogen/kg/24 hours and between about 20% and about 40% of non-protein Calories as lipids) of either total parenteral, combined parenteral/enteral or full enteral feeding, the latter mode attempted as early as possible. Other concomitant ICU therapy can be left to the discretion of attending physicians.

Alternatively, the following procedure can be used or it is possible to use a combination or variant of these procedures, as the physician considers advantageous for the patient:

A critically ill patient may be fed, on the admission day, using, for example, a 20% glucose infusion and from day 2 onward by using a standardised feeding schedule consisting of normal caloric intake (for example, about 25-35 Calories/kgBW/24 h) and balanced composition (for example, about 20%-40% of the non-protein Calories as lipids and about 1-2 g/kgBW/24h protein and about 0,01-100 mg/kg spermidine BW/24h) of either total parenteral, combined parenteral/enteral or full enteral feeding, the route of administration of feeding depending on assessment of feasibility of early enteral feeding by the attending physician. All other treatments, including feeding regimens, were according to standing orders currently applied within the ICU.

The polyamine compound and optionally another therapeutic agent are administered at an effective dose. The dosing and regimen most appropriate for patient treatment will vary with the disease or condition to be treated, and in accordance with the patient's weight and with other parameters.

An effective dosage and treatment protocol can be determined by conventional means, comprising the steps of starting with a low dose in laboratory animals, increasing the dosage while monitoring the effects (e.g., histology, disease activity scores), and systematically varying the dosage regimen. Several factors may be taken into consideration by a clinician when determining an optimal dosage for a given patient. Additional factors include, but are not limited to, the size of the patient, the age of the patient, the general condition of the patient, the particular disease being treated, the severity of the disease, the presence of other drugs in the patient, and the in vivo activity of the polyamine compound.

A typical effective human dose of a polyamine compound would be from about 10 µg/kg body weight/day to about 100 mg/kg/day, preferably from about 50 µg/kg/day to about 50 mg/kg/day, and most preferably about 100 µg/kg/day to 20 mg/kg/day. As analogs of the polyamine compound disclosed herein can be 2 to 100 times more potent than naturally occurring counterparts, a typical effective dose of such an analog

can be lower, for example, from about 100 ng/kg body weight/day to 1 mg/kg/day, preferably 10 µg/kg/day to 900 µg/kg/day, and even more preferably 20 µg/kg/day to 250 µg/kg/day.

In another embodiment, the effective dose of a polyamine compound of present is less than 10 µg/kg/day. In yet another embodiment the effective dose of a polyamine compound of present is greater than 10 mg/kg/day.

The specific dosage for a particular patient, of course, has to be adjusted to the degree of response, the route of administration, the patient's weight, and the patient's general condition, and is finally dependent upon the judgment of the treating physician. Especially the highly critical condition of ICU patients requires a specific dosage and dosage regime.

It is understandable that the ideal dosage per serving to have the health effect will have to vary according the body weight of the subject who consumes the oral ingestible dosage form which comprises the polyamine compound of present invention. A beneficial effect can be obtained in a subject with about 50 kg body weight by an orally ingestible dosage form comprising between 5 mg and 2,5 gram, preferably 15 mg to 2 gram, more preferably between 25 mg and 1,5 gram, more preferably between 50 mg and 750 mg of the polyamine compound of present invention per administration (as demonstrated in Table 1).

Table 1. Possible amount of the polyamine compound active ingredient of present invention per serving by a subject (BW: body weight).

	BW/kg									
	50	60	70	80	90	100	110	120	130	140
Dose mg/kg										
	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
0,1	5	6	7	8	9	10	11	12	13	14
0,2	10	12	14	16	18	20	22	24	26	28
0,3	15	18	21	24	27	30	33	36	39	42
0,4	20	24	28	32	36	40	44	48	52	56
0,5	25	30	35	40	45	50	55	60	65	70
1	50	60	70	80	90	100	110	120	130	140
5	250	300	350	400	450	500	550	600	650	700
10	500	600	700	800	900	1000	1100	1200	1300	1400
15	750	900	1050	1200	1350	1500	1650	1800	1950	2100
20	1000	1200	1400	1600	1800	2000	2200	2400	2600	2800
25	1250	1500	1750	2000	2250	2500	2750	3000	3250	3500
30	1500	1800	2100	2400	2700	3000	3300	3600	3900	4200

35	1750	2100	2450	2800	3150	3500	3850	4200	4550	4900
40	2000	2400	2800	3200	3600	4000	4400	4800	5200	5600
45	2250	2700	3150	3600	4050	4500	4950	5400	5850	6300
50	2500	3000	3500	4000	4500	5000	5500	6000	6500	7000

A beneficial effect can also be obtained in a subject with about 50 kg body weight as part of a TPN therapy comprising between 5 mg and 2,5 gram, preferably 15 mg to 2 gram, more preferably between 25 mg and 1,5 gram, more preferably between 50 mg and 750 mg of the polyamine compound of present invention per administration.

Also contemplated are methods of prevention or treatment involving combination therapies comprising administering an effective amount of the polyamine compound molecule of present invention can be in combination with another therapeutic agent or agents. The other therapeutic agent or agent can be, for example, an anti-osteoporosis agent, a steroid hormones, a non-steroid hormone, growth factor, a selective estrogen receptor modulator, an insulin-releasing agent, an inhibitor of glucagon secretion, a glucagon antagonists, a circadian rhythm regulator, a growth hormone secretagogue, an agent that increase IGF-1 levels, an immunotherapeutic agent, a cytokine, a protease inhibitor, a vitronectin receptor antagonist, a bisphosphonate compound, a kinase inhibitor, an integrin receptor or antagonist thereof, an anti-obesity agent, a lipid-metabolism improving agent, a neuropeptide Y blocker, a kainate/AMPA receptor antagonist, a β-adrenergic receptor agonist, a compound that reduces caloric intake, an anti-diabetes agent, or a dietary nutrient. Examples of therapeutic agents include, but are not limited to:

- anti-osteoporosis agent, such as alendronate sodium, calcium L-threonate (e.g., C8H14O10Ca) clodronate, etidronate, gallium nitrate, mithramycin, norethindrone acetate (e.g., that which is commercially available as ACTIVELLA) osteoprotegerin pamidronate and risedronate sodium.
- steroid hormones, such as androgen (e.g., androstenedione, testosterone, dehydroepiandrosterone, dihydrotestosterone, 7-alpha-methyl-19-nortestosterone, 7-alpha-methyl-19-nortestosterone acetate, methandroil, oxymetholone, methanedione, oxymesterone, nordrolone phenylpropionate, noretbandrolone), glucocorticoids, estrogenic hormones (e.g., that which is commercially available as PREMARIN) and progestin
- non-steroid hormones such as calcitonin, calcitriol growth hormone (e.g., osteoclast-activating factor), melatonin, parathyroid hormone prostaglandin, thyroid hormone.

- growth factors such as epidermal growth factor, fibroblast growth factor, insulin-like growth factor 1, insulin-like growth factor 2, platelet-derived growth factor, vascular endothelial growth factor

- selective estrogen receptor modulator such as, BE-25327, CP-336156, clometherone, delmadinone, droloxifene, idoxifene, nafoxidine, nitromifene, ormeloxifene, raloxifene (e.g., that which is commercially available as EVISTA), tamoxifen, toremifene, trioxifene, [2-(4-hydroxyphenyl)-6-hydroxynaphthalen-1-yl][4-[2-(1-piperidinyl)-ethoxy]phenyl]-methane.

-Insulin-releasing agent such as GLP-1, nateglinide, repaglinide (e.g., that which is commercially available as PRANDIN), sulfonylurea (e.g., glyburide, glipizide, glimepiride)

- vasopressin

- inhibitor of glucagon secretion, such as somatostatin, glucagon antagonists, substituted glucagons having an alanine residue at position 1, 2, 3-5, 9-11, 21, or 29, des-His1-Ala2 glucagons, des-His1-[Ala2,11-Glu21]glucagon,

- circadian rhythm regulators such as alkylene dioxybenzene agonist, melatonin, neuropeptide Y, tachykinin agonist, visible light therapy, growth hormone secretagogue, cycloalkano[b]thien-4-ylurea, GHRP-1, GHRP-6, growth hormone releasing factor, hexarelin, thiourea, B-HT920, benzo-fused lactams (e.g., N-biphenyl-3-amido substituted benzolactams), benzo-fused macrocycles (e.g., 2-substituted piperidines, 2-substituted pyrrolidines, 2-substituted hexahydro-1H-azepines, di-substituted piperidines, di-substituted pyrrolidines, di-substituted hexahydro-1H-azepines, tri-substituted piperidines, tri-substituted pyrrolidines, tri-substituted hexahydro-1H-azepines, L-pyroglutamyl-pyridylalanyl-L-prolinamides)

- agents that increase IGF-1 levels such as L-acetylcamitine, L-isovalerylcamitine, L-propionylcarnitine.

- immunotherapeutic agents such as antibodies and immunomodulators

- cytokine such as endothelial monocyte activating protein, granulocyte colony.

- stimulating factor, such as interferon (e.g., IFN-γ), interleukin (e.g., IL-6).

- lymphokine such as, lymphotoxin-α, lymphotoxin-β.

- tumor necrosis factor, tumor necrosis-factor-like cytokine, macrophage inflammatory protein, monocyte colony stimulating factor, 4-1BBL, CD27 ligand, CD30 ligand, CD40 ligand, CD137 ligand, Fas ligand, OX40 ligand,

- protease inhibitors such as cysteine protease inhibitor (e.g., vinyl sulfone, peptidylfluoromethyl ketone, cystatin C, cystatin D, E-64), DPP IV antagonist, DPP IV

inhibitor (e.g., N-(substituted glycyl)-2-cyanopyrrolidines, N-Ala-Pro-Onitrobenzyl-hydroxylamine, and ε-(4-nitro)benzoxycarbonyl-Lys-Pro), serine-protease inhibitor (e,g., azapeptide, BMS232632, antipain, leupeptin),

- vitronectin receptor antagonist, anti-vitronectin receptor antibody (e.g., 23C6), cyclo-S,S-N α-acetyl-cysteinyl-N alpha-methyl-argininyl-glycyl-aspartyl penicillamine, RGD-containing peptide (e.g., echistatin), bisphosphonate compound, alendronate (e.g., that which is commercially available as FOSAMAX), aminoalkyl bisphosphonate, (e.g., alendronate, pamidronate (3-amino-1-hydroxypropylidene)bisphosphonic acid disodium salt, pamidronic acid, risedronate (1-hydroxy-2-(3-pyridinyl)ethylidene)bisphosphonate, YM 175 [(cycloheptylamino)methylene-bisphosphonic acid], piridronate, aminohexane-bisphosphonate, tiludronate, BM-210955, CGP-42446, EB-1053), risedronate (commercially available as ACTONEL).

- kinase inhibitors, such as Rho-kinase inhibitor (e.g., (+)-trans-4-(1-aminoethyl)-1-(4-pyridylcarbamoyl)cyclohexane, trans-N-(1H-pyrrolo[2,3-b]pyridin-4-yl)-4-guanidino-methylcyclohexanecarbox amide, 1-(5-isoquinolinesulfonyl)homopiperazine, 1-(5-isoquinolinesulfonyl)-2- methylpiperazine).

- integrin receptor, α subunit (e.g., subtype 1-9, D, M, L, X, V, IIb, IELb), β subunit (e.g., subtype 1-8)

- integrin receptor antagonists, ethyl 3(S)-(2,3-dihydro-benzofuran-6-yl)-3-{2-oxo-3-[3-(5,6,7,8-tetrahydro-[1,8]naphthyridin-2-yl)-propyl]-tetrahydro-pyrimidin-1-yl}-propionate; ethyl 3(S)-(3-fluorophenyl)-3-(2-oxo-3(S or R)-[3-(5,6,7,8-tetrahydro-[1,8]naphthyridin-2-yl)-propyl]-piperidin-1-yl)-propionate; ethyl 3(S)-(3-fluorophenyl)-3-(2-oxo-3® or S)-[3-(5,6,7,8-tetrahydro-[1,8]naphthyridin-2-yl)-propyl]-piperidin-1-yl)-propio nate; 3(S)-(2,3-dihydro-benzofuran-6-yl)-3-{2-oxo-3-[3-(5,6,7, 8-tetrahydro-[1,8]n aphthyridin-2-yl)-propyl]-tetrahydro-pyrimidin-1-yl}-propionic acid; 3(S)-(3-fluorophenyl)-3-(2-oxo-3® or R)-[3-(5,6,7,8-tetrahydro-[1,8]naphthyridin-2-yl)-propyl]-piperidin-1-yl)-propionic acid; 3(S)-(3-fluorophenyl)-3-(2-oxo-3(S or S)-[3-(5,6,7,8-tetrahydro-[1,8]naphthyridin-2-yl)-propyl]-piperidin-1-yl)-propionic acid.

- anti-obesity agents such as benzphetamine (commercially available as DIDREX), benzylisopropylamine (commercially available as IONAMIN), bupropion, dexfenfluramine (commercially available as REDUX), dextroamphetamine (commercially available as DEXEDRINE), diethylpropion (commercially available as TENUATE), dimethylphenethylamine (commercially available as ADIPEX or DESOXYN), evodamine, fenfluramine (commercially available as PONDIMIN), fluoxetine, mazindol (commercially available as SANOREX or MAZANOR),

methamphetamine, naltrexone, orlistat (commercially available as XENICAL), phendimetrazine (commercially available as BONTRIL or PLEGINE), phentermine (commercially available as FASTIN), sibutramine (commercially available as MERIDIA), a lipid-metabolism improving agent, capsaicin, an neuropeptide Y blocker, NGD-95-1, kainate/AMPA receptor antagonist, β-adrenergic receptor agonist, compound that reduces caloric intake, fat substitute (e.g., that which is commercially available as OLESTRA), sugar substitute (e.g., that which is commercially available as ASPARTAME), anti-diabetes agent, insulin glargine (commercially available as LANTUS), pioglitazone (commercially available as ACTOS), rosiglitazone maleate (commercially available as AVANDIA),

- dietary nutrients such as sugar; dietary fatty acid, triglyceride, oligosaccharides (e.g., fructo-oligosaccharides, raffinose, galacto-oligosaccharides, xylo-oligosaccharides, beet sugar and soybean oligosaccharides), protein, vitamin (e.g., vitamin D), mineral (e.g., calcium, magnesium, phosphorus and iron)

The other therapeutic agents can be made and used at doses as disclosed previously. For example, an anti-osteoporosis agent (see e.g., U.S. Pat. Nos. 2,565,115 and 2,720,483), a non-steroid hormone (see, e.g., U.S. Pat. Nos. 6,121,253; 3,927,197; 6,124,314), a glucagon antagonists (see, e.g., U.S. 5,510,459), a growth hormone secretagogue (see, e.g., U.S. Pat. Nos. 3,239,345; 4,036,979; 4,411,890; 5,206,235; 5,283,241; 5,284,841; 5,310,737; 5,317,017; 5,374,721; 5,430,144; 5,434,261; 5,438,136; 5,494,919; 5,494,920; and 5,492,916; European Patent Nos. 144,230 and 513,974; International Patent Publication Nos. WO 89/07110; WO 89/07111; WO 93/04081; WO 94/07486; WO 94/08583; WO 94/11012; WO 94/13696; WO 94/19367; WO 95/03289; WO 95/03290; WO 95/09633; WO 95/11029; WO 95/12598; WO 95/13069; WO 95/14666; WO 95/16675; WO 95/16692; WO 95/17422; WO 95/17423; WO 95/34311; and WO 96/02530), an agent that increase IGF-1 levels (see, e.g., U.S. 6,166,077), a cytokine (see, e.g., U.S. 4,921,697), a vitronectin receptor antagonist (see e.g., U.S. 6,239,138 and Horton *et al.*, 1991, Exp. Cell Res. 195:368), a bisphosphonate compound (see e.g., U.S. 5,409,911), a kinase inhibitor (U.S. 6,218,410), and an integrin receptor or antagonist thereof (see, e.g., U.S. 6,211,191).

Examples

The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appending claims. This invention is not limited to the particular methodology, protocols, delivery forms and reagents described as these may vary.

Example 1: Animal model for critical illness

Our research group previously developed an animal model of critical illness that has shown to mimick the dynamic endocrine, immunological and metabolic changes characteristic of human critical illness. In this animal model we investigated the effect of critical illness on spermidine levels and the effects of spermidine-administration during critical illness on survival, organ function (clinical, biochemical, and cyto/histopathological effects), and on metabolic, inflammatory/immunological and cellular pathways. Animals were treated according to the "Principals of Laboratory Animal Care" formulated by the U.S. National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Institutes of Health. The protocol was approved by the K.U.Leuven Ethical Review Board for Animal Research. Adult male New Zealand White rabbits, weighing approximately 3 kg, were purchased from a local rabbitry, were housed individually with free access to water, hay and regular rabbit chow, and were exposed to artificial light for 14 h per day. This animal model of prolonged critical illness mimicks the human condition (Weekers F, *et al.* Endocrinol (2003) 144: 5329-5338). Indeed, the critically ill animals undergo the same metabolic, immunological and endocrine disturbances and development of organ failure and muscle wasting as the human counterpart. In this animal model, we previously demonstrated that parenteral feeding may be important in improving overall outcome (Derde *et al.* Crit Care Med 2009). Compared to starvation, a small dose of parenteral feeding in critically ill animals decreased muscle catabolism and did not induce significant lethality. A higher dose of parenteral feeding however holds risk of death, which thus reflects a trade-off for improved muscle preservation. As soon as hyperglycemia is allowed to develop, a higher lethality precludes any benefit from parenteral feeding (Derde *et al.* Crit Care Med 2009, in press).

Indeed, parenteral feeding has also disadvantages, one of which is development of hyperglycemia, which, if left untreated, leads to increased mortality, multiple organ failure and muscle breakdown. Our previous research indicates that even brief cellular

hyperglycemia and nutrient overload exerts direct toxic cellular effects in the setting of critical illness, leading to these disastrous effects (Van den Berghe G, *et al.* N Engl J Med 2001; 345: 1359-1367, Van den Berghe G, *et al.* N Engl J Med 2006; 354: 449-461, Vlasselaers D, *et al.* Lancet 2009; 373: 547-556, Ellger B, *et al.* Diabetes 2006; 55: 1096-1105, Vanhorebeek I, *et al.* Lancet 2005; 365: 53-59, Vanhorebeek I, *et al.* Crit Care Med 2009; 37: 1355-1364 and Vanhorebeek I *et al.* Kidney Int 2009; 76: 512-520). Prevention of hyperglycemia in the critically ill, however, has shown to be difficult to achieve (Finfer *et al.* N Engl J Med 2009; 360: 1283-1297), specifically since there is a risk of hypoglycemia, which could counteract any benefit.

We now demonstrate here that such lethal effects of parental feeding and hyperglycemia in critically ill animals can be abrogated by administration of spermidine.

Example 2: Induction of critical illness in a rabbit animal model

In our animal model, critical illness was induced by placing intravascular catheters, selectively destroying pancreatic β-cells by alloxan, followed by burn injury. As mentioned, this model revealed the dynamic endocrine and metabolic changes characteristic of human critical illness, including hyperglycemia and endogenous insulin deficiency. Alloxan is a toxic glucose analogue, which selectively destroys insulin-producing cells in the pancreas when administered to rodents and many other animal species. The administration of alloxan was necessary to control both blood glucose and plasma insulin levels independently. The application of the burn wound is done 48 hours after alloxan-injection, at which time alloxan has done irreversible damage to the β-cells (selective β-cell necrosis, phase 4 after alloxan-injection). After imposing a burn wound, animals were brought to hyperinsulinaemia, because this reflects most the human situation of critical illness. Non-injured, healthy rabbits served as control.

At 09:00 ± 1 h of Day -2 (Fig. 1), animals were weighed, and randomized into three groups by sealed envelopes: group 1 (Burn/Hyperglycemia), group 2 (Burn/Normoglycemia), and group 3 (Control). The protocol was designed to reach at least a target of eight rabbits per group surviving until day 7. Under general anesthesia (30 mg/kg ketamine i.m. [Imalgene 1000; Merial, Lyon, France]; 0,15 ml/kg medetomidine i.m. [Orion, Espoo, Finland]), an ice-cold 10% solution of alloxan-monohydrate (150 mg/kg; Alloxan; Sigma-Aldrich, Bornem, Belgium) was injected slowly via a marginal ear vein. Afterward, the animals had free access to regular rabbit chow and drinking water enriched with glucose to face alloxan-induced acute

hypoglycemia. Control rabbits were left untouched in the cage, had free access to regular rabbit chow, hay and received water and hay ad libitum.

At 10:00 ± 1 h of Day 0 (Fig. 1), glycemia was measured in the burn groups to confirm hyperglycemia after alloxan (irreversible phase 4 after alloxan-injection). When glycemia exceeded 300 mg/dl, animals were considered eligible for the study. Under general anesthesia (see above) supplemented with 1.5 volume % isoflurane (Isoba Vet.; Schering-Plough, Brussels, Belgium) inhalation, animals were shaved and catheters were placed into the right jugular vein for intravenous infusion (4F; Vygon, Ecouen, France) and into the right carotid artery for blood sampling (5 Ch; Sherwood Medical, Tullamore, Ireland). A paravertebral block (5 ml Xylocaine 1%; Astra Zeneca, Brussels, Belgium) was performed and a full thickness burn injury of 20% body-surface area was imposed. Animals were then fitted to a homemade jacket to secure catheters and immediately returned to their cages. Continuous fluid resuscitation (16 ml/h Hartmann solution [Baxter, Lessiness, Belgium] supplemented with 25 g glucose/500 ml) was started via a volumetric pump (Infusomat secura; B.Braun, Melsungen, Germany) using a homemade swiffle device to allow free moving in the cage. Insulin (Actrapid; Novo Nordisk, Begsvaerd, Denmark) was continuously administered intravenously via a syringe pump (Perfusor secura; B.Braun), at a minimum dose of 4U/kg/24h. The two preset levels of blood glucose were achieved by adjusting a continuous glucose infusion (50% glucose via a syringe pump; Baxter) supplementing basal glucose intake (fig 2). Glycemic target was 80-110 mg/dl in the normoglycemic group and 300-315 mg/dl in the hyperglycemic group. Burn-injured animals were deprived of regular rabbit chow and received water and hay ad libitum. In the evening, a supplementary dose of piritramide was given subcutaneously (0,2 mg/kg Dipidolor; Janssen-Cilag, Beerse, Belgium). Control rabbits were left untouched in the cage until day 7, had free access to regular rabbit chow, and received water and hay ad libitum.

Example 3: Measurement of spermidine levels in critically ill rabbits

At 13:00 1 h of Day 1. (Fig. 2), Hartmann solution was replaced by parenteral nutrition infused at 10 ml/h. We chose total intravenous nutrition because this is the only way to assure equal nutrient intake of the rabbits. Parenteral nutrition contained 35% Clinomel N7 (Baxter; Clinitec, Maurepas Cedex, France), 35% Hartmann solution, and 30% glucose 50%. All intravenous infusions were prepared daily under sterile conditions and weighed before and after administration for exact quantification of intake.

Parenteral nutrition was changed daily at 13:00 ± 1 h of Days 2–7 (Fig. 2) at which time the amount of parenteral nutrition and supplementary glucose, and the amount of insulin given was recorded.

At 14:00 ± 1 h of Day 7 (Fig. 2), animals were anesthetized using half of the above mentioned dose of anesthetics intravenously, and the animals were weighed. After tracheostomy, animals were normoventilated (small animal ventilator KTR4; Hugo Sachs, March-Hugstetten, Germany). Anesthesia was supplemented with 1.5 volume % isoflurane inhalation and 0,15 mg/kg piritramid i.v. Arterial blood pressure and central venous pressure (CVP) were monitored from the indwelling lines. Animals were sacrificed by cutting out the heart.

Peroperative on day -2 (before injecting alloxan), day 0 (after placing catheters), and thereafter daily at 09:00 ± 1h, arterial blood was sampled and immediately analyzed on a blood gas analyzer (ABL725, Radiometer Copenhagen, Denmark) to quantitate pH, hemoglobin, electrolytes, lactate, and glucose. After imposing the burn wound, glucose was measured minimum four times daily, and additional glucose measurements were carried out whenever blood glucose was unstable to allow tight adjustment of glucose/insulin infusion (Fig. 2). Supplementary, on day -2 (before injecting alloxan), day 0 (after placing catheters), and thereafter daily at 09:00 ± 1h, 3ml blood was collected and centrifuged for 10 min at 10,000 rpm, and plasma was stored at -80°C until further analysis. Healthy control rabbits underwent only 1 blood sampling (for blood gas analysis and plasma storage), i.e. before induction of anesthesia on day 7. Sampling was performed by puncturing the central ear artery. Urine was collected in a bucket under every cage. Daily at 13:00 ± 1 h, 3 ml urine was sampled and stored at -80°C until further analysis, and total urinary volume was recorded. On day 7, all organs were sampled under anesthesia and stored at -80°C until further analysis.

48 hours after alloxan injection, all animals had glucose levels >300 mg/dl, confirming permanent diabetic status. At day 7, spermidine levels in plasma of critically ill rabbits were significantly different compared to spermidine levels in plasma of healthy control rabbits (Fig. 9, Fig. 10). Day 7 spermidine levels of hyperglycemic rabbits were significantly different than levels of normoglycemic counterparts. Likewise, spermidine levels in tissue were significantly different in critically ill rabbits, compared to healthy controls. Hyperglycemic rabbits had significantly different tissue spermidine levels than normoglycemic rabbits.

Example 4: Effects of spermidine administration in critical illness

The effects of spermidine administration were investigated in parentally fed, hyperglycemic, burn-injured rabbits. The rabbits were purchased from a local rabbitry and weighed approximately 3-3,5kg. The burn injury experiments were performed analoguous to those described above. After imposing the burn wound, animals received a continuous infusion of saline or spermidine (low dose ranging from 0,3-3mg/day or high dose ranging from 30-300mg/day). Hence, we tested a dose range of spermidine approximately from 0,01 to 100mg/kg/day.

At 09:00 ± 1 h of Day -1 (Fig. 3) animals were weighed, and under general anesthesia (30 mg/kg ketamine i.m. [Imalgene 1000; Merial, Lyon, France]; 0,15 ml/kg medetomidine i.m. [Orion, Espoo, Finland]), an ice-cold 10% solution of alloxan-monohydrate (150 mg/kg; Alloxan; Sigma-Aldrich, Bornem, Belgium) was injected slowly via a marginal ear vein. Afterward, the animals had free access to regular rabbit chow, hay and drinking water enriched with glucose to face alloxan-induced acute hypoglycemia.

At 10:00 ± 1 h of Day 0 (Fig. 3), glycemia was measured to confirm hyperglycemia after alloxan (irreversible phase 4 after alloxan-injection). When glycemia exceeded 300 mg/dl, animals were considered eligible for the study. Under general anesthesia (see above), supplemented with 1.5 volume % isoflurane (Isoba Vet.; Schering-Plough, Brussels, Belgium) inhalation, animals were shaved and catheters were placed into the right jugular vein for intravenous infusion (4F; Vygon, Ecouen, France) and into the right carotid artery for blood sampling (5 Ch; Sherwood Medical, Tullamore, Ireland). A paravertebral block (5 ml Xylocaine 1%; Astra Zeneca, Brussels, Belgium) was performed and a full thickness burn injury of 20% body-surface area was imposed. Animals were then fitted to a homemade jacket to secure catheters and immediately returned to their cages. The animals were then randomized into six groups by sealed envelopes: group A (Spermidine 300mg/day), group B (Spermidine 100mg/day), group C (Spermidine 30mg/day), group D (Spermidine 3mg/day), group E (Spermidine 0,3mg/day), or group F (Saline). The vials containing spermidine and saline were prepared in a sterile way by the hospital pharmacy, and the investigators were blinded to what the animals received.

Continuous fluid resuscitation (16 ml/h Hartmann solution [Baxter, Lessiness, Belgium] supplemented with 25 g glucose/500 ml) was started via a volumetric pump (Infusomat secura; B.Braun, Melsungen, Germany) using a homemade swivel device to allow free moving in the cage. Insulin (Actrapid; Novo Nordisk, Begsvaerd, Denmark) was continuously administered intravenously via a syringe pump (Perfusor secura;

B.Braun), at a minimum dose of 2U/kg/24h. The preset levels of blood glucose were achieved by adjusting a continuous glucose infusion (50% glucose via a syringe pump; Baxter) supplementing basal glucose intake (fig 2). Glycemic target was 300-315 mg/dl. Animals were deprived of regular rabbit chow and received water and hay ad libitum. Animals received a continuous infusion of saline or spermidine via a syringe pump. In the evening, a supplementary dose of piritramide was given subcutaneously (0,2 mg/kg Dipidolor; Janssen-Cilag, Beerse, Belgium).

Example 5: Effects of spermidine administration in critical illness

At 13:00 ± 1 h of Day 1 (Fig. 4), Hartmann solution was replaced by parenteral nutrition infused at 10 ml/h. We chose total intravenous nutrition because this is the only way to assure equal nutrient intake of the rabbits. Parenteral nutrition contained 35% Clinomel N7 (Baxter; Clinitec, Maurepas Cedex, France), 35% Hartmann solution, and 30% glucose 50%. All intravenous infusions were prepared daily under sterile conditions and weighed before and after administration for exact quantification of intake.

Parenteral nutrition was changed daily at 13:00 ± 1 h of Days 2–7 (Fig. 4), at which time the amount of parenteral nutrition and supplementary glucose, the amount of spermidine /saline, and the amount of insulin given was recorded.

At 14:00 ± 1 h of Day 7 (Fig. 4), animals were anesthetized using half of the above mentioned dose of anesthetics intravenously, and the animals were weighed. After tracheostomy, animals were normoventilated (small animal ventilator KTR4; Hugo Sachs, March-Hugstetten, Germany). Anesthesia was supplemented with 1.5 volume % isoflurane inhalation and 0,15 mg/kg piritramid i.v. Arterial blood pressure and central venous pressure (CVP) were monitored from the indwelling lines. Animals were sacrificed by cutting out the heart.

Peroperative on day -1 (before injecting alloxan), day 0 (after placing catheters), and thereafter daily at 09:00 ± 1h, arterial blood was sampled and immediately analyzed on a blood gas analyzer (ABL725, Radiometer Copenhagen, Denmark) to quantitate pH, hemoglobin, electrolytes, lactate, and glucose.

From day 0 on, glucose was measured minimum four times daily, and additional glucose measurements were carried out whenever blood glucose was unstable to allow tight adjustment of glucose/insulin infusion (Fig. 4). Supplementary, on day -1 (before injection of alloxan), day 0 (after placing catheters), and thereafter daily at 09:00 ± 1h, 3ml blood was collected and centrifuged for 10 min at 10,000 rpm, and plasma was stored at -80°C until further analysis. Urine was collected in a bucket under every cage.

Daily at 13:00 ± 1 h, 3 ml urine was sampled and stored at -80°C until further analysis, and total urinary volume was recorded. On day 7, all organs were sampled under anesthesia and stored at -80°C until further analysis.

A beneficial effect of spermidine was observed on overall survival, and the organ function (clinical, biochemical, morphological/cyto- and histopathological) and metabolic, inflammatory/immunological and cellular pathways were improved or restored. Thus, spermidine administration during critical illness could restore plasma and tissue levels of spermidine. Spermidine administration during critical illness resulted in decreased mortality, improvement of organ function, and affected multiple metabolic, inflammatory/immunological and cellular pathways. Blocking the effects of spermidine with an analogue had the opposite effects on survival, organ function and other morbidity.

Spermidine administration, given to these parenterally fed hyperglycemic hyperinsulinemic critically ill animals, resulted in a marked improved survival rate, with the benefit most pronounced for the higher doses of spermidine (Fig 11).

Figure 11 shows the mortality of 3 groups of 8 animals receiving doses between 30 and 300 mg spermidine per day (group A, B and C). In these groups the mortality is 12,5 %. 2 groups of 7 animals received doses between 0.3 and 3 mg spermidine per day (group D and E). In these groups the mortality is 28 %. A control group (F) of 4 animals received a saline solution without spermidine. In this group the mortality is 50 %. At any time point, there were more survivors in the groups receiving spermidine (Fig 12).

A considerable number of critically ill patients develop lactic acidosis as a result of increased anaerobic metabolism. The development of lactic acidosis (lowering of blood pH and an increase in lactate) is associated with poor outcome in patients, so blood pH and lactate can be used as markers of illness severity and predictors of outcome (Gunnerson *et al.* Crit Care 2006; 10: R22, Mizock *et al.* Crit Care Med 1992; 20: 80-93.and Smith *et al.* Base excess and lactate as prognostic indicators for patients admitted to intensive care. Intensive Care Med 2001; 27: 74-83). Also in our rabbits, the development of lactic acidosis is associated with poor chances of survival. Already on day 3 of critical illness, some time before the first animals die because of illness, the plasma lactate of future non-survivors is different from lactate of survivors (Fig. 13) The evolution of pH and lactate over time is totally different between randomization groups. Rabbits who receive spermidine-infusion during illness are able to maintain a normal pH and normal lactate levels during their illness, and consequently have a higher

survival. In rabbits receiving saline, lactate accumulates over time, pH decreases over time, and mortality is higher (Fig. 14).

Thus, the better survival in the spermidine groups was associated with a better preservation of blood pH and lactate, both being good markers of illness severity.

Plasma creatinine, a marker of kidney function, spontaneously rose in our animals, indicating the development of renal failure. This rise in creatinine could be prevented by administration of spermidine (Fig. 15A, 15B, 15C) Even low doses of spermidine resulted in renoprotection.

The rise in ureum, another marker of kidney dysfunction -- as well as muscle wasting-- could also be prevented by spermidine infusion (FIG 16)

After the initial hit, our critically ill animals develop early liver dysfunction, as indicated by increased AST, a marker of liver dysfunction. In the days following, animals receiving spermidine have a rapid and profound decrease in AST-levels, indicating a rapid resolution of liver dysfunction after the initial hit. In animals receiving saline, recovery of liver function is hampered, as shown by less decrease in AST-levels (Fig 17) .

We also measured plasma and tissue levels of spermidine and other polyamines. Spermidine-administration could restore both plasma and tissue levels of spermidine, an effect already seen with doses of 1 – 200 mg spermidine per kg per day, or doses of 5 – 120 mg spermidine / kg per day , preferably doses of 10 – 30 mg spermidine / kg per day.

Thus, counteracting the spontaneous decrease in spermidine, which we observed during critical illness (see above), strikingly reduced mortality and morbidity in our animal model of critical illness.

A number of proteins involved in autophagy such as citrate synthase, and mitochondrial enzyme complex activity in liver was measured. In addition, EM-analysis on tissue samples is performed. Spermidine administration resulted in an increase in autophagy. Expression of key autophagic proteins was stimulated by spermidine-administration, and the number of autophagosomes increased. Mitochondrial enzyme complex activity (complexes I to V) in liver, expressed per number of mitochondria, increased by spermidine-administration, indicating that autophagy resulted in increased clearance of less functional or damaged mitochondria. (see figure 18).

Example 6: Effects of spermidine administration in critical illness

Critically ill patients requiring prolonged intensive care are characterized by a profound decrease of lean body mass but a preservation of adipose tissue. Furthermore, obese critically ill patients, with a BMI between 30 and 40, have a lower risk of death than patients with a normal BMI. Metabolic activity of adipose tissue in critical illness has hitherto not been studied. We hypothesized that critical illness, hallmarked by severe hyperglycemia, hyperinsulinemia and hypertriglyceridemia, changes adipose tissue substrate handling. We therefore studied glucose transport and metabolization, fatty acid metabolization and the anatomy of adipose tissue in subcutaneous and omental adipose tissue biopsies of 61 prolonged critical ill patients (taken minutes after death) and of 20 non-critically ill patients (taken during abdominal surgery).

The studied critically ill patients were included in a large randomized controlled trial on glucose control. Patients who had been randomly assigned to conventional insulin therapy (CIT) received insulin only when glucose concentrations exceeded 215 mg/dl, resulting in mean blood glucose of 157 mg/dl (hyperglycemia). IIT maintained blood glucose levels between 80 and 110 mg/dl resulting in mean blood glucose of 110 mg/dl (normoglycemia).

Glucose transporters (GLUT1, GLUT3) mRNA and protein expression was increased in adipose tissue of critically ill patients. Glucokinase mRNA expression was upregulated. Glucose tissue levels werey increased but G-6-P and glycogen adipose tissue levels were low in adipose tissue of critically ill patients, levels of acetyl CoA carboxylase and activity of fatty acid synthase was strongly upregulated. Also expression of stearoyl-coA desaturase, involved in the biosynthesis of mono-unsaturated fatty acids, was increased in critical illness. The cell area of adipocytes decreased in critical illness, as did the expression of perilipin, which is a lipid droplet coating protein in adipocytes. Furthermore, adipose tissue of more then 95% of the studied critically ill patients stained positive for CD68, a macrophage marker, while only 33% of the healthy control tissues did.

Together these results indicate a change in substrate handling in adipose tissue of critical ill patients. Our data suggest that glucose uptake in adipose tissue may be increased in critical illness, followed by an increased metabolization of glucose to fatty acids. Intensive insulin therapy only has very minor effects on these pathways. Concomitantly with increased lipogenesis, adipocyte cell number, rather then cell size, increased. The presence of macrophages in adipose tissue of critically ill patients might

support an increased turnover of adipocytes. These changes turn adipose tissue into a functional 'waist bin' for toxic metabolites such as glucose during critical illness.

Example 7: Effects of spermidine administration in critical illness

Rabbit studies are carried out to elucidate whether pre-operative supplementation with spermidine improves post-operative organ function and decreases multiple organ dysfunction-associated risk factors.

One group of rabbits is fasted for 16 hours (water ad libitum), prior to clamping the SMA. The intervention group receives 113 g of dextrin and 12.7 g fructose per litre, plus an isotonic mix of salts and citric acid in drinking water, starting 5 days before the operation and continuing until the day of operation. Ad libitum water serves as control. The animals are sacrificed by exsanguinations. Intestinal permeability and translocation of bacteria are measured immediately. Plasma and different organ samples are frozen in liquid nitrogen for organ function parameters measurements. Sham-fasted animals serve as controls.

Ischemia reperfusion (IR) in the fasted animals results in a significant increased intestinal permeability. Preoperative ad libitum administration of a spermidine drink shows to preserve a significantly better intestinal barrier function when compared to overnight fasted ischemic rabbits.

Fasted operated rabbits show an increased bacterial translocation to the liver, kidney and mesenteric lymph nodes when compared to sham fasted rabbits or sham fed rabbits. Preoperative supplementation of the spermidine drink significantly decreases bacterial translocation to the liver, kidney and mesenteric lymph nodes as compared to IR fasted animals. Furthermore, a trend to decreased bacterial translocation is seen in the spleen of preoperative fed animals.

The lung shows increased neutrophil infiltration as indicated by myeloperoxidase activity in the IR fasted group in comparison with the sham-fasted group. The group, pre-operatively supplemented with the spermidine mixture, shows a significant decrease in comparison to the IR fasted rabbits. Moreover, the IR fasted group shows significantly decreased GSH concentration in comparison with the pre-operative supplemented group. In contrast, the GSH concentration of the IR supplemented group is almost retained at the level of the sham fasted animals. Oxidative stress, indicated as MDA concentration, shows a trend to decrease in comparison with IR fasted animals.

Rabbits that are allowed ad libitum pre-operative access to the spermidine drink show a significant decrease in urea concentration in comparison with IR fasted rabbits.

Asymmetrical dimethylarginine (ADMA) concentration, recently suggested to be a risk factor for organ dysfunction, is significantly increased in the IR fasted rabbits in comparison with sham-fasted. Importantly, the pre-operative supplemented group shows significantly decreased ADMA concentration and are shown to be deprived from an increase in ADMA in comparison with IR fasted and sham-fasted animals respectively. Another parameter that has concentration-dependently been linked to the incidence and severity of single and multiple organ dysfunction ((S)MOD) is IL-6, a pro-inflammatory cytokine. The IL-6 concentration shows a significant decrease in the group pre-operatively supplemented with the spermidine mixture in comparison with the IR fasted rabbits.

In conclusion, pre-operative administration of spermidine decreases MOD. This decrease is shown by improved intestinal barrier function and lowered bacterial translocation. Furthermore, lung inflammation pulmonary oxidative stress and plasma urea are decreased. These improvements in organ function parameters in the spermidine-fed rabbits are paralleled by a simultaneous decrease in ADMA and IL-6 concentration. The beneficial effects of preoperative spermidine supplementation on decreasing MOD and MOD associated factors suggest an important role for preoperative nutrition to improve post-operative recovery.

Example 8 Materials and methods for in vitro experiments and experiments with yeast and Drosophila.

Blood Samples, Preparation, and Culture of Peripheral Blood Mononuclear Cells (PBMC)

Sixty ml of peripheral full blood were obtained from healthy young (<35 years) persons, registered at the Institute for Biomedical Aging Research as blood donors. Informed written consent was obtained and the study was approved by the local ethical committee. PBMC were purified from heparinized blood by Ficoll Paque density gradient centrifugation (Pharmacia, Uppsala, Sweden). PBMC were cultured for 12 days in RPMI 1640 (Life Technologies, New York, USA) supplemented with 10% fetal calf serum (Sigma-Aldrich, Austria), 1% penicillin-streptomycin (Gibco, Invitrogen Corporation, UK) and phytohemagglutinin (PHA) (1 µg/ml, Sigma, Vienna, Austria) using 24 well-plates (BD, Franklin Lakes, NJ, USA) for 12 days. PBMC were kept at a density of 10^6 cells per well at 37 °C, 5% CO_2. Spermidine (Sigma, Austria) was added

after 1 and 7 days of culture (0 nM, 0,2 nM, 2 nM, 20 nM, and 2 µM). Survival of cells was measured after 6 and 12 days.

Immunofluorescence Staining of PBMC

Cells were washed (1500 rpm, 10 min, RT) with phosphate buffered saline (PBS) and resuspended in 50 µl PBS / 10^6 cells. Necrosis staining of cells was performed by adding the DNA intercalator 7-Aminoactinomycin D (7-AAD), which is visible in the Phycoerythrin (PE) channel, at a concentration of 0,5 µl/ 50 µl PBS and incubated for 30 minutes at 4 °C. Cells were then washed with PBS and resuspended in 100 µl Annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). 5 µl of the Fluoresceinisothiocyanate (FITC) labeled Annexin V (BD Pharmingen, San Jose, CA, USA) were added and samples were incubated for 15 min at RT, in the dark. Finally, 400µl of Annexin binding buffer were added, samples were kept on ice and measured at the FACS immediately. Cells which were negative for both staining were considered as viable (Fabrizio *et al.*, *J Cell Biol* **166**, 1055-1067 (2004)).

Cell Culture Conditions, Plasmid Transfection, and Autophagy Measurements

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 1 mM pyruvate and 10 mM Hepes at 37°C under 5% CO2. For plasmid transfection cells were cultured in six-well plates and transfected at 80% confluence. Transient transfections with LC3-GFP plasmid was performed with Lipofectamine 2000 reagent (Invitrogen) and cells were used 24 h after transfection. For fluorescence microscopy HeLa cells transfected with LC3-GFP were fixed with paraformaldehyde (4%, w/v) and nuclei were labeled with 10 mg/ml Hoechst 33342 (Molecular Probes-Invitrogen). Fluorescence microscopy was analyzed with a Leica IRE2 equipped with a DC300F camera. For western blot analysis cells were washed with cold PBS at 4°C and lysed as previously described (Criollo *et al.*, *Cell Death Differ* **14**, 1029-1039 (2007)). Fifty µg of protein were loaded on a 10% SDS-PAGE precasted gels (Invitrogen) and transferred to Immobilon membrane (Millipore). The membrane was incubated for 1 h in TBS-Tween 20 (0,05%) containing 5% non-fat milk. Primary antibodies including anti-LC3 I/II (Cell Signaling) or anti-p53 (DO-1; SantaCruz) were incubated overnight at 4°C and revealed with the appropriate horseradish peroxidase-labeled secondary antibodies (Southern Biotechnologies Associates) plus the SuperSignal West Pico chemoluminiscent substrate (Pierce). Anti-GAPDH (Chemicon CA) antibody was used to control equal loading.

Drosophila Life Span Experiments

Flies from an isogenized w^{1118} strain were used in all the experiments. They were kept in a 25 °C, 70% humidity, 12 h light/ 12 h dark incubator. Spermidine (S4139, Sigma, Austria) was prepared as a 1 M stock solution in sterile distilled water, aliquoted in single use portions and stored at -20 °C. New stock solution was prepared once a month. Spermidine was mixed to liquid food medium (2.2% sugar beet syrup, 8% malt extract, 1.8% yeast, 1.2% nipagine). In a preliminary experiment, we tested that the flies ate normally when fed with spermidine supplemented food to exclude a dietary restriction effect on life span. Food colorant was added to normal food and food mixed with 10 µM, 100 µM, 1 mM and 10 mM spermidine. The intensity of the color in the flies' abdomens was checked regularly for 24 hours. We could not detect any difference between the control group and the groups fed spermidine at all concentrations. Where indicated, the preservatives propionic acid (0,84%) and phosphoric acid (0,05%) were added to the food.

Newly enclosed flies were collected for a total of 60 flies in each group. Both males and females were studied. Data presented are from experiments with female flies, the effects on male flies were smaller but significant (data not shown). Twenty flies of the same sex were put in an empty vial closed with a foam plug in which a cut was made to insert a filter paper soaked with 400 µl of food. The filters were replaced by new ones and dead flies were counted every weekday. Over the weekend, 1.2 ml of food was given to the flies. Comparison of survivorship data was performed using log rank and Wilcoxon survival tests and corrected for multiple comparisons against the control group. Each sex and replicate was analyzed separately.

The lines for the generation of the *Atg7* homozygous and heterozygous flies were kindly provided by Dr. T. Neufeld (University of Minnesota, USA; (Juhasz, *et al. Genes Dev* **21**, 3061-3066 (2007).)). The homozygote mutants, $Atg7^{d14}/Atg7^{d77}$ are homozygous mutant for *Atg7*, heterozygous for *Sec6* and *CG5335*. The flies of the genotype $CG5335^{d30}/Atg7^{d14}$ (heterozygous for *Atg7, Sec6* and *CG5335*) were used as controls. For life span experiments of *Atg7* mutants, an *yw* control (obtained from The Bloomington Stock Center, Indiana, USA) was included as the closest genetic background for the *Atg7* lines. Similar results were obtained as compared to $CG5335^{d30}/Atg7^{d14}$ flies (data not shown). The w^{1118} line was also included to control for the replicability of the results.

Autophagy Measurements in *Drosophila* Tissue

W^{1118} females were kept for 48 hours on normal food (control), food supplemented with 1 mM spermidine (spermidine) or on a 10% glucose solution (starved). Muscles from

the thorax and sections of the esophagus were dissected in PBS and then transferred for two minutes in a PBS solution containing the fluorescent dyes Hoechst 4432 (dilution 1:1000) and LysoTracker Red DND-99 (Invitrogen, Ref L7528) diluted 1:10000, After staining, the tissues were transferred on a microscope slide (SuperFrost *Ultra*Plus Menzel-Gläser Nr. J4800AMNZ), covered with a cover slip and immediately imaged with a fluorescence microscope Zeiss axioplan 2 imaging / Coolsnap HQ. At least 20 flies were imaged for each group. On each picture from the muscles, the number of autophagic vesicles recognized by the LysoTracker dye and the nuclei were manually counted. The ratio of vesicles to nuclei was calculated for each picture and analyzed with a Kruskal-Wallis test corrected with a Bonferroni post-hoc tests.

Experimental Animals and Determination of Thiol Groups in Mice Serum

Male and female C57BL/6 mice were purchased from the Institut für Labortierkunde und -genetik, Himberg, Austria. All animals were used at an age between 12 and 16 weeks. All mice were kept and treated according to institutional guidelines and Austrian law and the experiments were approved by the responsible governmental commission. For each group, one male and two female mice were housed singly and fed *ad libitum* with regular food (pellets) and spermidine was supplemented to drinking water in concentrations of 0,3 and 3 mM for 200 days. Controls were given pure drinking water. Drinking water was replaced every 2-3 days and spermidine freshly added from 1 M aqueous stock (spermidine/HCl pH 7.4), which was kept at -20°C for no longer than one month. Food and body weight, calculated on a weekly basis, remained unaffected by supplementation of spermidine (data not shown), indicating that not calorie restriction could account for the observed effects. At the end of the experiment, the animals were anesthetized by ether inhalation, and exsanguinated by heart puncture. Peripheral blood was allowed to clot for 20 min, and serum was obtained by centrifugation at 200 g for 10 min. The spleens and livers (shock frozen in liquid nitrogen and stored at -80 °C upon further use) were immediately excised. Serum was used for determination of free thiol groups by Ellmans' reaction (*Ellman, Arch Biochem Biophys 82, 70-77 (1959) and Riener et al., Anal Bioanal Chem 373, 266-276 (2002).*) as described previously (Schraml *et al.*, *Exp Gerontol* **42**, 1072-1078 (2007).). Spleen weight, which was similar in all groups, indicated that all mice were of similar general health (data not shown).

Extraction of Polyamines for LC/MS/MS Measurements

For acid extraction of polyamines from yeast cells (Balasundaram *et al. Proc Natl Acad Sci U S A* **88**, 5872-5876 (1991)) culture equivalents of 20 OD_{600} were washed three times with ddH_2O, resuspended in 400 µl ice-cold 5% TCA, and incubated on ice for one hour with vortexing every 15 min. Supernatants were neutralized with 100 µl of 2 M K_2HPO_4 and stored at -80 °C upon polyamine measurements using LC/MS/MS.

Extraction of polyamines from mice liver tissue and from flies was performed according to the freeze/ thaw- method described by Minocha *et al.* (Minocha, *et al.*, *J Plant Growth Regul* **13**, 187-193 (1994).) with slight modifications. Briefly, about 50-75 mg of mice liver tissue or 15-20 mg of whole flies were semi-homogenized using Fisherbrand Disposable Pestle System (Fisher scientific) and polyamines extracted with 400 µl 5% TCA by three repeated freeze-thaw cycles. After extraction 100 µl of 2 M ammonium formiate were added to supernatants and stored at -80 °C upon polyamine measurements using LC/MS/MS.

Polyamine Measurements using LC/MS/MS

Polyamines were determined according to the method described previously by Gianotti *et al.* (V. Gianotti *et al.*, *J Chromatogr A* **1185**, 296-300 (2008).). All experiments were carried out on an Ultimate 3000 System (Dionex, LCPackings) coupled to a Quantum TSQ Ultra AM (ThermoFinnigan) using an APCI ion source. The system was controlled by Xcalibur Software 1.4. The stationary phase was a Sequent ZIC-HILIC column (150 x 2.1 mm, 3µm, 100 Å). The elution solvent A was 50 mM ammonium formiate in ultra pure water and solvent B was acetonitrile. Separation was performed with 15% acetonitrile for 2 min. Thereafter, the acetonitrile content was linearly decreased to 5% over 2 min. After 1 min, acetonitrile content was increased to 15% for column equilibration. Flow rate was set to 300 µl/min.

Polyamines were detected in MRM mode using following transitions: spermidine (m/z 146 -> 72, CE 34 eV), putrescine (m/z 89 -> 72, CE 28 eV), bis(hexamethylene)-triamine as internal standard (m/z 216 -> 100, CE 36 eV). Calibration standards were prepared by spiking extraction buffer with specific concentrations of spermidine, putrescine and internal standard. 20 µl of each sample were injected.

Yeast Strains and Molecular Biology

Experiments were carried out in BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and respective null mutants, obtained from Euroscarf. The double mutant *Δiki3Δsas3* was generated according to Gueldener *et al. Nucleic Acids Res. 2002 Mar 15;30(6):e23* by using gene-specific URA3-knockout cassette, amplified by PCR with pUG72 as template (Lovaas & Carlin, *Free Radic Biol Med* **11**, 455-461 (1991).). The double

mutant phenotype was confirmed using a strain generated by mating and sporulation of the respective single mutants (BY4742 *Δiki3* MATα and BY4741 *Δsas3* MATa). All *spe1* double mutant strains were obtained through mating and sporulation of BY4741 *Δspe1* with the respective BY4742 (Matα) single mutant strains. Single and double mutant strains were verified for correct gene deletion by PCR and further checked for consistent auxotrophies. Notably, at least three different clones of each generated mutant were tested for the survival plating during aging to rule out clonogenic variation. Strains were grown at 28 °C on SC medium containing 0,17% yeast nitrogen base (Difco), 0,5% $(NH_4)_2SO_4$ and 30 mg/l of all amino acids (except 80 mg/l histidine and 200 mg/l leucine), 30 mg/l adenine, and 320 mg/l uracil with 2% glucose (SCD). To demonstrate the complete requirement of polyamines for life span extension upon media alkalinization, experiments were carried out in polyamine-free SCD, obtained by sterile filtering and special treatment of glass ware as described (Longo, E. B. Gralla, J. S. Valentine, *J Biol Chem* **271**, 12275-12280 (1996).). To construct *NHP6A*-EGFP in pUG35-Ura (giving rise to a C-terminally tagged chimeric fusion protein under control of the met25-Promotor) the insert was amplified by PCR using genomic DNA from BY4741 as template and cloned into pUG35 using the *EcoRI* restriction site. The EGFP-*ATG8* construct in pUG36-Ura (N-terminally tagged fusion protein) was similarly generated, using *EcoRI* and *ClaI* restriction sites. -

Yeast Survival Plating and Test for Cell Death Markers

For chronological aging experiments, cultures were inoculated from fresh overnight cultures to OD_{600} of 0,1 (~$1 \cdot 10^6$ cells/ml) with culture volume being 10% of flask volume and aliquots were taken out to perform survival plating at indicated time points (B. Liu, A. Sutton, R. Sternglanz, *J Biol Chem* **280**, 16659-16664 (2005).). Survival at day 1 of wild type control cultures was set to 100% and other samples calculated accordingly. If not otherwise indicated, representative aging experiments are shown with at least three independent samples (as indicated) aged at the same time, which have been repeated at least twice with similar outcome. In case of experiments with *Δspe1* (Fig. 25 A-E), all strains were inoculated to $5 \cdot 10^4$ cells/ml. Upon deletion of both *IKI3* and *SAS3* we observed slight aggregation of cells possibly due to a defect in late budding events. Therefore, for calculation of survival rates in experiments using *Δiki3Δsas3*, cell numbers of each sample were determined after two pulse of sonication on ice with Sonifier 250 from Benson (Duty Cycle: 35; Output Control: 2.5). Tests for apoptotic (TUNEL and Annexin V staining) and necrotic (PI staining) markers as well as markers for oxidative stress (DHE staining) were performed as described (B. Dod, A. Kervabon,

J. Parello, *Eur J Biochem* **121**, 401-405 (1982)). For quantifications using flow cytometry (BD FACSAria), 30,000 cells were evaluated and analyzed with BD FACSDiva software. Spermidine (S4139, Sigma, Austria) and putrescine (P5780, Sigma, Austria; 1 mM final concentration) were added to stationary cultures at day 1 of the aging experiments (24 h after inoculation). 1 M aqueous stock solution of spermidine was stored in one use aliquots at -20 °C for no longer than 1 month. For adjustment of extracellular pH (pH_{ex}) to 6 (± 0,5), the required amount of sodium hydroxide was added 30 h after inoculation. The pH_{ex} was maintained at approximately 6 (± 0,5) throughout the aging.

As a further marker for necrosis, nuclear release of the yeast HMGB1 homolog (Nhp6Ap) was monitored by epifluorescence microscopy of ectopically expressed chimeric fusion protein Nhp6Ap-EGFP. Therefore, yeast strains transformed with pUG35/*NHP6A* were grown on SCD lacking uracil and aged until indicated time points. Cells were washed once with PBS and directly applied to epifluorescence microscopy with the use of small-band EGFP filter (Zeiss) on a Zeiss Axioskop microscope in order to monitor intracellular localization of Nhp6A-EGFP. Expression during aging was verified by immunoblotting (data not shown). Notably, release of Nhp6A-EGFP to the extracellular space, which has been reported for mammalian HMGB1 (Lotze &. Tracey, *Nat Rev Immunol* **5**, 331-342 (2005)), could not be detected in yeast after 100x concentration of culture media (data not shown).

Yeast Replicative Life Span Determination

For replicative life span analysis of BY4741 synthetic complete glucose medium (SC-glucose) containing 2% (w/v) D-glucose, 0,17% yeast nitrogen base (Difco), 0,5% $(NH_4)_2SO_4$, and 10 ml complete dropout was used. Complete dropout contains: 0,2% Arg, 0,1% His, 0,6% Ile, 0,6% Leu, 0,4% Lys, 0,1% Met, 0,6% Phe, 0,5% Thr, 0,4% Trp, 0,1% Ade, 0,4% Ura, 0,5% Tyr. Agar plates were made by adding 2% (w/v) agar to the media. Where necessary, spermidine from a freshly prepared aqueous stock solution (0,2 M, pH 7.0) was added to a final concentration of 1 mM. Pre-tests showed that this concentration does not influence growth properties of fraction V cells (data not shown). Preparation of senescent yeast cells (fraction V) by elutriation was performed as described in Laun *et al.* (Laun *et al.*, *Mol Microbiol* **39**, 1166-1173 (2001).). To determine the remaining life span of fraction II and fraction V cells, cohorts of 80 randomly chosen cells per fraction were taken directly after elutriation and, for each cell, the number of remaining cell cycles was determined by micromanipulation. Cells

that never budded were excluded from analysis. Statistical analysis was performed as described in Laun *et al.* (Laun *et al.*, *Mol Microbiol* **39**, 1166-1173 (2001).).

Electron Microscopy

Yeast cells were aged to day 20 or logarithmically grown (day 0), transformed into spheroblasts and fixed in 2% glutaraldehyde (Sigma, Austria) for 1 hour. Spheroblastation was performed using zymolyase (20 U/ml), lyticase (100 U/ml), and glucoronidase/arylsulfatase (7 µl/ml) (Roche, Austria) in 20 mM potassium phosphate buffer (pH 7.4) with 1.2 M sorbitol for 70 minutes at 28 °C. Glutaraldehyde fixation was done in 20 mM potassium phosphate buffer (pH 7.4) with addition of 0,4 M potassium chloride for osmotic stabilisation of spheroblasts. Fixed cells were postfixed in osmium tetroxide and prepared for electron microscopy as described (Fahrenkrog *et al. J Cell Biol* **143**, 577-588 (1998)). Thin sections were cut on a Reichert Ultracut microtome (Reichert-Jung Optische Werke, Vienna, Austria) using a diamond knife (Diatome, Biel, Switzerland). The sections were collected on parlodion coated copper grids and stained with 6% uranyl acetate for 1 hour followed by 2% lead citrate for 2 minutes. Electron micrographs were recorded with a Hitachi H-7000 transmission electron microscope (Hitachi Ltd., Tokyo, Japan) operated at an acceleration voltage of 100 kV.

Immunoblotting and Quantification of Histone Acetylation

Trichloroacetic acid whole-cell extracts were prepared according to the method described by Kao *et al.* (Howe *et al.*, *Genes Dev* **15**, 3144-3154 (2001).). Proteins were separated on 15% SDS-PAGE for Western blot analysis on PVDF membrane (Millipore) as described (Madeo *et al.*, *Mol Cell* **9**, 911-917 (2002).) using CAPS buffer (10 mM 3-(Cyclohexylamino)-1-propanesulfonic acid, 10% methanol) for transfer of proteins. Blots were probed with the rabbit polyclonal antibody against histone H3 (ab1791, Abcam) (1:5,000), which served as a loading control for total histone H3, as well as the following histone H3 modification antibodies (Upstate Biotechnology): K56ac (1:6,000) (Recht *et al.*, *Proc Natl Acad Sci U S A* **103**, 6988-6993 (2006).); K18ac (1:10,000); and K9+14ac (1:10,000). Peroxidase-conjugated affinity-purified secondary antibody was obtained from Sigma. For quantification of relative acetylation blots were scanned using a densitometer (Molecular Dynamics, Model P.D. 300) and quantified with ImageQuant Version 5.1 (Molecular Dynamics). Band densities of acetylation specific blots were normalized to the respective densities of total histone H3 blots in order to obtain specific acetylation rates for each sample. Acetylation rates of wild type control cultures were normalised to 1 and the relative acetylation of each sample was calculated accordingly.

Yeast Intracellular pH Measurement

Intracellular pH (pH_i) of aging yeast cells was assessed by FACS analysis of cells stained with the pH-dependent fluorescent dye SNARF-4F (Invitrogen, Austria), following the method described by Valli *et al.* (Valli *et al.*, *Appl Environ Microbiol* **71**, 1515-1521 (2005)) with slight modifications. The dye is applied as its acetomethyl ester (SNARF-4F-AM) and needs to be activated by intracellular esterases. In order to ensure sufficient activation in aging cells the incubation time for dye loading was increased to 30 minutes.

Spontaneous Mutation Frequency and Budding Index

Spontaneous mutation frequency was determined based on the appearance of mutants able to form colonies on agar plates containing 60 mg/l L-canavanine sulfate according to Fabrizio *et al.* (Fabrizio *et al.*, *J Cell Biol* **166**, 1055-1067 (2004).). Mutation rates were calculated per 10^6 living (colony forming on YEPD) cells. Budding index was assessed by counting the percentage of budded cells after 10 seconds of sonication on ice using Sonifier 250 from Benson (Duty Cycle: 35; Output Control: 2.5) in micrographs of no more than 40 cells. For each sample, at least 500 cells were evaluated.

Oxygen consumption

Oxygen consumption was directly determined in 1.7 ml of chronologically aged yeast cultures transferred to a recording chamber by measuring the decline of oxygen concentration under anaerobic conditions using an oxygen electrode. Slopes were calculated over 15 min within the linear decrease of oxygen (minute 2 – 17) and normalized to living cells as determined by plating on YEPD agar plates.

Fractionation of "upper" and "lower" (quiescence) cells

Cells were cultured in SCD media as described in section on "Yeast Strains and Molecular Biology". Percoll density gradient centrifugation was performed according to Allen *et al.* (Allen *et al.*, *J Cell Biol* **174**, 89-100 (2006)). Cell counts for each fraction were determined using a CASY cell counter (Innovatis).

Yeast Nuclear Extract Preparation

Yeast nuclei were isolated from 200 ml BY4741 wild type culture (grown for 24 h in SCD to stationary phase) as described previously (Buttner *et al.*, *Mol Cell* **25**, 233-246 (2007)). Nuclear extract was prepared using nuclear extraction buffer from BioVision's Nuclear/ Cytosol Fractionation Kit (Bio Vision, K266-25) without DTT addition, according to the manufacturer's protocol. Incubation time was doubled to 80 min with vortexing every 8 minutes. Protein concentration was determined via Bradford, giving

yields of approximately 1 mg/ml protein. Yeast nuclear extract was immediately subjected to HAT activity assays.

HAT Activity Assay

For HAT-activity determination the commercially available HAT Activity Colorimetric Assay KIT from BioVision (Bio Vision K332-100) was employed. HAT assays were performed according to the manufacturer's protocol. In brief, assays were performed with each 15 µg of yeast nuclear extract or nuclear extract of HeLa-cells (Bio Vision K332-100-4), respectively. Spermidine was added at a final concentration of 100 mM 15 minutes after assay initiation. Development of tetrazolium dye was measured by absorption at 440 nm using a GeniosPro plate reader (Tecan). Background readings were done with samples without NADH generating enzyme, giving the nuclear extracts unspecific background activity and eliminate any possible negative effects of spermidine addition on the assay itself. For calculation of relative HAT activity linear regression over 100 minutes within the suggested assay time (minute 95 to 195) was performed to determine the slope of dye development. Regression coefficients of R^2 > 0,99 were obtained. Calculated slopes of spermidine treated samples were compared to slopes of untreated samples which were set to a relative activity of 100%.

Yeast RNA Isolation and Affymetrix Array Analyses

Total RNA extraction from chronologically aged yeast cells (with or without spermidine application) by glass bead disruption were performed using RNeasy MiniKit (Qiagen) according to the manufacturers' instructions. 10^8 cells were used after shock freezing in liquid nitrogen and storage at -80 °C upon preparation. RNA of two independent aging experiments at day 3 and 10 of the aging experiment (biological replicates) was applied to Affymetrix Array Analyses.

Syntheses of cDNA and hybridization experiments were outsourced to the Microarray Facility Tuebingen, Germany, an authorized Affymetrix Service Provider. Hybridization was done onto high-density oligonucleotide arrays Yeast Genome 2.0 (Affymetrix). Both, experimental and data analysis workflow were fully compliant with the MIAME 2.0 Standard. Annotation Data for the Yeast Genome 2.0 Array were supplied by Affymetrix Inc. Raw data were normalized with GCRMA (Wu *et al. Journal of the American Statistical Association* **99**, 909-918 (2004)) using CarmaWeb (Rainer *et al. Nucleic Acids Res* **34**, W498-503 (2006).). P-values were calculated with a paired T-Test comparing untreated (controls) with treated (spermidine supplemented) samples at the respective time points using TM4 MeV software (Saeed *et al.*, *Biotechniques* **34**, 374-378 (2003).).

Yeast Autophagy Measurements

Autophagy was monitored either by vacuolar localization of Atg8p using fluorescence microscopy of cells expressing an EGFP-Atg8 fusion protein (T. Kirisako *et al.*, *J Cell Biol* **147**, 435-446 (1999).) or by alkaline phosphatase (ALP) activity according to Kissova *et al.* (Kissova *et al. J Biol Chem* **279**, 39068-39074 (2004).) using BY4741 wild type strain transformed with and selected for stable insertion of pTN9 HindIII fragment (confirmed by PCR). In order to correct for intrinsic (background) ALP activity, BY4741 (without pTN9) have been simultaneously processed and ALP activity subtracted. For generation of EGFP-*ATG8* constructs see section on Molecular Biology.

Statistical Analyses

Except for *Drosophila* experiments (see section on *Drosophila* life span experiments and autophagy measurements) and for replicative aging (see section on replicative life span of yeast), statistical analyses were performed using Students T-Test (one-tailed, unpaired).

Example 9: Results

Histone H3 acetylation is regulated by intracellular polyamines in part mediated through Iki3p and Sas3p, as shown in Figure 24 with: **(A)** Immunoblot of whole cell extracts of wild type cells chronologically aged to designated time points with (+) or without (-) spermidine application. Blots were probed with antibodies against total histone H3 or H3 acetylation sites at the indicated lysine residues; **(B)** Relative acetylation of histone H3 lysine 9+14 of *Δspe1* cells compared to wild type cells chronologically aged to day 5 with (open bars) or without (closed bars) adjustment of pH_{ex} to 6. Data represent means ± SEM of three independent experiments. **p < 0,01; **(C)** Quantification (FACS analysis) of phosphatidylserine externalization and loss of membrane integrity using AnnexinV/PI co-staining performed at day 20 of the chronological aging experiment shown in (Fig. 22 D). For each staining 30,000 cells were evaluated. ***p < 0,001; **(D)** Immunoblot of whole cell extracts of wild type and *Δiki3Δsas3* cells with (+) or without (-) spermidine application obtained at day 20 of the aging experiment shown in (Fig. 22D). Blots were probed with antibodies against total histone H3 or H3 acetylation sites at lysine 9+14 (Lys9+14).

Exogenous supply of spermidine to chronologically aging (BY4741 wild type) cells (at day 1) caused a drastic increase in yeast life span by a factor of up to 4 times, as determined in clonogenic assays that monitored the frequency of viable cells (Fig.

19B). Similar results were obtained using the wild type strain DBY746 (data not shown). Supplementation of spermidine led to a stable increase in the intracellular spermidine level in aging cells that otherwise would exhibit a decrease in spermidine levels (Fig. 21 A, C).

While chronological aging serves as a model for aging of post-mitotic tissues, replicative aging of yeast models the life span of dividing cells in higher eukaryotes. Both aging systems are interrelated, since replicatively old cells die early during chronological aging (Allen *et al.*, *J Cell Biol* **174**, 89-100 (2006)). We therefore analyzed the differential effect of spermidine on replicatively young and old cells obtained by elutriation (Laun *et al.*, *Mol Microbiol* **39**, 1166-1173 (2001)). The remaining replicative life span of old cells was significantly increased by spermidine (Fig. 21 D, fraction V cells, $p < 0,02$) while no apparent effect was observable on the remaining life span of replicatively young cells (fraction II cells, Fig. 21 D). These aged yeast cells treated with spermidine did not only exhibit an increased life span, but also a strong resistance against stress inflicted by heat shock or H_2O_2 treatment (Fig. 22). Present invention also demonstrates that orally delivered spermidine is actively taken up and can be used to increase the intracellular levels of bioactive spermidine .Ordinary food (full media) of the fruit fly *Drosophila melanogaster* with spermidine. Low doses of spermidine increased the mean life span of flies up to 30% (Fig. 21E, $p = 0,0002$ for 1 mM; for mean life spans and replicates see Fig. 21). Measurement of endogenous polyamines confirmed that intracellular spermidine levels were stably increased by spermidine supplementation of ~20% compared to controls. Of note, putrescine (a polyamine interconvertable with spermidine) was not detectable in control samples but clearly present in spermidine fed flies (~ 100 nmol/g) indicating that spermidine was indeed taken up and metabolized by the flies cells (data not shown).

We next asked whether polyamines also enhanced the lifespan of human peripheral blood mononuclear cells (PBMC), and monitored survival by means of co-staining with Annexin V/7-AAD (unstained cells were regarded as viable) After 12 days, only 15% of the cells survived in PBMC control cultures, whereas up to 50% of the cells survived after addition of 20 nM spermidine (Fig. 21 B). Unexpectedly, the rescuing effect was not due to inhibition of apoptotic cell death, as the percentage of apoptotic cells (positive for Annexin V but negative for 7-AAD) remained unaltered. Instead, cell death associated with membrane rupture indicative of necrosis (as evident by dual 7-AAD and Annexin V staining) was markedly reduced (Fig. 23).

One of the most widely accepted theories of aging is the free radical theory that explains aging by accumulating oxidative stress (Harman, *J Gerontol* **11**, 298-300 (1956)). Consistently, in rodents the level of oxidative stress and protein damage increases with age, observable in the serum by a decline of free thiol groups (Schraml *et al.*, *Exp Gerontol* **42**, 1072-1078 (2007)). Feeding mice with 3 mM spermidine (supplemented to drinking water) for 200 days increased the serum level of free thiol groups by ~30%, indicative of reduced age-related oxidative stress (Fig. 21F). Notably, such an increase of free thiol groups is comparable to the natural decline that has been observed during the course of aging (between young and old rodents) (Schraml *et al.*, *Exp Gerontol* **42**, 1072-1078 (2007)). Again, intracellular spermidine levels were significantly increased by exogenous spermidine supplementation as determined in liver cells (Fig. 21G).

Next, we investigated the effect of polyamine depletion on aging, using a yeast strain deleted in *SPE1* (Δ*spe1*) and hence unable to synthesize polyamines. Polyamine depletion, as confirmed by measurement of intracellular spermidine (Fig. 17A), caused a drastic drop in yeast chronological life span (Fig. 25B), which can be restored by supplementation with spermidine or putrescine, the obligate precursor of spermidine (data not shown). Consistent with the free radical theory of aging (Harman, *J Gerontol* **11**, 298-300 (1956).), we observed an enhanced accumulation of oxygen radicals upon disruption of *SPE1,* as measured by dihydroethidium (DHE) staining (Fig. 25C, 25D). An enhancement of radicals upon *SPE2* deletion has also been shown in growing cells (Chattopadhyay *et al. Yeast* **23**, 751-761 (2006)). Since oxidative stress can cause apoptosis in yeast (Madeo *et al.*, *J Cell Biol* **145**, 757-767 (1999)) we determined apoptotic markers of wild type and polyamine depleted Δ*spe1* cells. Surprisingly, the frequency of apoptotic events (that is cells that exhibit DNA-fragmentation detectable by TUNEL or phosphatidylserine externalization detectable with Annexin V) was not affected by *SPE1* deletion (Fig. 25E). Instead, we observed an increase in necrotic, PI positive cells in *Δspe1* cultures as compared to wild type controls (Fig. 25E). Accordingly, deletion of apoptotic effector molecules (including the yeast caspase, Yca1p; apoptosis-inducing factor, Aif1p; endonuclease G, Nuc1p; or the serine protease HtrA2/OMI, Nma111p) in the background of Δ*spe1* did not prevent the aging-associated death accelerated by polyamine depletion (data not shown). We therefore conclude that depletion of intracellular polyamines can precipitate premature chronological aging via non-apoptotic, possibly necrotic death of yeast cells.

Very few studies have addressed the mechanisms of necrotic cell death in a systematic fashion. Using *C. elegans* as a model, it has been demonstrated that acidification of the cytosol is required for necrotic cell death, whereas alkalinization has a cytoprotective effect (Artal-Sanz *et al. J Cell Biol* **173**, 231-239 (2006), Syntichaki *et al. Curr Biol* **15**, 1249-1254 (2005)). In yeast, adjustment of the extracellular pH from normally 3.5 to 6.5 not only extends chronological life span (Fabrizio *et al.*, *J Cell Biol* **166**, 1055-1067 (2004)) but also stabilizes the intracellular pH in a polyamine dependent manner (Fig. 30 B), corroborating the assumption that cytosolic acidification limits cellular life span. Since addition of 4 mM spermidine to chronologically aging yeast increases the extracellular pH to 6 (± 0,5), we asked, if indeed intracellular polyamines (e.g. spermidine) were responsible for life span extension under these conditions. Making use of polyamine depleted cells (Δ*spe1*), we demonstrate that the protective effect of external alkalinization on longevity, and thus of spermidine application, is strictly dependent on intracellular polyamines (Fig. 30A).

Consistent with an anti-necrotic effect of alkalinization in *C. elegans,* spermidine treatment procures a drastic reversion of age-associated necrosis in yeast. Determination of cell death markers revealed that markers of necrosis and oxidative stress (DHE positivity) were drastically diminished upon spermidine treatment (Fig. 27C). In contrast, externalization of phosphatidylserine, an early apoptotic marker (Annexin V^+ PI^- cells), remained largely unaltered. Instead, loss of membrane integrity due to primary necrosis (PI positivity) and late apoptosis resulting in secondary necrosis (Annexin V^+ PI^+) was reduced from 50% to less than 10% in spermidine-treated cultures as late as after 18 days of aging (Fig. 27C). We therefore conclude that death associated with chronological aging of yeast is mainly mediated by spermidine-inhibitable necrosis-like cell death. Consistently, electron microscopy of old cells (day 20) showed necrotic disintegration of subcellular structures and plasma membrane ruptures, while the ultrastructure of spermidine-treated samples of the same age resembled that of young cells (Fig. 27 E; overview in Fig. 28). Nuclear release of the high mobility group box 1 protein (HMGB1), a chromatin bound non-histone protein, is a defining feature of necrosis in mammalian cells (Scaffidi *et al.* Bianchi, *Nature* **418**, 191-195 (2002)). Fluorescence microscopy detected a nucleo-cytosolic translocation of the yeast HMGB1 homolog Nhp6Ap (which was rendered visible with an EGFP tag) after 14 days of aging (Fig. 27D). Again, this necrotic feature was prevented by application of spermidine (Fig. 27D). Thus, spermidine treatment protects against aging by inhibition of necrosis.

As the extended life span of spermidine-treated cultures was neither associated with an increased mutation frequency nor a higher budding index (Fig. 29), we speculated that epigenetic modifications rather than genetic changes (such as the regrowth of death-resistant mutants) were responsible for the positive impact of spermidine on longevity. We could also exclude that a simple direct anti-oxidant effect of polyamines Lovaas & Carlin, *Free Radic Biol Med* **11**, 455-461 (1991)) could account for the observed life span extension.

Global histone deacetylation, a key event of epigenetic chromatin modification, is associated with prolonged life span and healthy aging in a wide range of organisms (Longo *et al. Cell* **126**, 257-268 (2006), Sauve *et al. Annu Rev Biochem* **75**, 435-465 (2006).). Since (de)acetylation of lysyl residues of histone H3 is critical for yeast longevity, at least during replicative aging (Imai *et al. Nature* **403**, 795-800 (2000)), we analyzed the effects of spermidine on the level of histone acetylation by means of a panel of specific antibodies that detect H3 acetylation at 4 different lysyl residues. The improved life span of aging wild type cells treated with spermidine correlated with hypoacetylation of histone H3 at all monitored acetylation sites (Fig. 19A, Fig. 30). Conversely, premature death of aging *SPE1*-deleted cells was accompanied by hyperacetylation of histone H3 (Fig. 19B). These results hint to an obligatory role for polyamines in the regulation of histone acetylation during aging. In line with the strict requirement of polyamines for life span extension upon extracellular alkalinization, we observed hypoacetylation of the chromatin only in wild type, but not in *SPE1* knockout cells upon alkalinization of the culture medium (Fig. 30B). These results suggest that global deacetylation and polyamines are connected to the extension of chronological life span in yeast.

As the role of the Sir2p deacetylase is well established in replicative aging (Longo & *Cell* **126**, 257-268 (2006), Lin *et al. Science* **289**, 2126-2128 (2000)), we tested its potential involvement in polyamine-promoted longevity during chronological aging. However, deletion of *SIR2* did not abrogate the ability of spermidine to extend the chronological life span -. Thus, the observed hypoacetylation during chronological life span extension is not due to the sole induction of Sir2p activity. Similarly, single deletion of all other known yeast sirtuins (*HST1, HST2, HST3, HST4*) did not affect longevity upon spermidine application (Table 2). This result is compatible with previous findings suggesting that chronological life span extension by calorie restriction is not mediated by Sir2p activity (Fabrizio *et al.*, *Cell* **123**, 655-667 (2005)) nor by any of the other yeast sirtuins (Smith *et al. Aging Cell* **6**, 649-662 (2007).).

Theoretically, spermidine treatment could lead to hypoacetylation either via activation of histone deacetylases or via inhibition of histone acetyltransferases (HATs). Therefore, we determined the effects on aging of the disruption of 28 genes involved in histone (de)acetylation in the presence or absence of spermidine. The anti-aging (pro-survival) effect of spermidine was partially abrogated in two of these strains, namely Δ*iki3* and Δ*sas3* (data not shown, Table 2). Interestingly, the histone acetyl transferase Sas3p preferentially acetylates histone H3 at lysine 14 (Howe *et al.*, *Genes Dev* **15**, 3144-3154 (2001)), one of the acetylation sites that is profoundly influenced by the availability of intracellular polyamines (see above). Deletion of *IKI3*, an essential subunit of the histone acetylating elongator complex, has been reported to reduce histone H3 acetylation at lysine 14 as well (Winkler *et al. Proc Natl Acad Sci U S A* **99**, 3517-3522 (2002)). Consequently, we generated the double mutant Δ*iki3*Δ*sas3* in an attempt to diminish these overlapping HAT-activities converging on lysine 14 of histone H3.

Chronologically aged Δ*iki3*Δ*sas3* cells responded significantly less to the anti-aging effect of spermidine than wild type cells (Fig. 19D, p = 0,002 for day 20). At day 20, spermidine treatment increased survival of wild type cells by 5-fold compared to only 1.3-fold for Δ*iki3*Δ*sas3* cells, suggesting that Iki3p and Sas3p are, at least to some extent, required for the life span prolonging effects of spermidine. Moreover, the untreated double mutant showed an improved survival during chronological aging as compared to wild type controls (Fig. 19D, p < 0,001 for day 20), indicating that histone acetylation activity is responsible for age-induced cell death. Accordingly, histone H3 acetylation was significantly reduced upon deletion of *IKI3* and *SAS3,* and spermidine application barely reduced the level of acetylation in this mutant (Fig. 19E). Evaluation of cell death markers revealed that increased survival of Δ*iki3*Δ*sas3* cells is clearly due to the inhibition of necrotic death (PI staining) while apoptotic markers (Annexin V) remained constant (Fig. 20C). The combined knockout of *IKI3* and *SAS3* did increase the life span of yeast cells, yet failed to mimic the life span prolongation of spermidine in quantitative terms, presumably because epigenetic aging processes are likewise regulated by more than just two proteins that modify the level of histone H3 acetylation on one lysyl residue. An in vitro assay for HAT-activity revealed that spermidine efficiently inhibited general HAT activity in extracts of isolated yeast and mammalian nuclei (Fig. 19C, data not shown). These results suggest that spermidine-mediated anti-aging effects are achieved via direct inhibition of HAT-activity.

Autophagy is believed to be essential for healthy aging and longevity, and the autophagy-regulatory Tor-pathway constitutes one of three highly conserved signaling pathways controlling aging of various organisms (Powers *et al. Genes Dev* **20**, 174-184 (2006)). Spermidine induced signs of autophagy in flies (Fig. 19H) and in cultured human cells (Fig. 19G). Staining of lysosomes by LysoTracker Red in esophagus-tissue obtained from flies fed with 1 mM spermidine for 48 h exhibited a strong increase in the number of lysosomes indicative of autophagy, which even exceeded that of starved flies (Fig. 19F, Fig. 31A for quantification). HeLa cells treated with 100 µM spermidine for 6 h showed a clear relocalization of LC3-GFP (the mammalian orthologue of Atg8p) into cytoplasmic puncta with a frequency of 40% (Fig. 19G and fig. 31B). Immunoblot detection of accumulating LC3-II, the palmitoylated form of LC3, confirmed induction of autophagy by spermidine (fig. 31C, D). Finally, deletion of ATG7-dependent autophagy completely abrogated spermidine-induced life span extension in flies (Fig. 19G).

Altogether, our results demonstrate that epigenetic regulation of necrotic death determines the chronological life span of yeast and that this epigenetic regulation is mediated by proteins involved in histone acetylation (such as Iki3p and Sas3p), which in turn are inhibited by spermidine. Additionally, histone (de)acetylation (and subsequent regulation of cell death) might also be regulated by polyamines depending on their acetylation state thereby directly modifying chromatin accessibility (Liu *et al. J Biol Chem* **280**, 16659-16664 (2005)). Consistently, we observed that deletion of *PAA1*, the sole known polyamine acetyl transferase (Liu *et al. J Biol Chem* **280**, 16659-16664 (2005)), effectively shortens yeast chronological life span accompanied by enhanced ROS levels (Fig. 32).

We showed that spermidine strongly induces autophagy in, flies and cultured human cells. Autophagy constitutes the major lysosomal degradation pathway recycling damaged and potentially harmful cellular material (such as damaged mitochondria). Of note, autophagy counteracts cell death and prolongs life span in various ageing models (Galluzzi *et al.*, *Curr Mol Med* **8**, 78-91 (2008)). Therefore, inhibition of necrotic cell death by autophagy could facilitate the long-term survival of spermidine-treated cells.

It is generally accepted that hypoacetylation is a key event of gene silencing (Guarente, *Genes Dev* **14**, 1021-1026 (2000)). Silencing might be concomitantly linked to lower metabolic rates causing less ROS (i.e. superoxide) and longevity (Guarente, *Genes Dev* **14**, 1021-1026 (2000)). This could be of high importance, especially in old cells, which suffer from damaged and inefficient mitochondria and therefore generate high

ROS levels when respiration is active. In support of this hypothesis, we demonstrate that spermidine-treated cells, which showed largely hypoacetylated histones, reduced their oxygen consumption (Fig. 32, day 20). Intriguingly, these cells resemble the status of quiescence as we demonstrated by sucrose-gradient separation of upper (non-quiescent) and lower (quiescent) cells (Fig. 33B). Quiescent cells are unbudded cells, exhibiting low ROS, reduced markers of apoptosis and necrosis, and low metabolic rates (Allen *et al.*, *J Cell Biol* **174**, 89-100 (2006).).

Interestingly, TOR depletion or rapamycin treatment, which also induces autophagy, similarly causes cells to enter a quiescent (G0-like) state (Jacinto & Hall, *Nat Rev Mol Cell Biol* **4**, 117-126 (2003)). Thus, autophagic processes as well as hypoacetylation-induced silencing might cooperate to promote longevity of non-growing cells by promoting a low-metabolic quiescent state.

Aging-associated necrotic death can be inhibited by simple spermidine application to yeast, flies, and human immune cells or by genetic modification of the HAT machinery in yeast, arguing in favor of programmed rather than accidental necrotic death. Necrotic cell death culminates in the leakage of intracellular compounds and consequent local inflammation, which in turn is suspected to be a driving force of aging ("inflammaging"). Recently, Franceschi *et al.* proposed that chronic inflammation may be one of the driving forces of human aging, causing immunosenescence (C. Franceschi *et al.*, *Mech Ageing Dev* **128**, 92-105 (2007)). In support of this theory, we showed that spermidine potently inhibits necrotic death during aging of human PBMCs and protects mice from oxidative stress. Thus, programmed necrotic processes might be of cardinal importance to understand the mechanisms of organismal aging in general.

Interestingly, polyamine concentrations decline during aging of various organisms, including humans (Scalabrino & Ferioli, *Mech Ageing Dev* **26**, 149-164 (1984).) and plants (Kaur-Sawhney *et al. Plant Physiol* **69**, 405-410 (1982)), and external application of spermidine inhibits oat leaf senescence (Altman *et al. Plant Physiol* **60**, 570-574 (1977)). Moreover, anti-oxidant as well as anti-inflammatory activities of polyamines have been described in human cells (Lovaas & Carlin, *Free Radic Biol Med* **11**, 455-461 (1991)).

Thus, our findings may have implications ranging from basic aging processes to human aging research.

Table 2. Effects on chronological aging of single disruption of genes involved in histone (de)acetylation.

The effects on chronological aging of 28 single deletion strains of genes involved in histone acetylation (***bold italic*** characters) or deacetylation (*italic* characters) are presented. All strains were aged with and without application of 4 mM spermidine and survival was determined by clonogenicity. Deletion strains were assigned to one of five categories, depending on the effects on survival during aging and the ability of spermidine to improve this survival.

Survival during chronological aging (compared to WT)	**Pro-survival effect of spermidine application (compared to WT)**	**Single deletion of…**
increased during early aging (day 5 to 15)	reduced	***SAS3, IKI3, ELP3***[1]
strongly reduced	increased during early aging (due to fast death of control cultures), BUT diminished or absent at later time points (day 15 to 25)	***GCN5, SGF73****, HDA2*
not affected	slightly reduced during early aging (day 5 to15)	***AHC1***
slightly increased	not affected	*HDA1*
slightly reduced	not affected	***SPT10,*** *RXT2, SDS3, SAP30*
not affected	not affected	***HAT1, HPA2, HPA3, YNG1,*** *HDA3, HOS1, HOS2, HOS3, HOS4, RPD3, PHO23, SIR2, HST1, HST2, SET3, SIF2*

[1]The pro-survival effect of spermidine in the ***ELP3*** deleted strain was only reduced until day 10 of aging.

CLAIMS

1. Use of a polyamine or a salt, solvate, or derivative thereof for the treatment or prevention of a life threatening condition in a critically ill patient with a non-infectuous disorder.

2. The use according to claim 1, wherein the polyamine is a metabolisable polyamine.

3. The use according to claim 1 or 2, wherein the polyamine is a substrate for the enzyme Spermine/Spermidine Acetyltranferase (SSAT).

4. The use according to any one of claim 1 to 3, wherein the polyamine is not modified at one or more of the NH_2 or NH groups.

5. The use according to any one of claim 1 to 4, wherein the polyamine is selected from the group consisting of putrescine (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, L-arginyl-3,4-spermidine and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride.

6. The use according to any one of claim 1 to 5, wherein the polyamine is spermine or spermidine.

7. The use according to any one of claim 1 to 6, wherein the life threatening condition is selected from the group consisting of lactic acidosis, muscle weakening, hyperglycemia, multiple organ failure and failed or disturbed homeostasis.

8. The use according to any one of claim 1 to 7, wherein the critically ill patient is a patient receiving enteral or parenteral nutrition.

9. The use according to any one of claim 1 to 8, wherein the polyamine is administered together with an enteral or parenteral nutritient composition.

10. The use according to any one of claim 1 to 9, wherein the disorder of the critically ill patient is selected from the group consting of severe or multiple trauma, high risk or extensive surgery, cerebral trauma or bleeding, respiratory insufficiency, abdominal peritonitis, acute kidney injury, acute liver injury, severe burns and critical illness polyneuropathy.

11. Use of a polyamine, or a salt, solvate, or derivative thereof for manufacture of a medicament for the treatment or prevention of a life threatening condition in a critically ill patient with a non-infectuous disorder.

12. The use according to claim 11, wherein the polyamine is a metabolisable polyamine.

13. The use according to claim 11 or 12, wherein the polyamine is a substrate for the enzyme Spermine/Spermidine Acetyltranferase (SSAT).

14. The use according to any one of claim 11 to 13, wherein the polyamine is not modified at one or more of the NH_2 or NH groups.

15. The use according to any one of claim 11 to 14, wherein the polyamine is selected from the group consisting of putrescine (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, L-arginyl-3,4-spermidine and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride.

16. The use according to any one of claim 11 to 15, wherein the polyamine is spermine or spermidine.

17. The use according to any one of claim 11 to 16, wherein the life threatening condition is selected from the group consisting of lactic acidosis, muscle weakening, hyperglycemia, multiple organ failure and failed or disturbed homeostasis.

18. The use according to any one of claim 11 to 17, wherein the critically ill patient is a patient receiving enteral or parenteral nutrition.

19. The use according to any one of claim 11 to 18, wherein the polyamine is administered together with a enteral or parenteral nutritient composition.

20. The use according to any one of claim 11 to 19, wherein the disorder of the critically ill patient is selected from the group consting of severe or multiple trauma, high risk or extensive surgery, cerebral trauma or bleeding, respiratory insufficiency, abdominal peritonitis, acute kidney injury, acute liver injury, severe burns and critical illness polyneuropathy.

21. A nutrient solution suitable for parenteral administration, said nutrient solution comprising a saccharide, characterised in that said solution further comprises a polyamine or a salt, solvate, or derivative thereof.

22. The nutrient solution according to claim 21, wherein said solution is suitable for intravenous administration.

23. The nutrient solution according to claim 21 or 22, wherein said saccharide is present in said solution in a concentration between 10 and 20 % (w/v).

24. The nutrient solution according to any one of claim 21 to 23, wherein said saccharide is glucose.

25. The nutrient solution according to any one of claim 21 to 24, wherein said polyamine is present in said solution in a concentration between 0,05 to 4 (w/v), between 0,5 to 2 (w/v), or between 1 to 1,5 % (w/v).

26. The solution according any one of claim 21 to 25, wherein the polyamine is a metabolisable polyamine.

27. The solution according to any one of claim 21 to 26, wherein the polyamine is a substrate for the enzyme Spermine/Spermidine Acetyltranferase (SSAT).

28. The solution according to any one of claim 21 to 27, wherein the polyamine is not modified at one or more of the NH_2 or NH groups.

29. The solution according to any one of claim 21 to 28, wherein the polyamine is selected from the group consisting of putrescine (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, L-arginyl-3,4-spermidine and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride.

30. The solution according to any one of claim 21 to 29, wherein the polyamine is spermine or spermidine.

31. The nutrient solution according to any one of claim 21 to 30, further comprising lipids.

32. Use of a saccharide and a polyamine or a salt, solvate, or derivative thereof as a medicament.

33. The use according to claim 32, wherein the polyamine is a metabolisable polyamine.

34. The use to claim 32 or 33, wherein the polyamine is a substrate for the enzyme Spermine/Spermidine Acetyltranferase (SSAT).

35. The use according to any one of claim 32 to 34, wherein the polyamine is not modified at one or more of the NH_2 or NH groups.

36. The use according to any one of claims 32 to 35, wherein said polyamine is selected from the group consisting of putrescine (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate, spermidine phosphate hexahydrate, L-arginyl-3,4-spermidine and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride.

37. Use of a saccharide and a polyamine or a salt, solvate, or derivative thereof for the treatment or prevention of a life threatening condition in a critically ill patient with a non-infectuous disorder.

38. Use of a saccharide and a polyamine or a salt, solvate, or derivative for the manufacture of a medicament for the treatment or prevention of a life threatening condition in a critically ill patient with a non infectuous disorder.

39. The use according to claim 38, wherein said medicament is a solution suitable for intravenous administration.

40. The use according to any one of claim 37 to 39, wherein said saccharide is present in said solution in a concentration between 10 and 20 % (w/v).

41. The use according to any one of claims 37 to 40, wherein said saccharide is glucose.

42. The use according to any one of claims 37 to 41, wherein said polyamine is present in said solution in a concentration between 0,5 to 4 (w/v), between 0,5 to 2 (w/v), or between 1 to 1,5 % (w/v).

43. The solution according any one of claim 37 to 42, wherein the polyamine is a metabolisable polyamine.

44. The solution according to any one of claim 37 to 43, wherein the polyamine is a substrate for the enzyme Spermine/Spermidine Acetyltranferase (SSAT).

45. The solution according to any one of claim 37 to 44, wherein the polyamine is not modified at one or more of the NH_2 or NH groups.

46. The use according to any one of claim 37 to 45, wherein the polyamine is selected from the group consisting of putrescine (1,4-diamino-butane), 1,3-diamino-propane, 1,7-diamino-heptane, 1,8-diamino-octane, spermine, spermidine, cholesteryl spermine, spermidine trihydrochloride, spermidine phosphate hexahydrate,

spermidine phosphate hexahydrate, L-arginyl-3,4-spermidine and 1,4-butanediamine N-(3-aminopropyl)-monohydrochloride.

47. The use according to any one of claim 37 to 46, wherein the metabolisable polyamine is spermine or spermidine.

48. A method of treating a life threatening condition in a critically ill patient with a non infectuous disorder comprising the step of adminstering to said patient of a polyamine, or a salt, solvate, or derivative thereof.

49. A pharmaceutical composition comprising a nutrient solution comprising a polyamine or a salt, solvate, or derivative thereof according to claim 21 for the treatment or prevention of a life threatening condition in a critically ill patient with a non-infectuous disorder, administering said a polyamine or a salt, solvate, or derivative thereof in one or more doses between 70 mg and 7 gram per day.

50. The pharmaceutical composition according to claim 49, wherein said polyamine is adminstered between 10 µg/kg body weight/day to about 100 mg/kg/day.

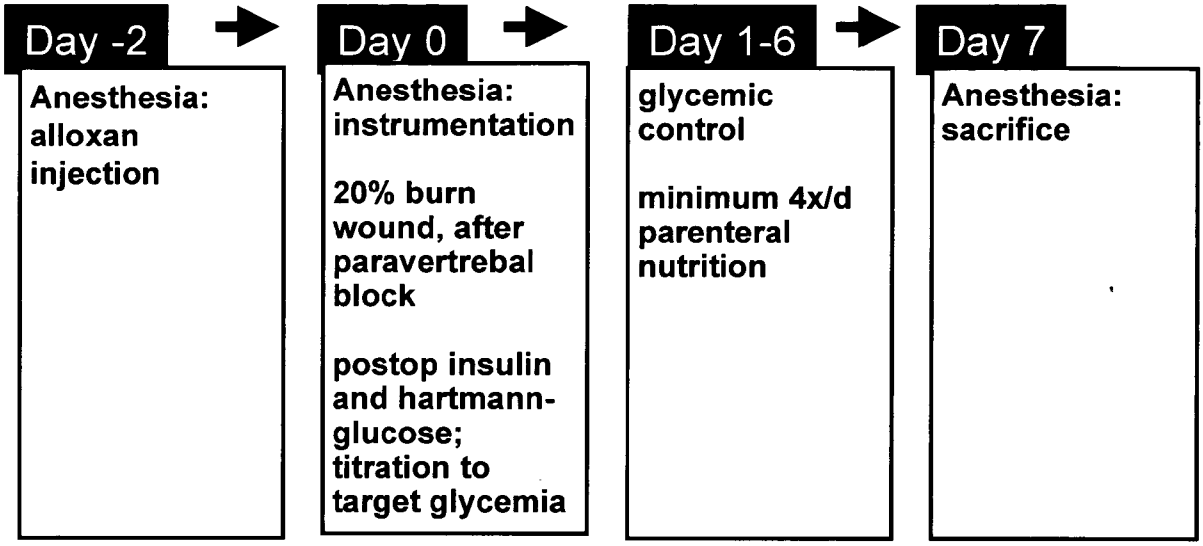


Figure 1

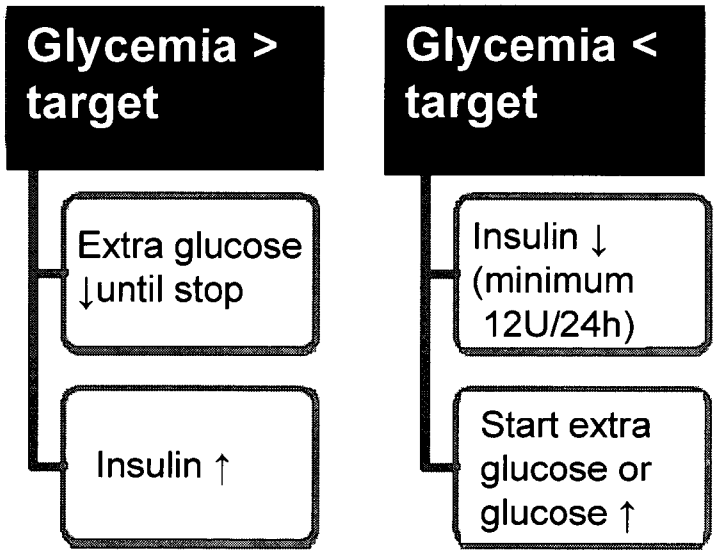


Figure 2

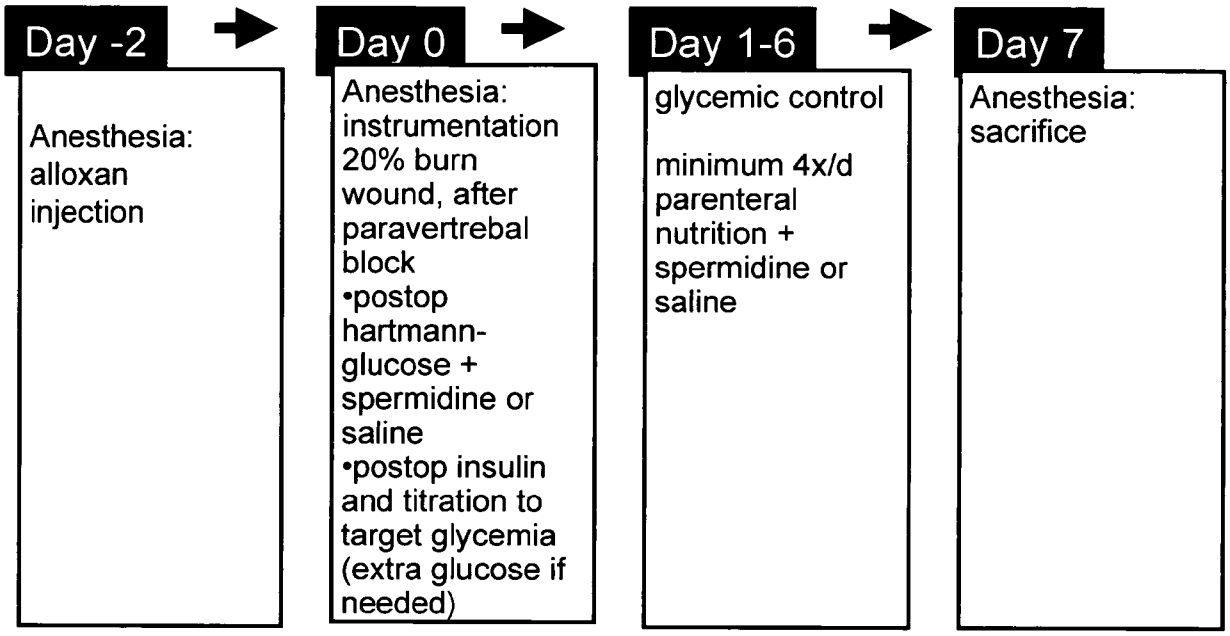


Figure 3

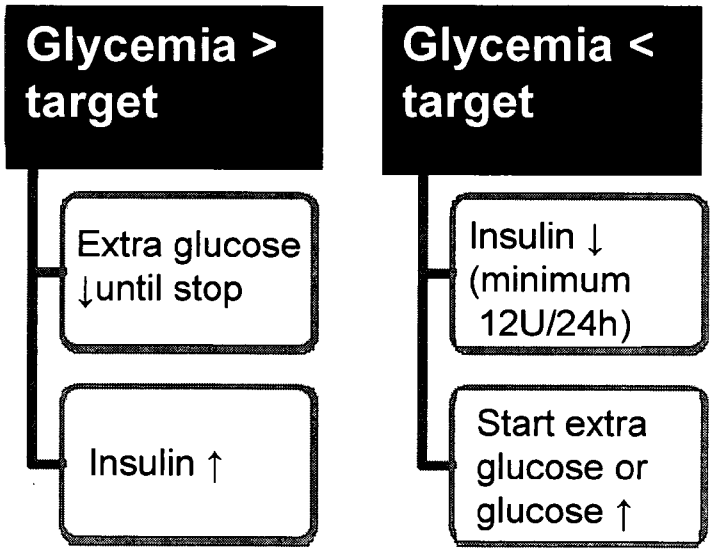


Figure 4

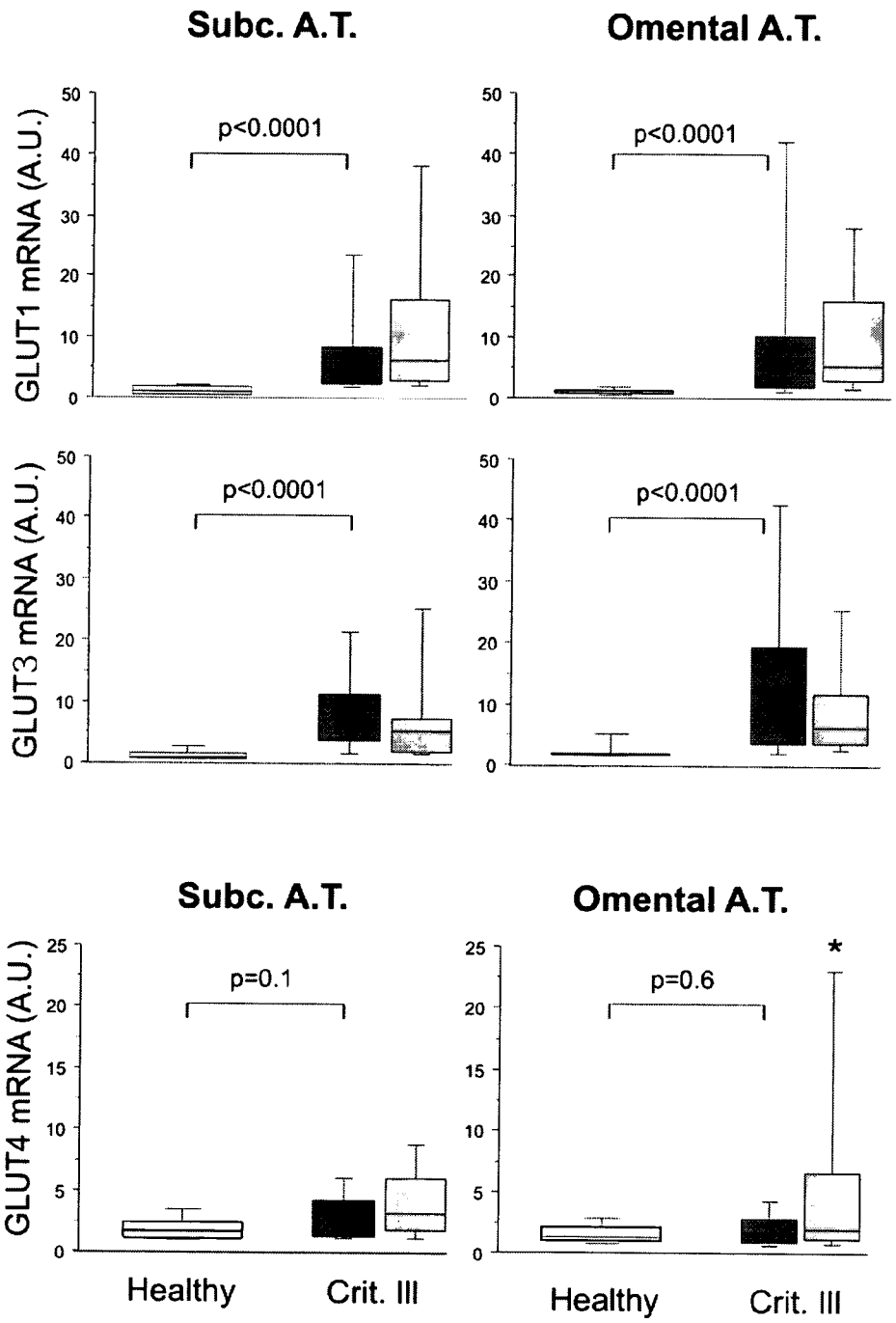
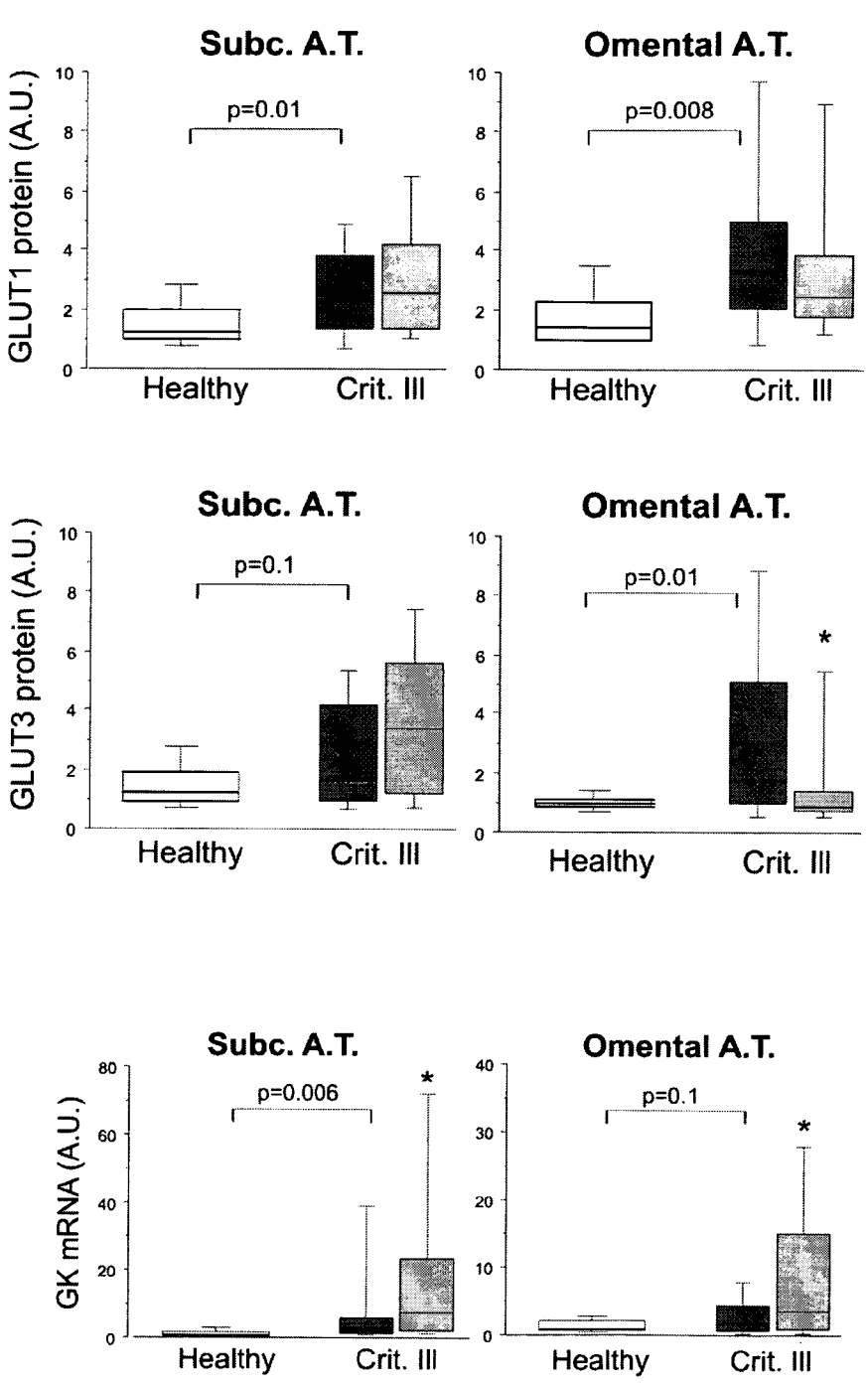

Subc. A.T.
Omental A.T.
A
GLUT1 mRNA (A.U.)
p<0.0001
p<0.0001
GLUT3 mRNA (A.U.)
p<0.0001
p<0.0001
Subc. A.T.
Omental A.T.
GLUT4 mRNA (A.U.)
p=0.1
p=0.6
*
Healthy
Crit. III
Healthy
Crit. III

Figure 5



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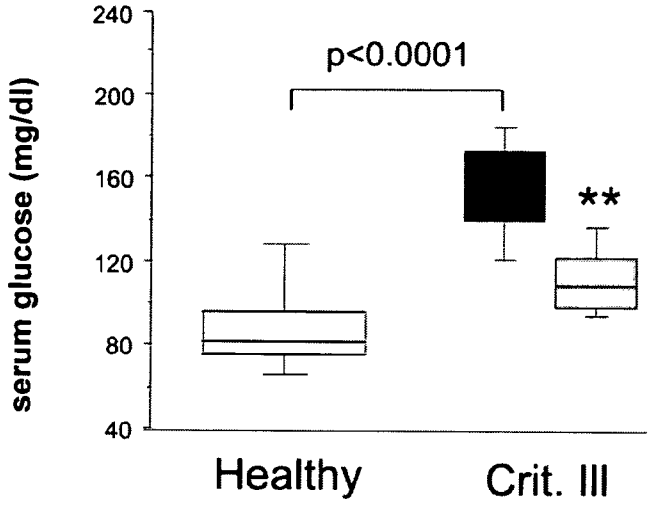

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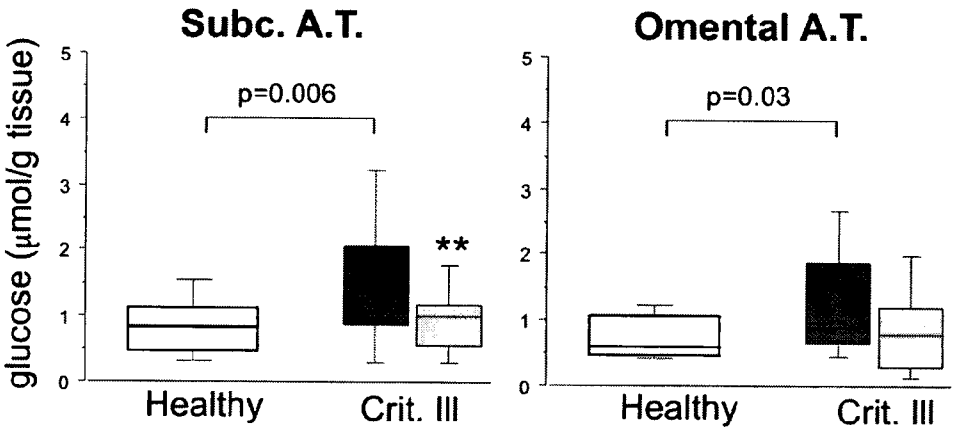

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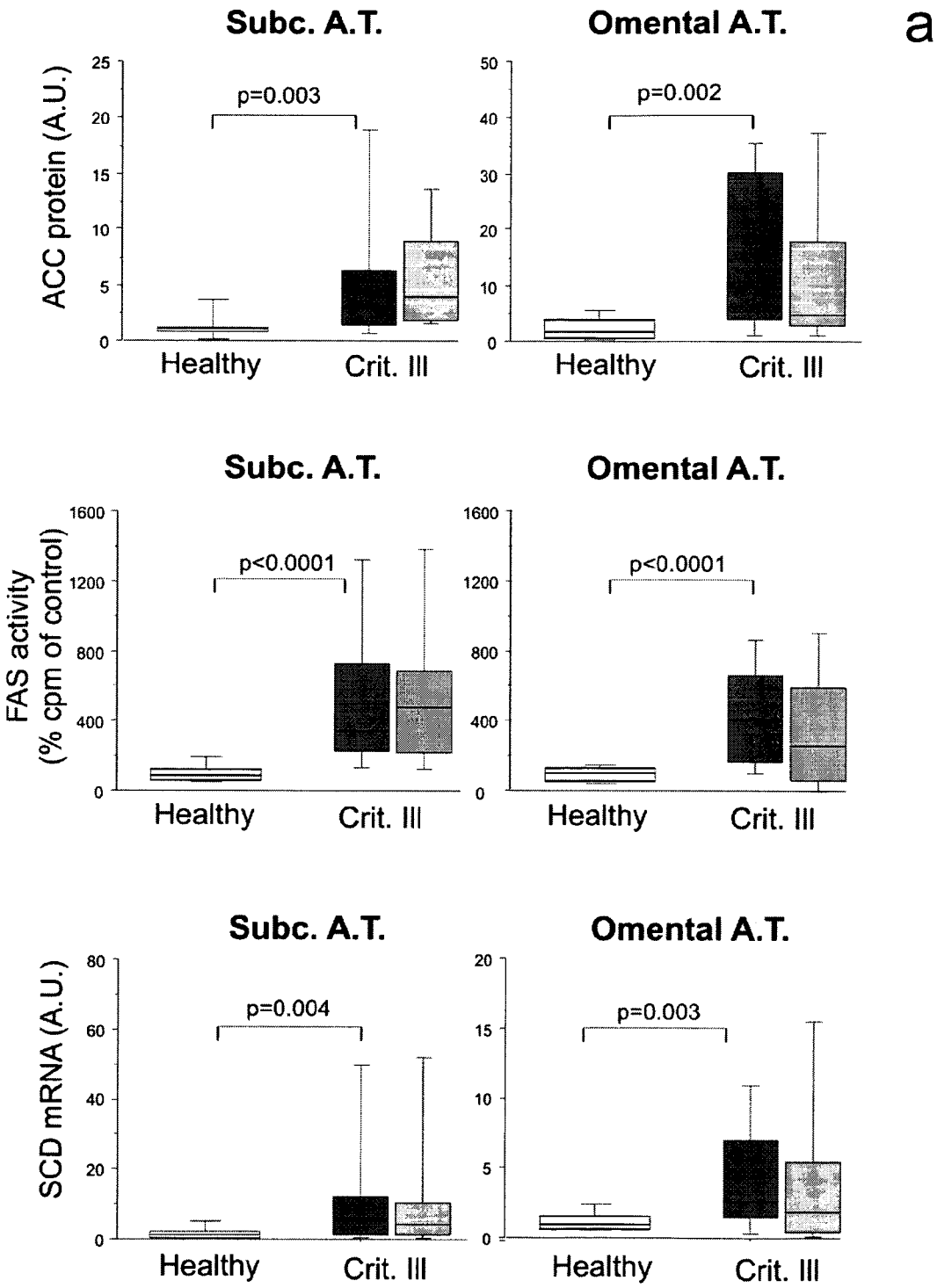
a
Subc. A.T.
Omental A.T.
ACC protein (A.U.)
p=0.003
p=0.002
Healthy
Crit. Ill
FAS activity (% cpm of control)
p<0.0001
SCD mRNA (A.U.)
p=0.004
p=0.003

Figure 6

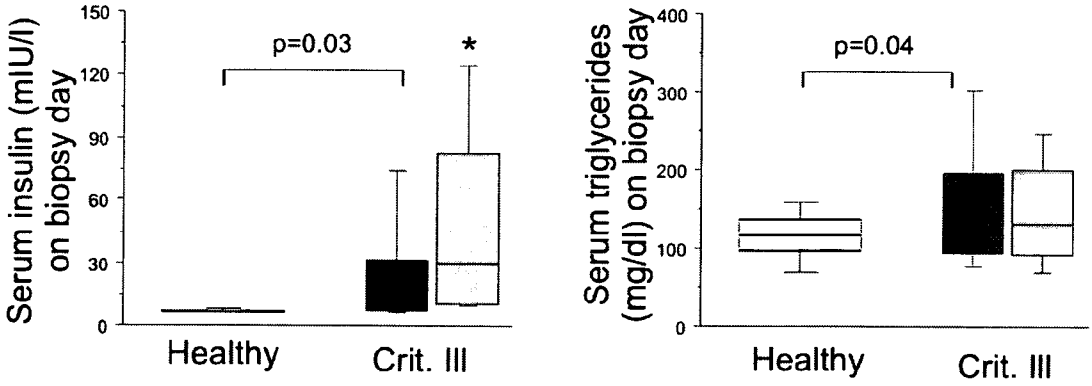

Figure 6 (continued)

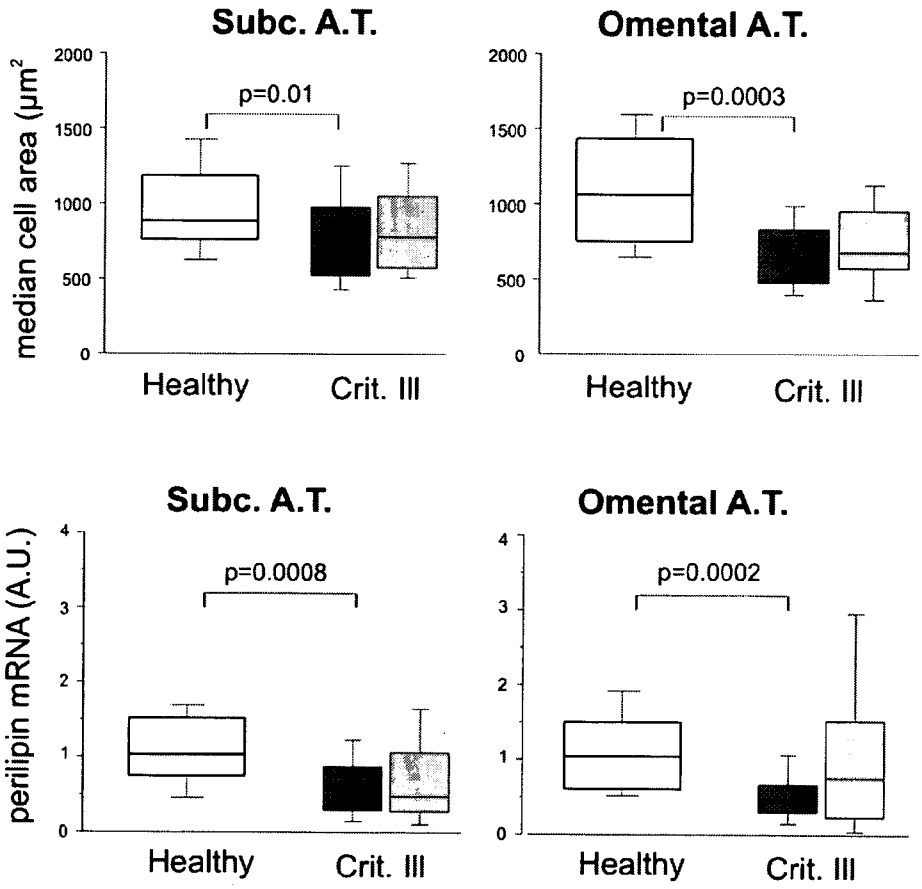

Figure 7

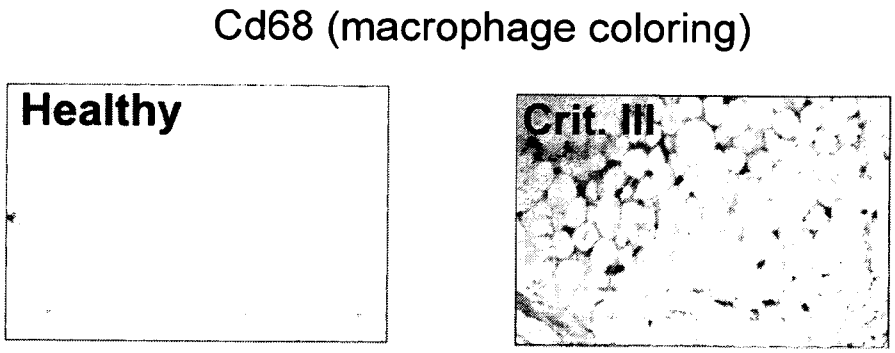

Figure 8

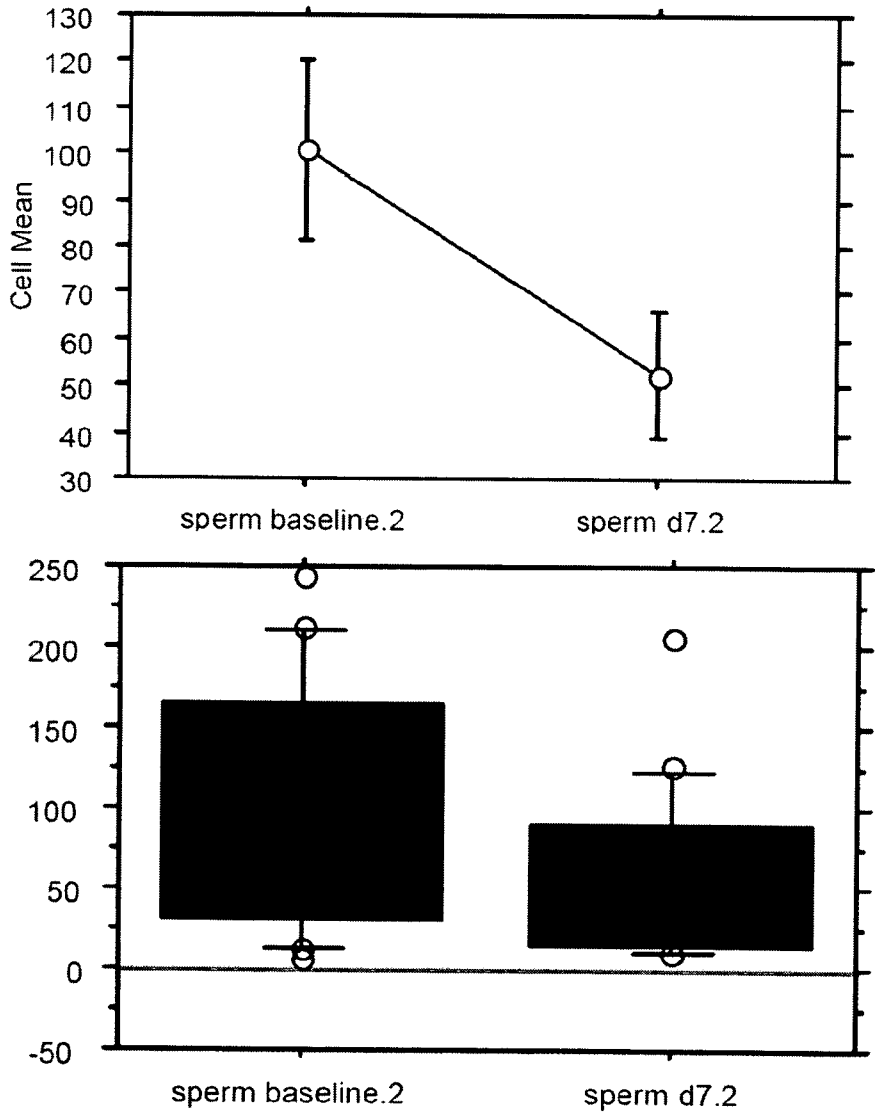

Figure 9

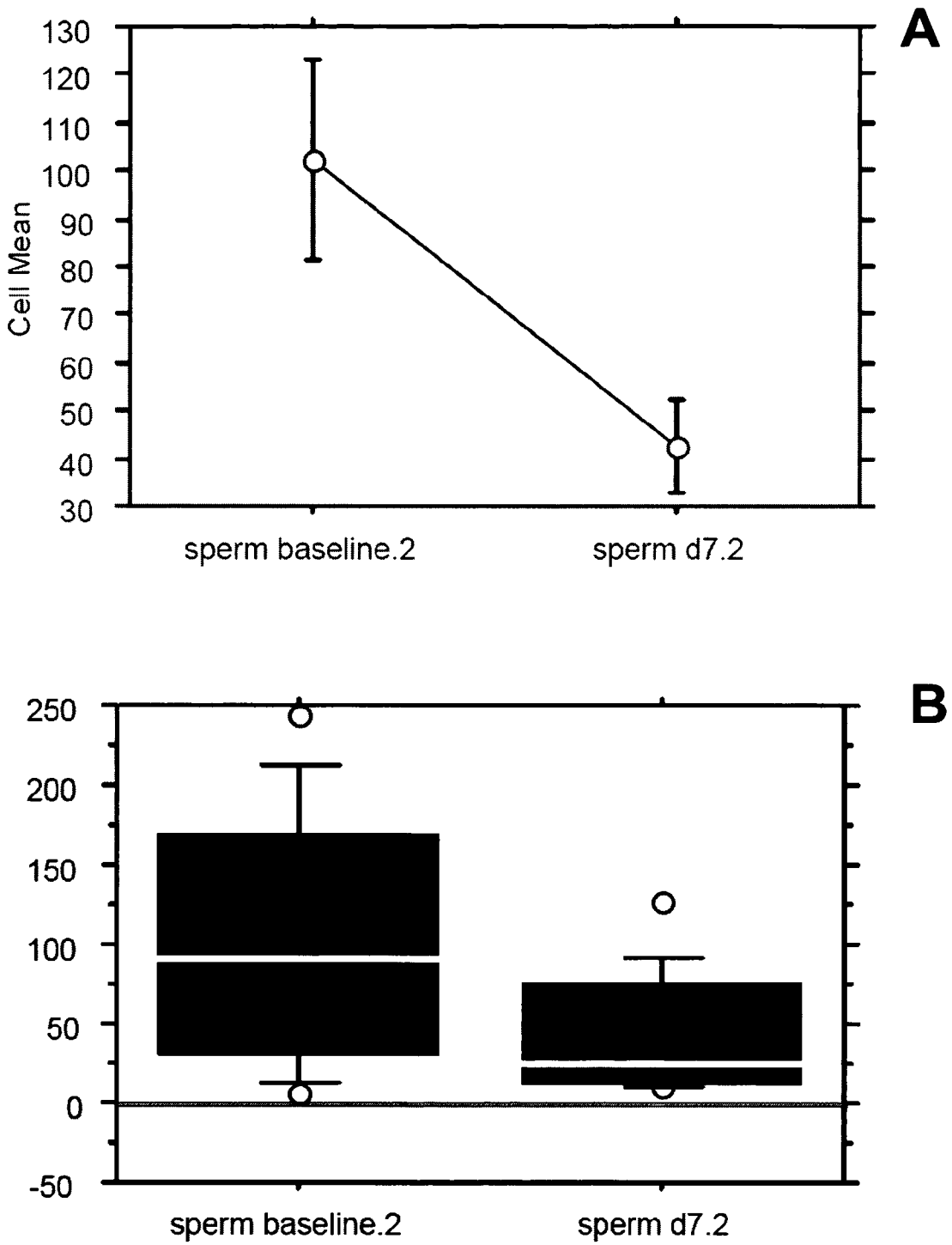
A
130
120
110
100
90
80
70
60
50
40
30
Cell Mean
sperm baseline.2
sperm d7.2
B
250
200
150
100
50
0
-50
sperm baseline.2
sperm d7.2

Figure 10

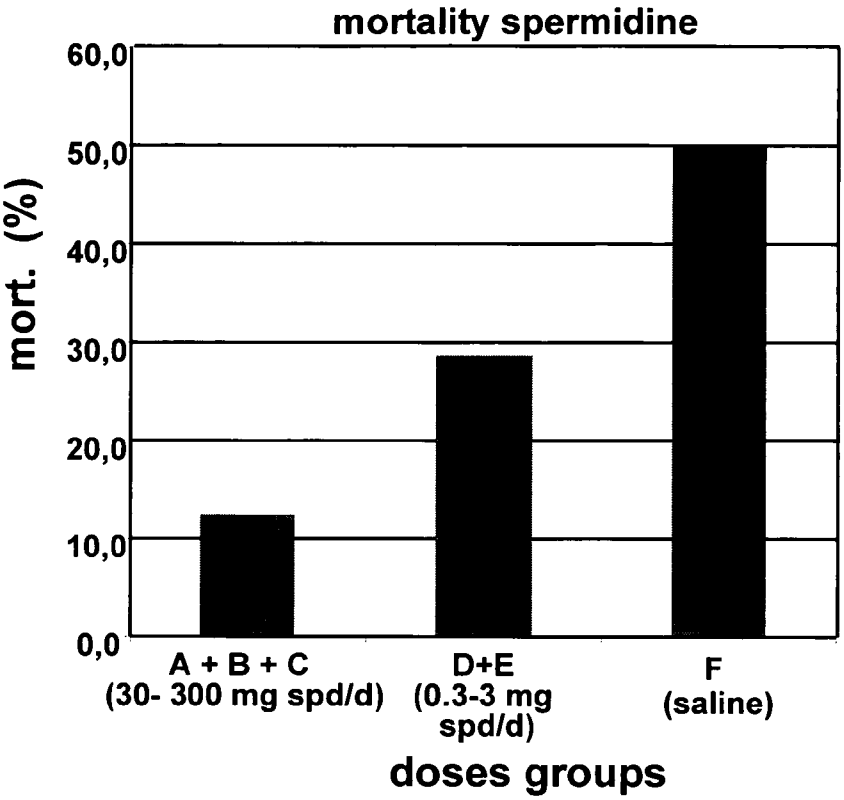

Figure 11

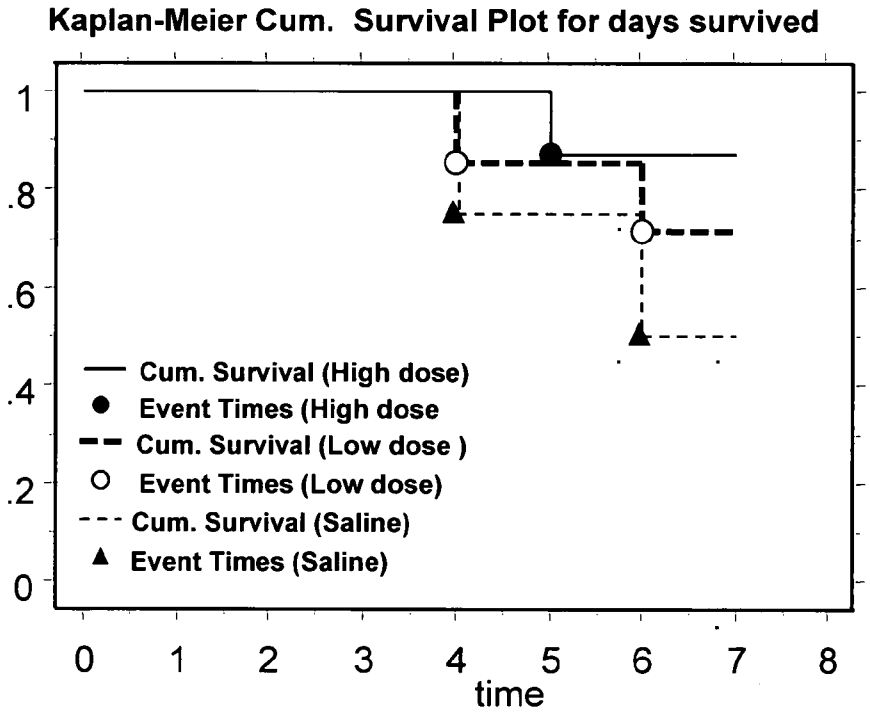

Figure 12

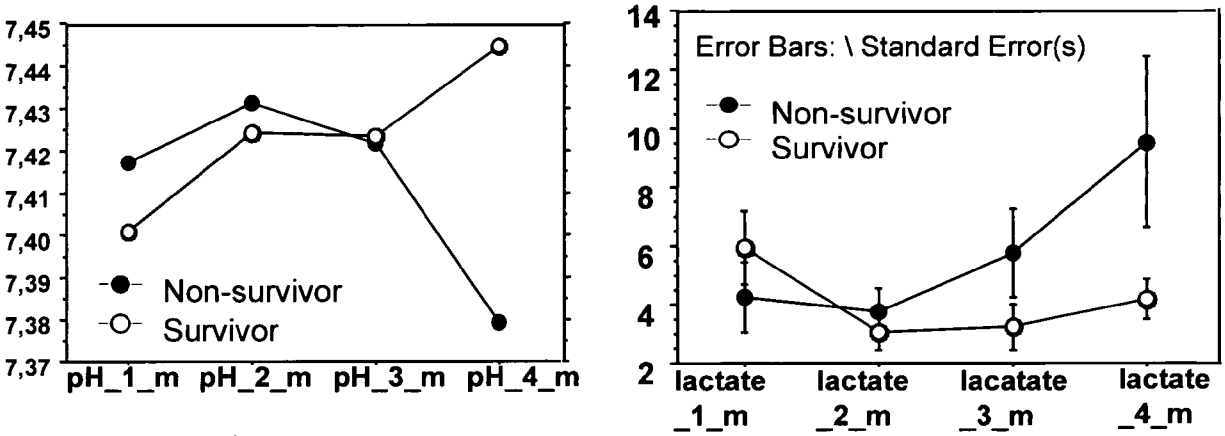

Figure 13

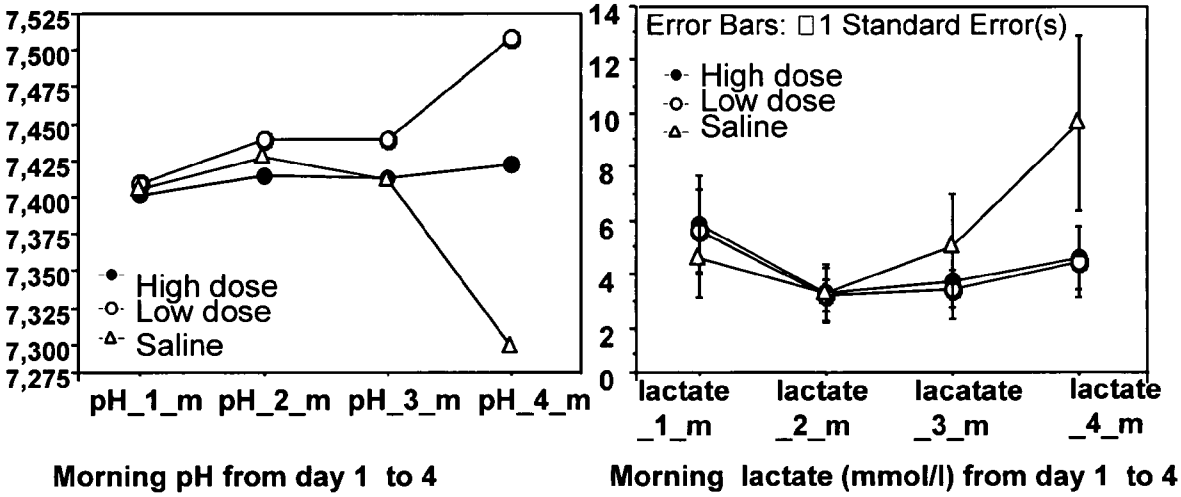

Figure 14

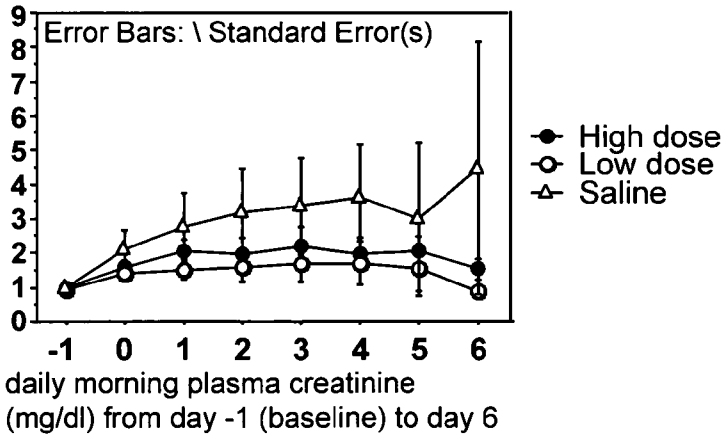

Figure 15

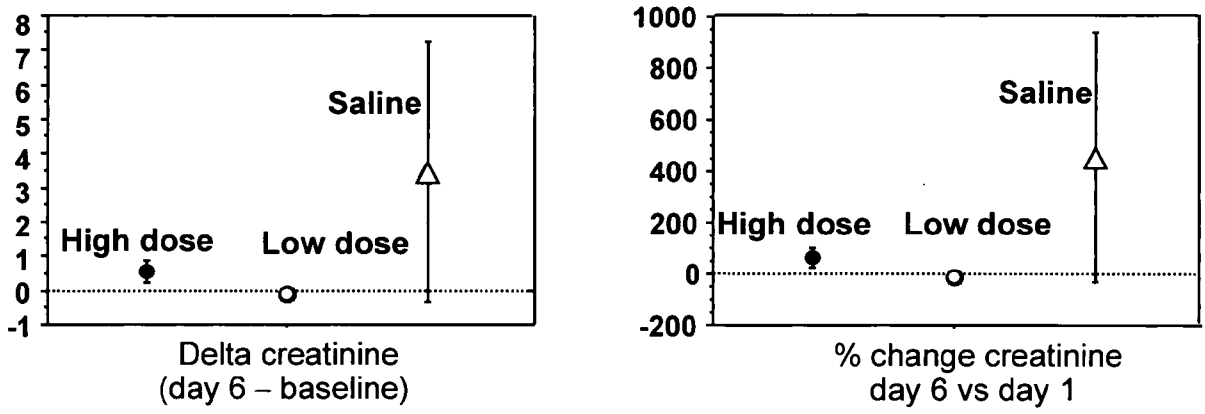

Figure 15 (continued)

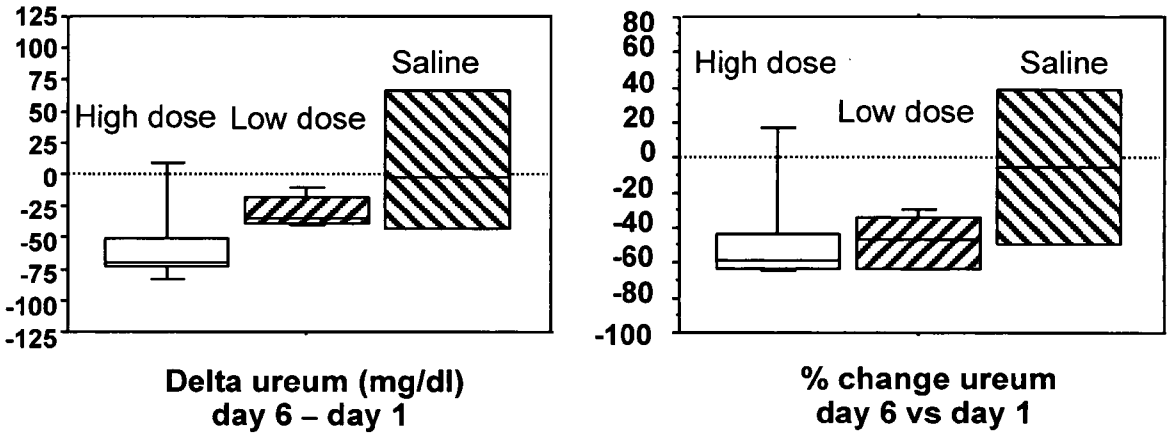

Figure 16

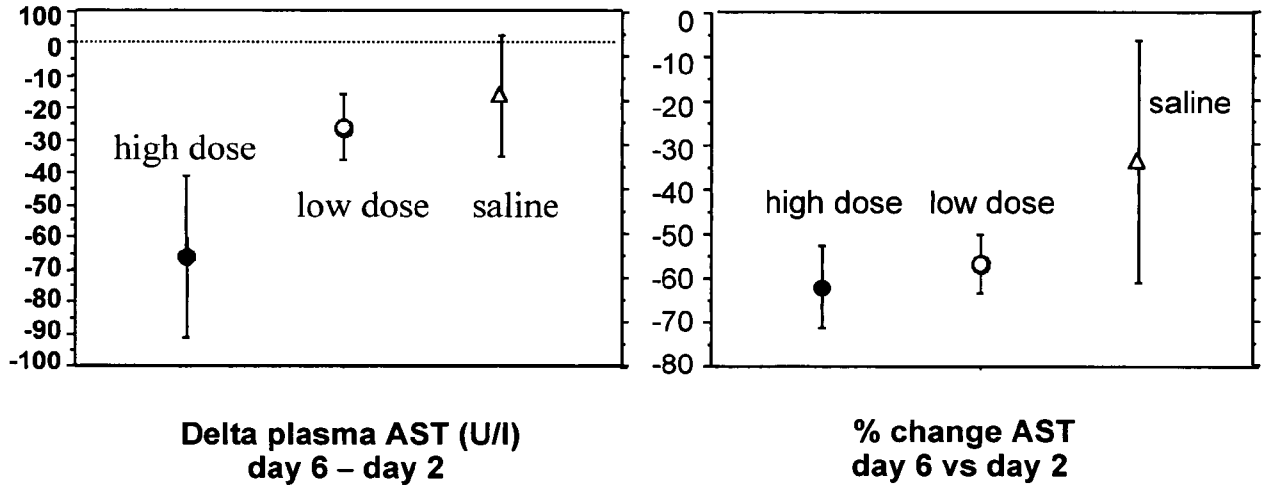

Figure 17

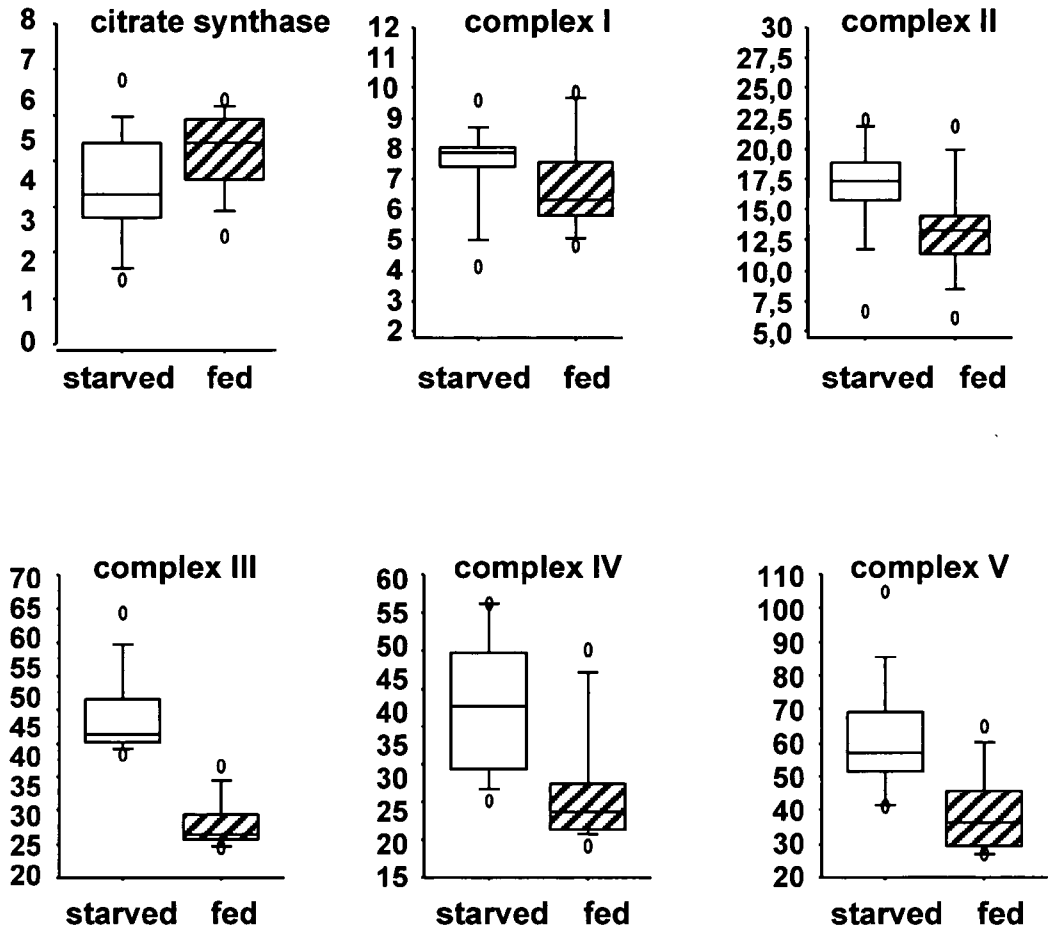
activity/ g liver
citrate synthase
complex I
complex II
complex III
complex IV
complex V
starved
fed

Figure 18

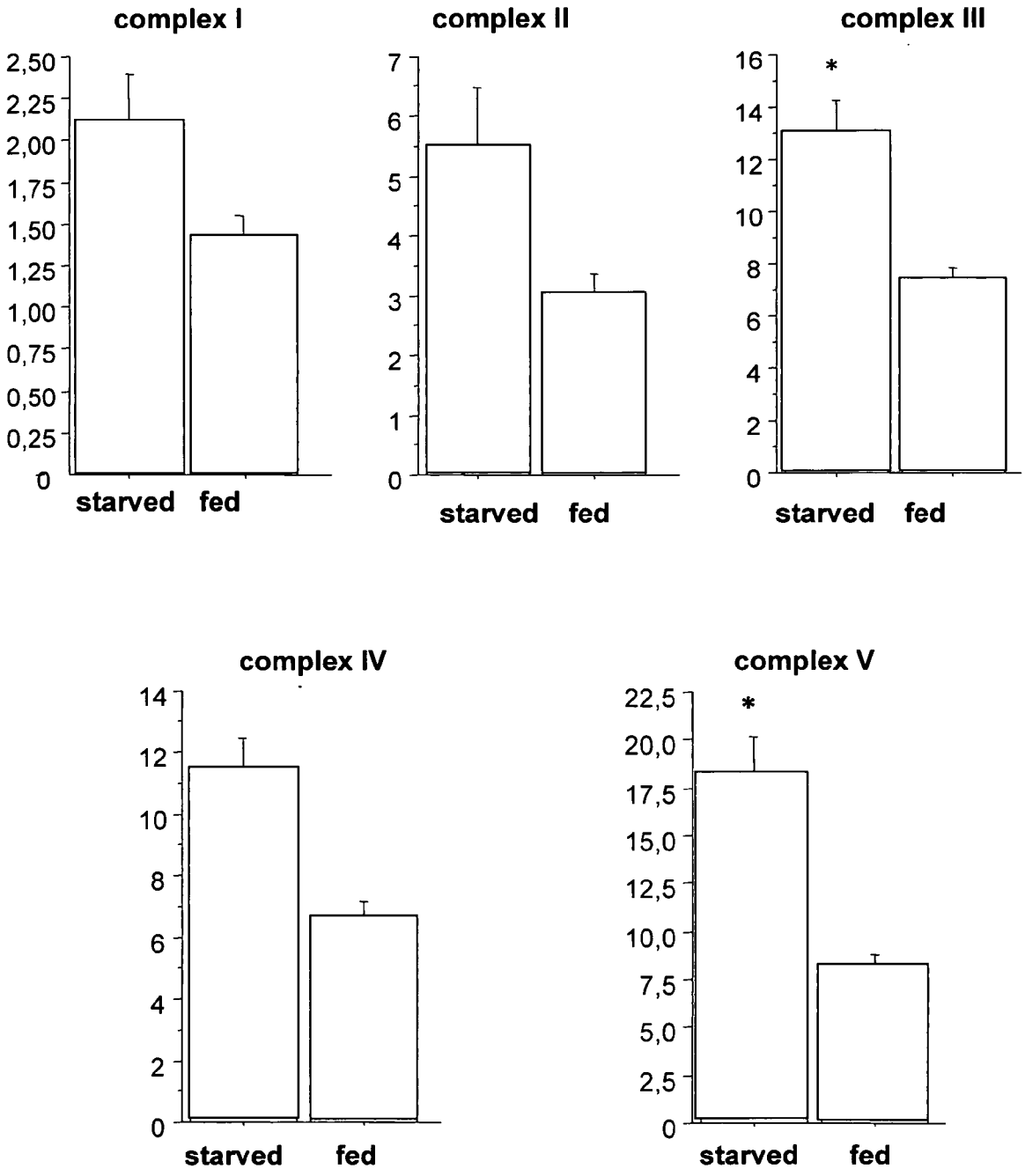

Figure 18 (continued)

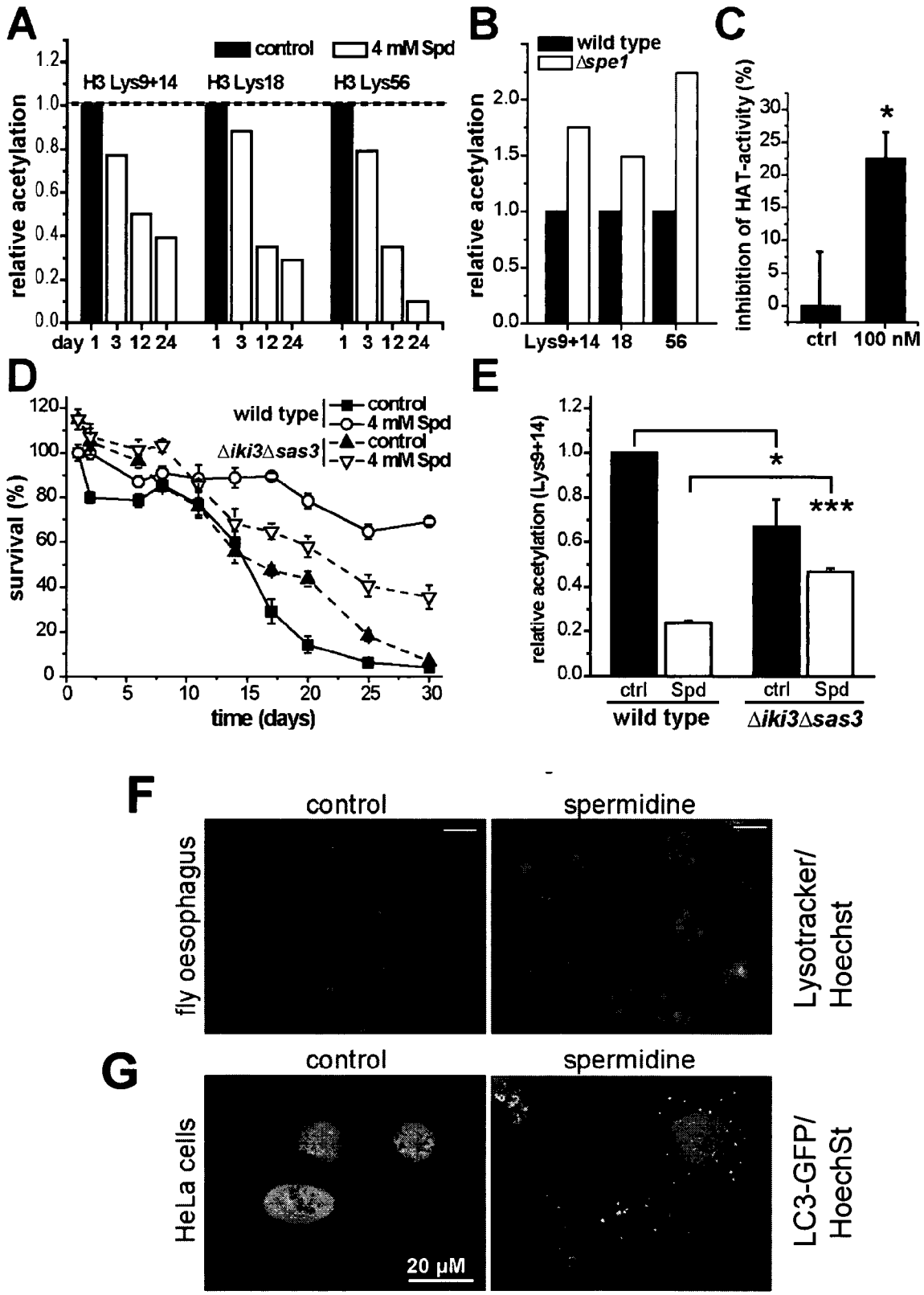
A
control
4 mM Spd
H3 Lys9+14
H3 Lys18
H3 Lys56
relative acetylation
day 1 3 12 24 1 3 12 24 1 3 12 24
B
wild type
Δspe1
relative acetylation
Lys9+14 18 56
C
inhibition of HAT-activity (%)
ctrl 100 nM
D
wild type
control
4 mM Spd
Δiki3Δsas3
control
4 mM Spd
survival (%)
time (days)
E
relative acetylation (Lys9+14)
ctrl Spd ctrl Spd
wild type
Δiki3Δsas3
F
control
spermidine
fly oesophagus
Lysotracker/ Hoechst
G
control
spermidine
HeLa cells
20 μM
LC3-GFP/ HoechSt

Figure 19

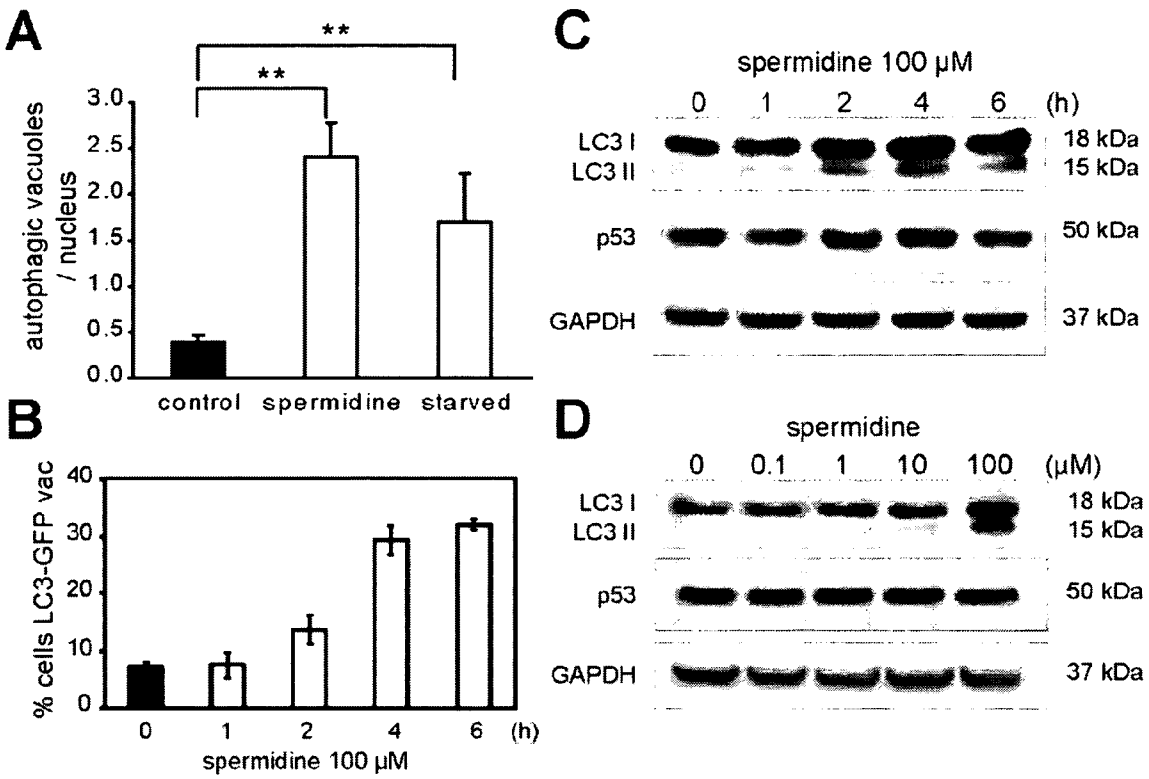
A
**
**
autophagic vacuoles / nucleus
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
spermidine
starved
B
% cells LC3-GFP vac
40
30
20
10
0
0
1
2
4
6
(h)
spermidine 100 µM
C
spermidine 100 µM
0
1
2
4
6
(h)
LC3 I
LC3 II
18 kDa
15 kDa
p53
50 kDa
GAPDH
37 kDa
D
spermidine
0
0.1
1
10
100
(µM)
LC3 I
LC3 II
18 kDa
15 kDa
p53
50 kDa
GAPDH
37 kDa

Figure 20

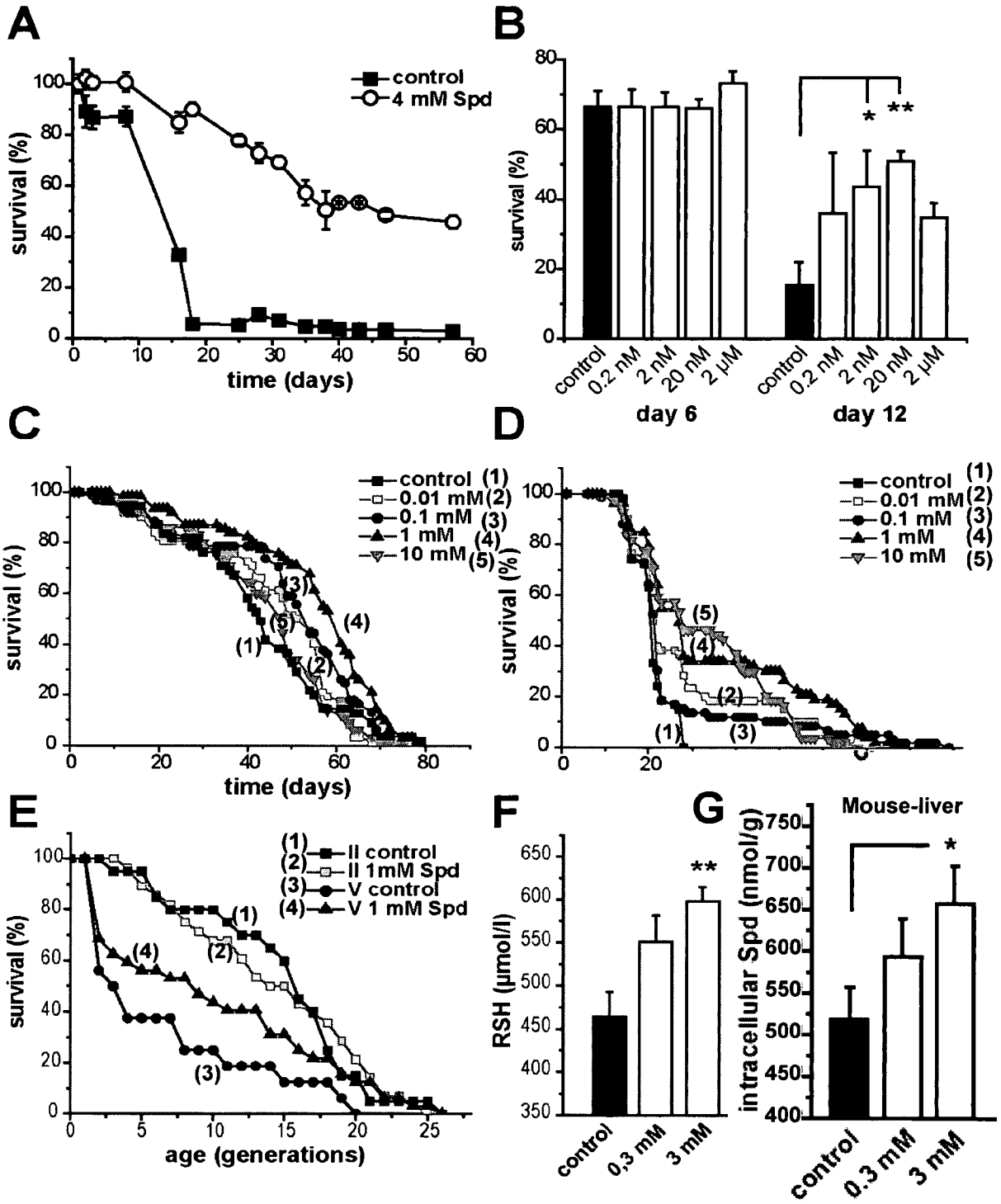
A
control
4 mM Spd
survival (%)
time (days)
B
survival (%)
control
0.2 nM
2 nM
20 nM
2 µM
day 6
day 12
C
control (1)
0.01 mM (2)
0.1 mM (3)
1 mM (4)
10 mM (5)
survival (%)
time (days)
D
control (1)
0.01 mM (2)
0.1 mM (3)
1 mM (4)
10 mM (5)
survival (%)
E
(1) II control
(2) II 1mM Spd
(3) V control
(4) V 1 mM Spd
survival (%)
age (generations)
F
RSH (µmol/l)
control
0,3 mM
3 mM
G
Mouse-liver
intracellular Spd (nmol/g)
control
0.3 mM
3 mM

Figure 21

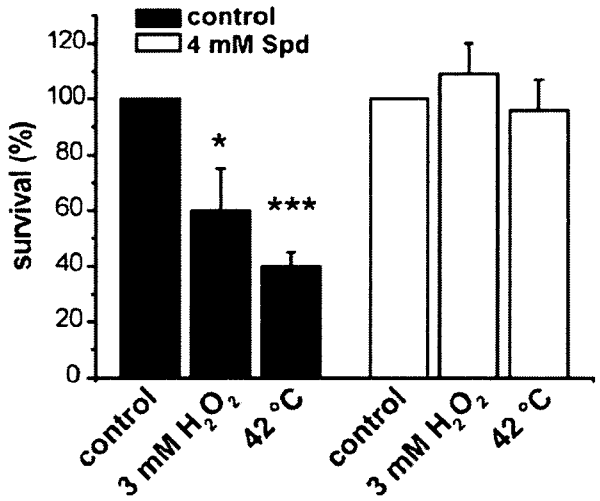

Figure 22

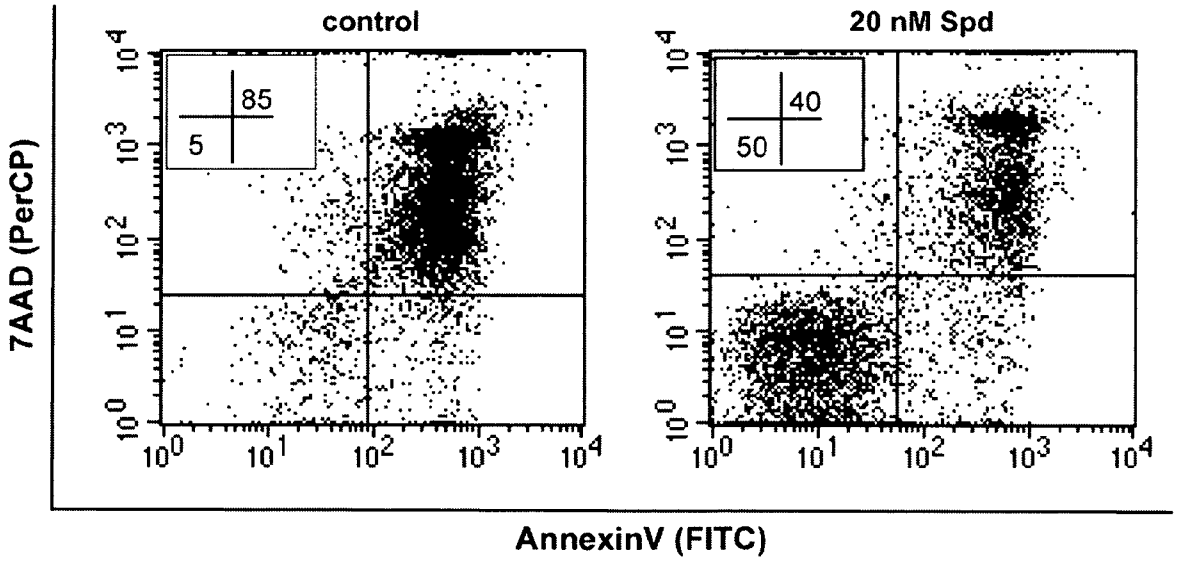

Figure 23

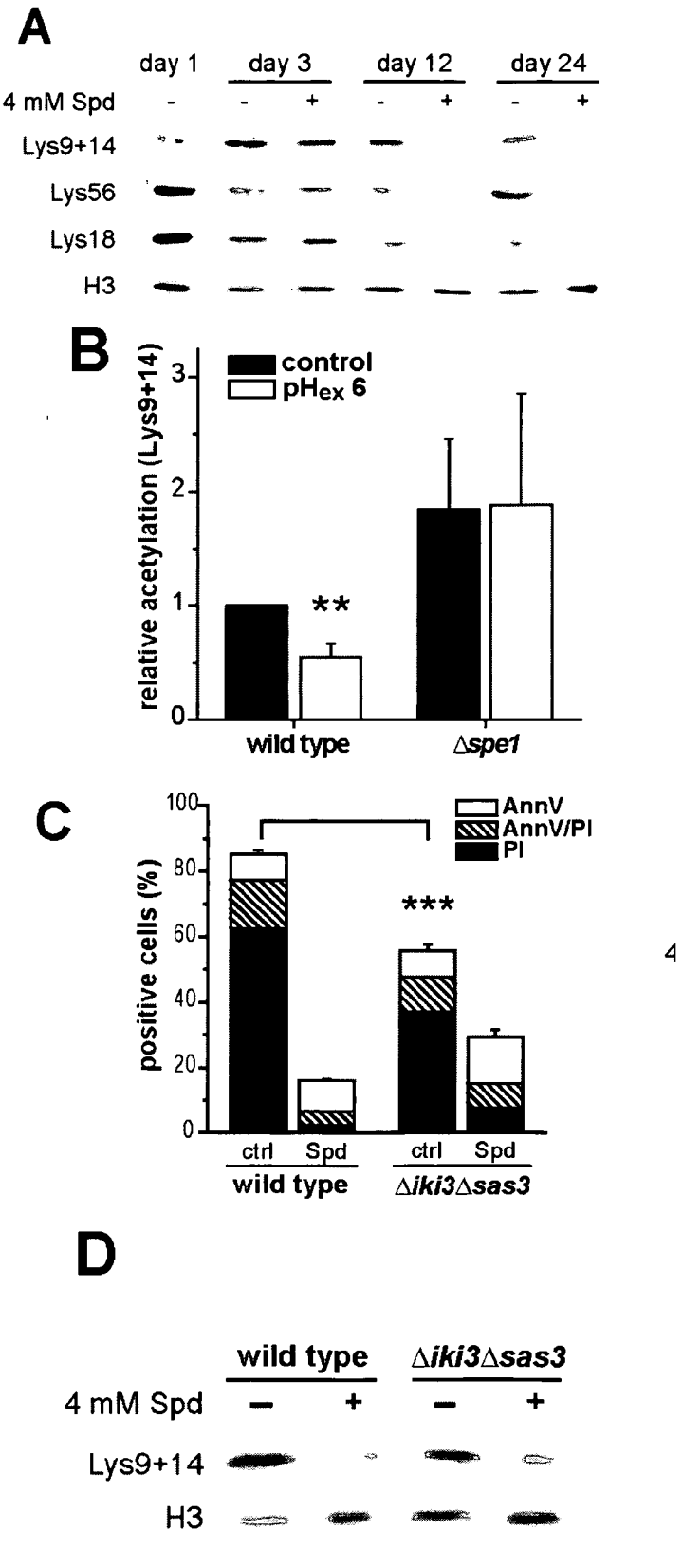
A
day 1
day 3
day 12
day 24
4 mM Spd
- - + - + - +
Lys9+14
Lys56
Lys18
H3
B
control
pHex 6
relative acetylation (Lys9+14)
0
1
2
3
**
wild type
Δspe1
C
AnnV
AnnV/PI
PI
positive cells (%)
0
20
40
60
80
100
***
ctrl Spd ctrl Spd
wild type
Δiki3Δsas3
D
wild type
Δiki3Δsas3
4 mM Spd
- + - +
Lys9+14
H3

Figure 24

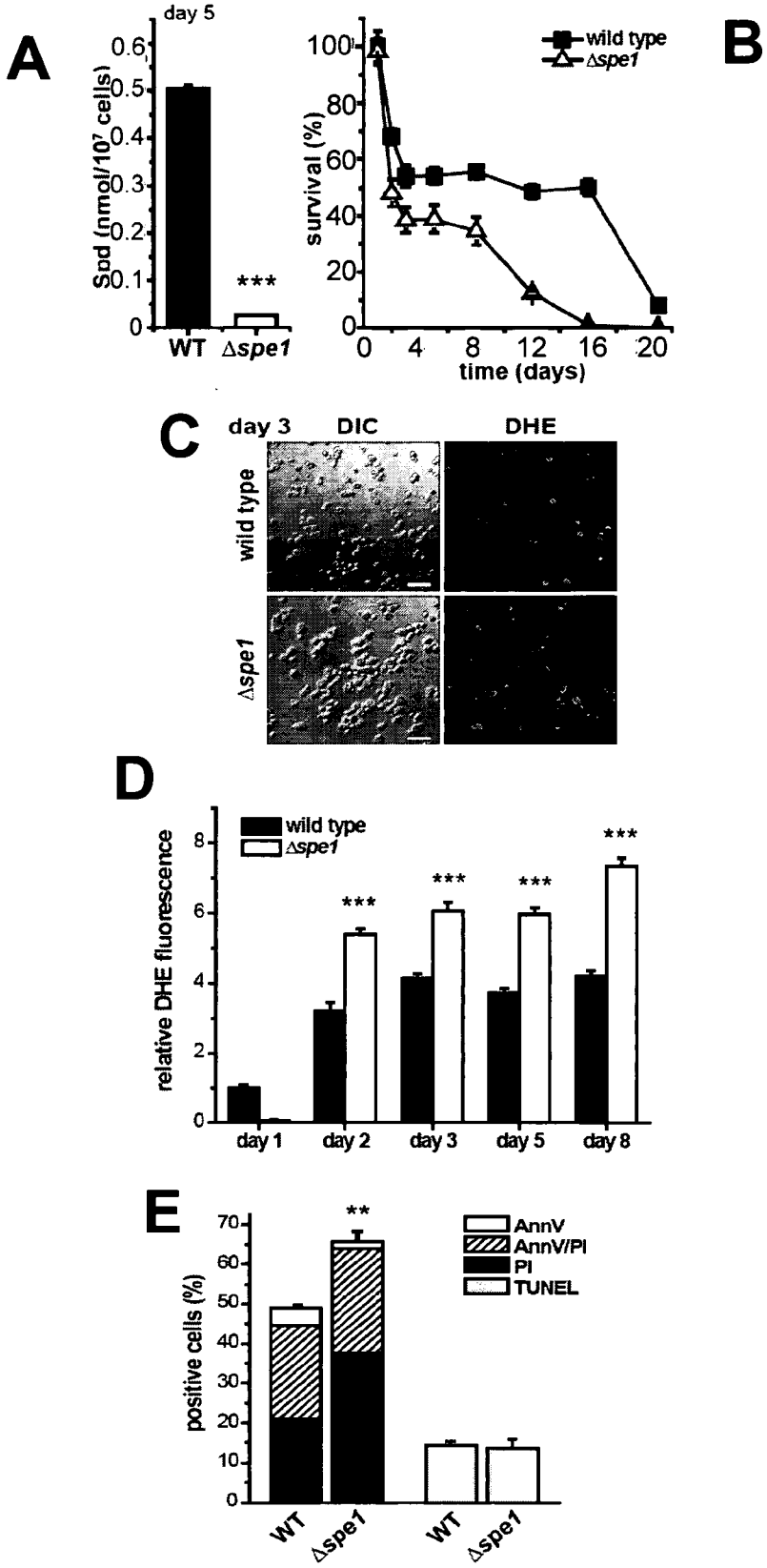
A
day 5
Spd (nmol/10^7 cells)
0.6
0.5
0.4
0.3
0.2
0.1
0
***
WT
Δspe1
B
wild type
Δspe1
survival (%)
100
80
60
40
20
0
0 4 8 12 16 20
time (days)
C
day 3
DIC
DHE
wild type
Δspe1
D
wild type
Δspe1
relative DHE fluorescence
8
6
4
2
0
***
***
***
***
day 1
day 2
day 3
day 5
day 8
E
positive cells (%)
70
60
50
40
30
20
10
0
**
AnnV
AnnV/PI
PI
TUNEL
WT
Δspe1
WT
Δspe1

Figure 25

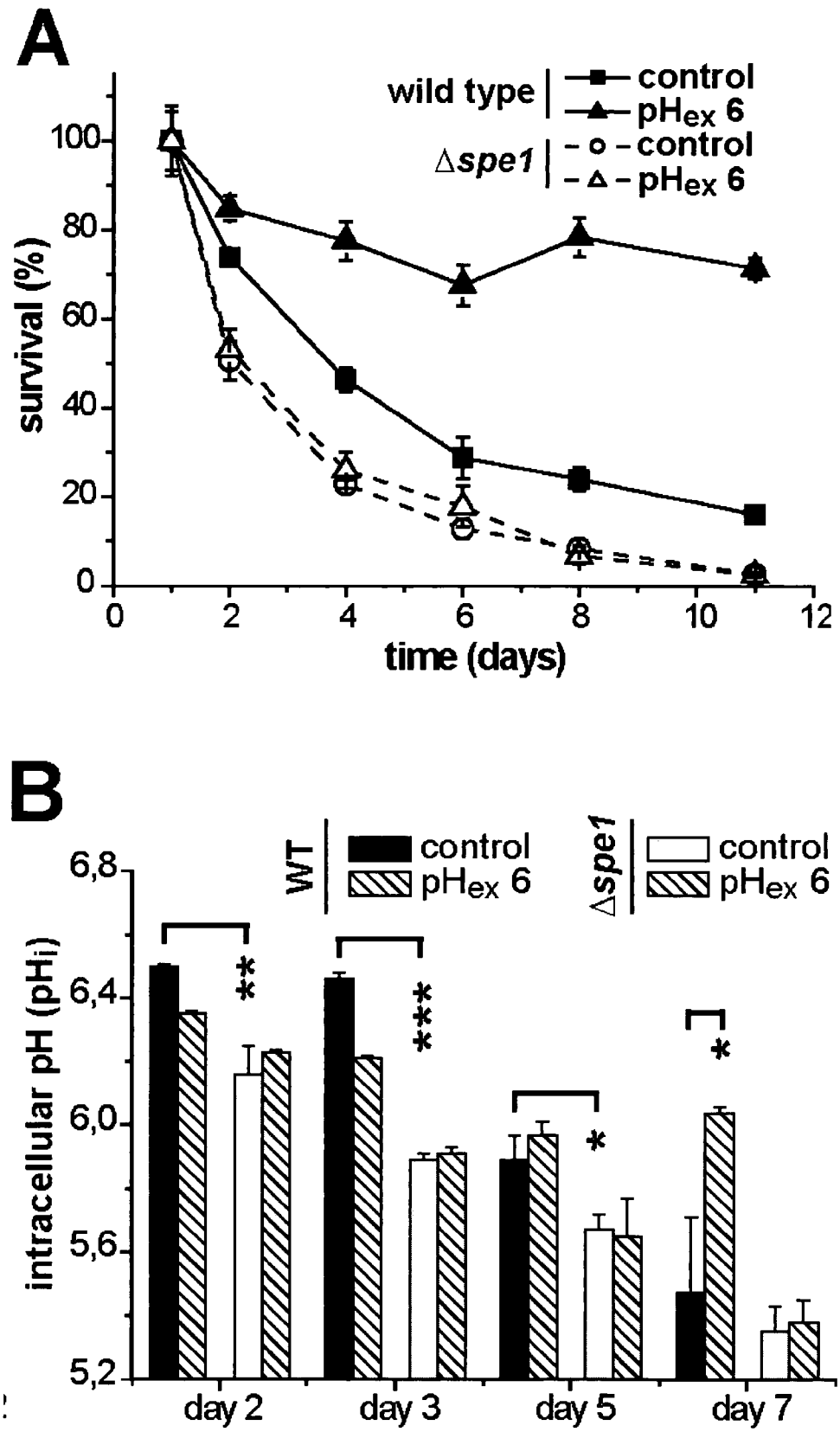
A
wild type
control
pHex 6
Δspe1
control
pHex 6
survival (%)
time (days)
B
WT
control
pHex 6
Δspe1
control
pHex 6
intracellular pH (pHi)
day 2
day 3
day 5
day 7

Figure 26

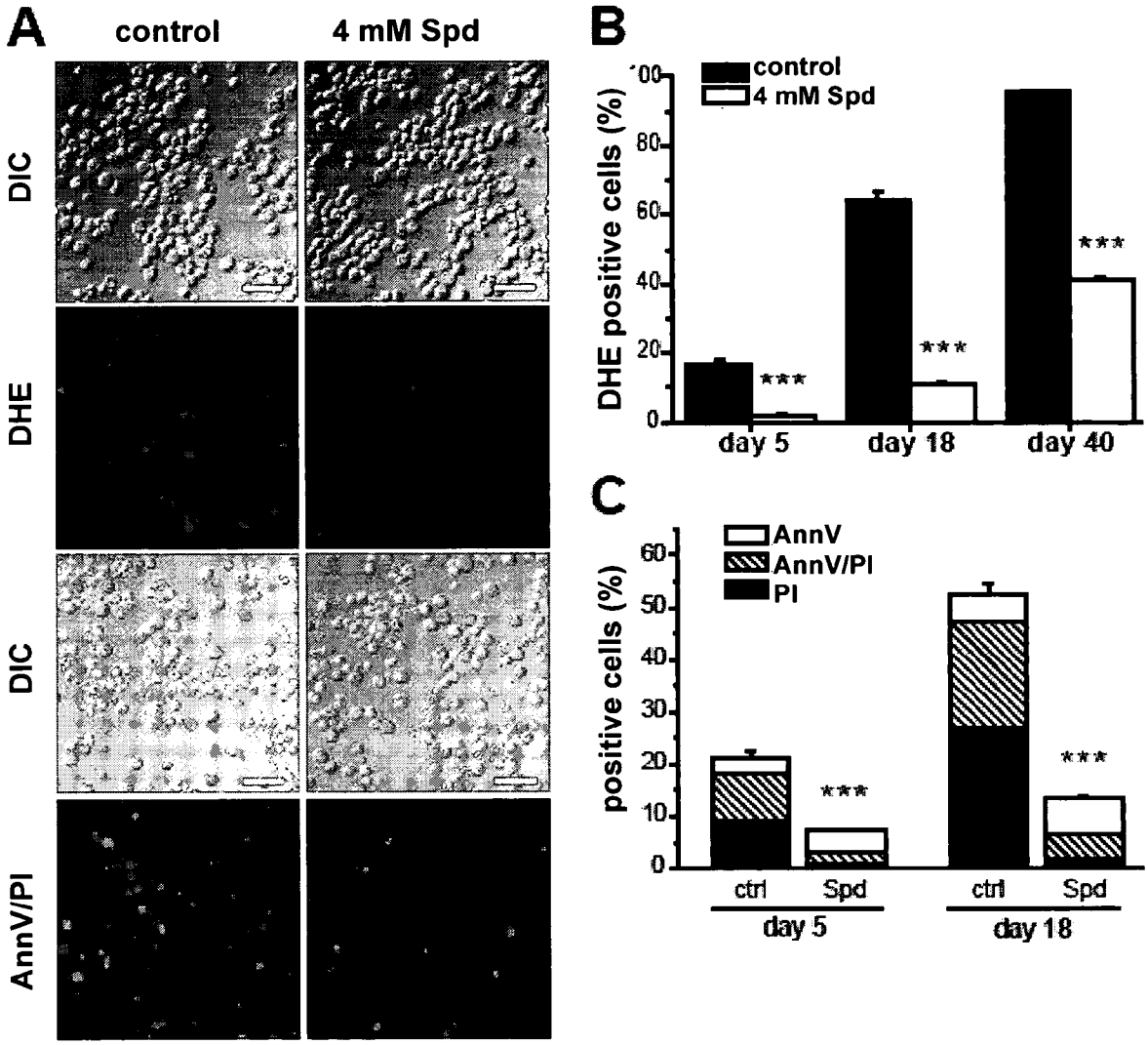
A
control
4 mM Spd
DIC
DHE
DIC
AnnV/PI
B
control
4 mM Spd
DHE positive cells (%)
100
80
60
40
20
0
***
***
***
day 5
day 18
day 40
C
AnnV
AnnV/PI
PI
positive cells (%)
60
50
40
30
20
10
0
***
***
ctrl
Spd
ctrl
Spd
day 5
day 18

Figure 27

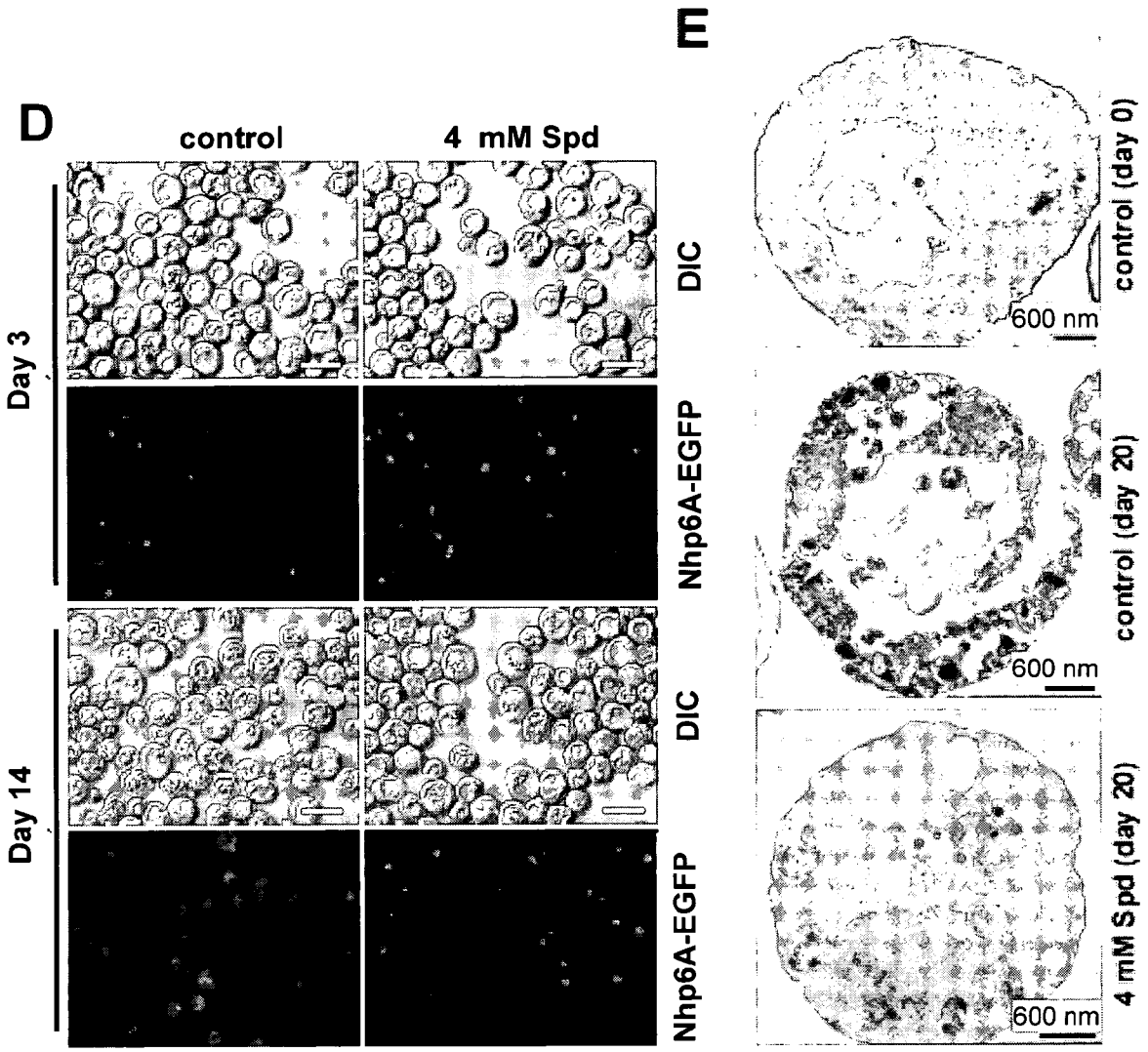

Figure 27 (continued)

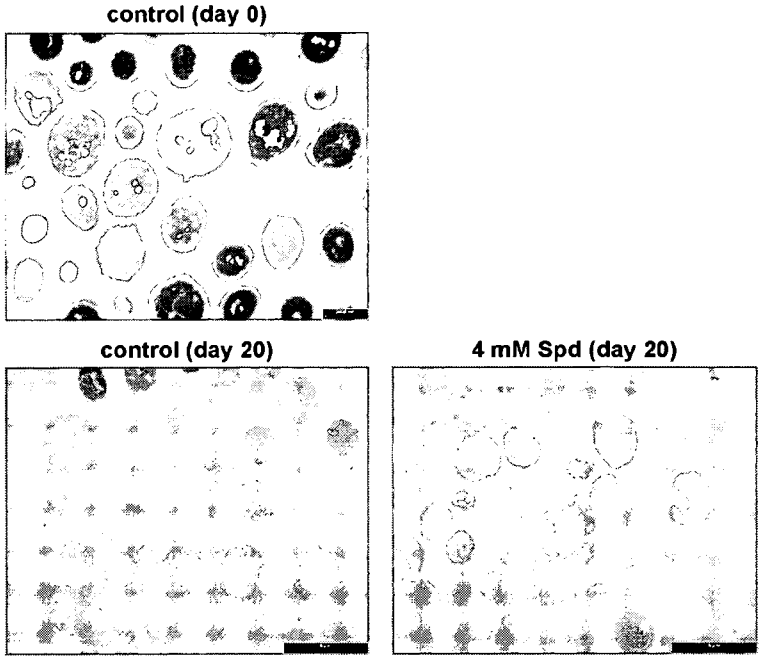

Figure 28

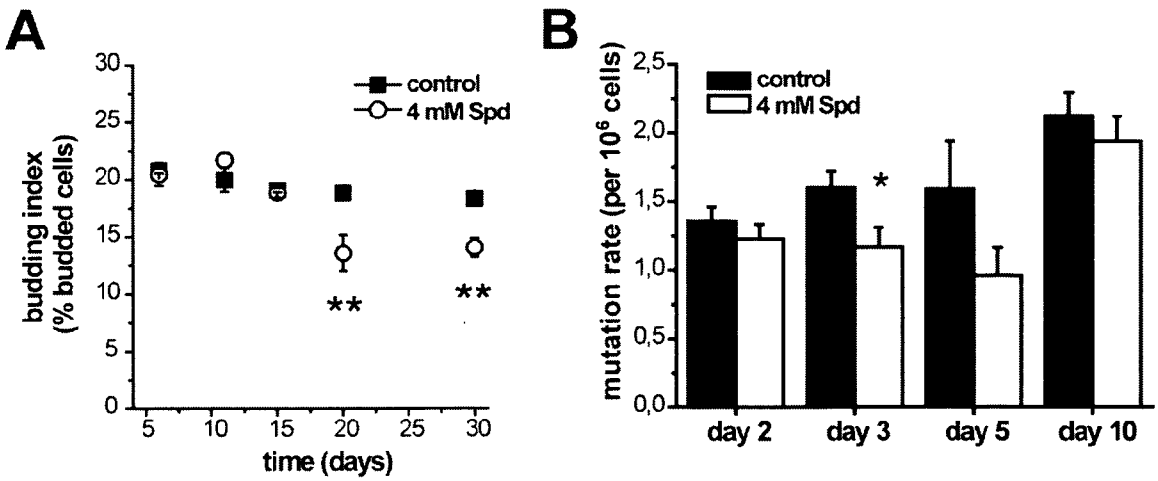

Figure 29

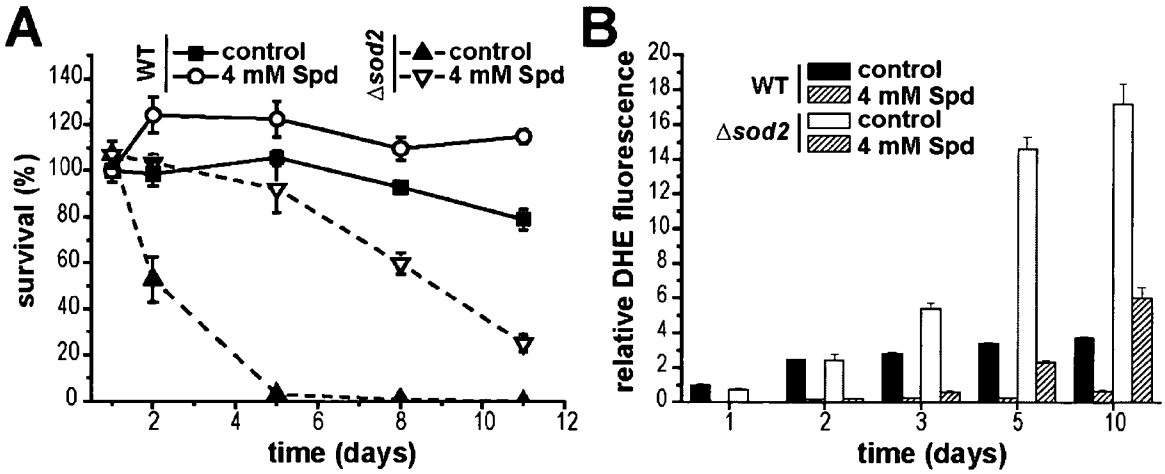

Figure 30

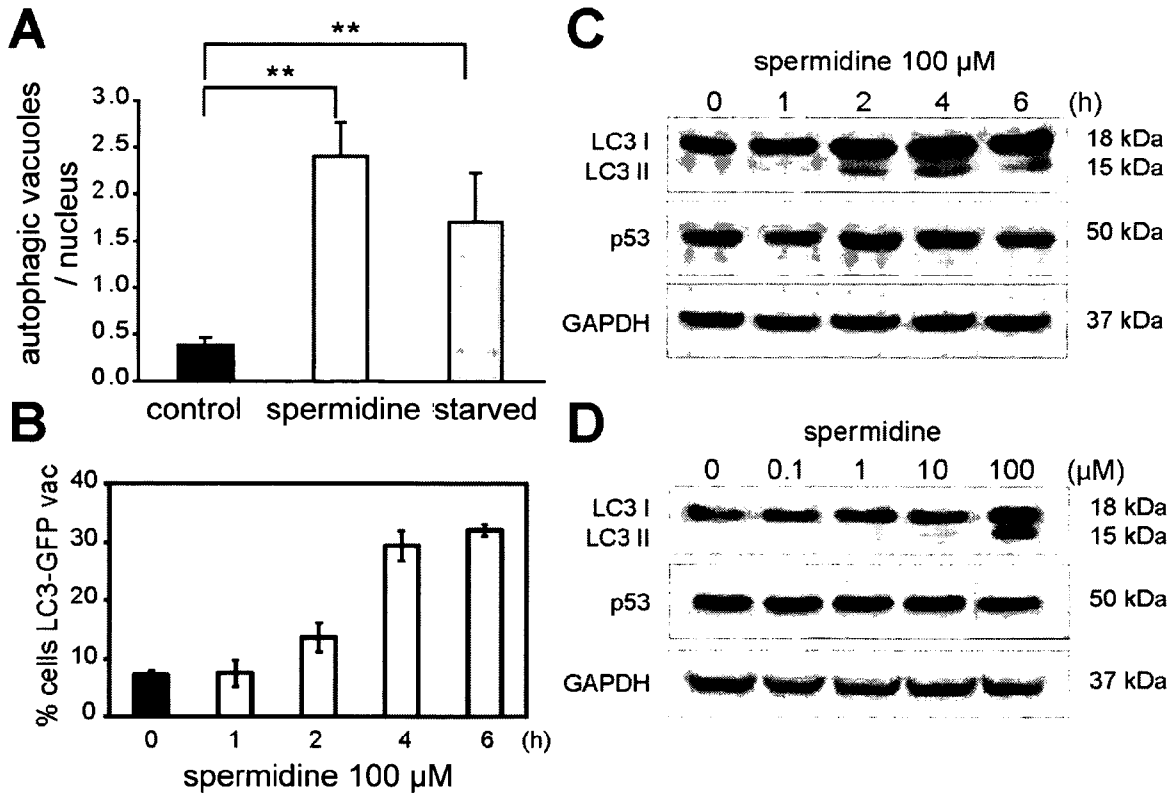

Figure 31

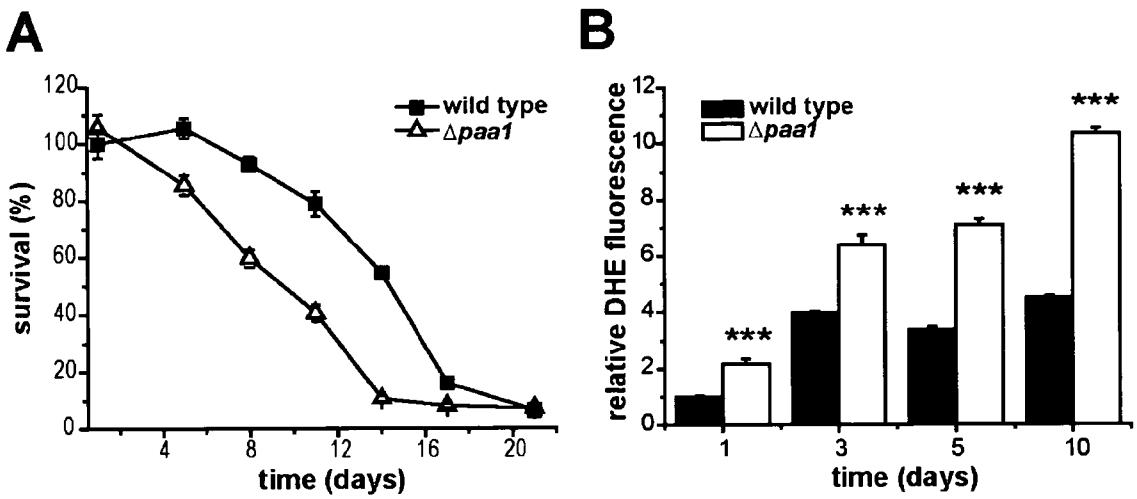

Figure 31

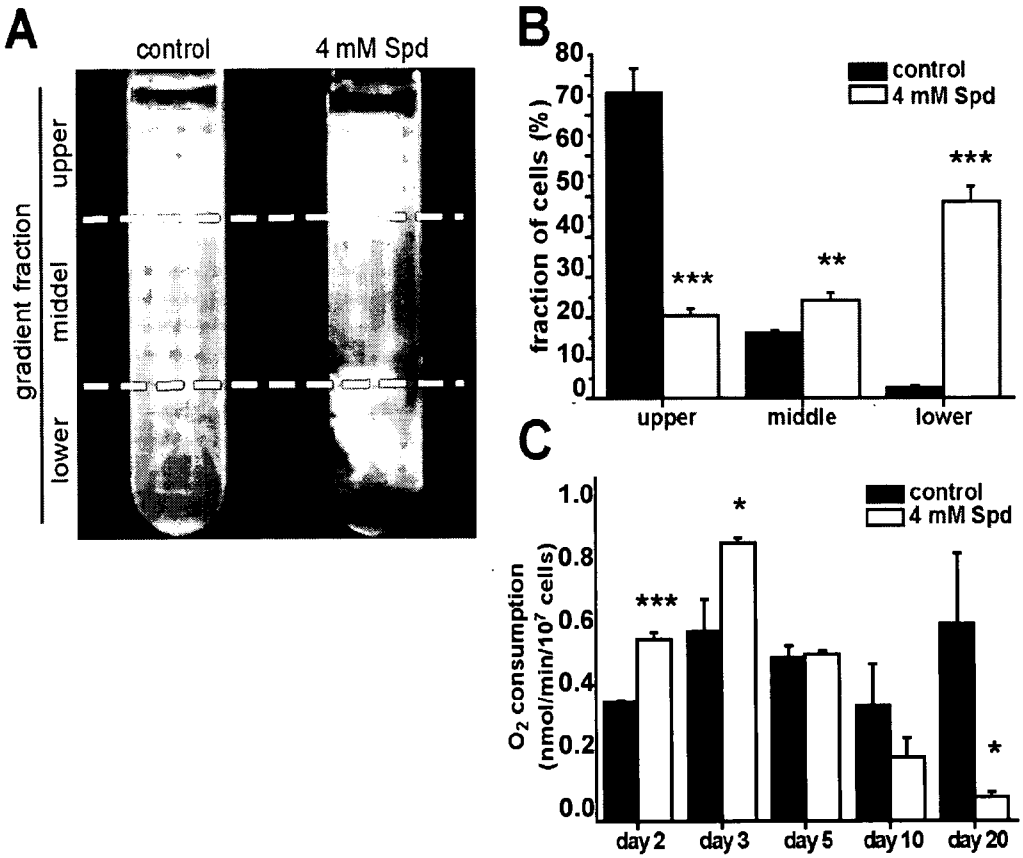

Figure 32