



- (51) **International Patent Classification:**
G01N 33/66 (2006.01)
- (21) **International Application Number:**
PCT/DK2012/050186
- (22) **International Filing Date:**
30 May 2012 (30.05.2012)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
PA 2011 70270 31 May 2011 (31.05.2011) DK
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- (81) **Designated States (unless otherwise indicated, for every
kind of national protection available):** AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD,
SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR,
TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) **Designated States (unless otherwise indicated, for every
kind of regional protection available):** ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments (Rule 48.2(h))

(54) **Title:** METHOD AND SYSTEM FOR IDENTIFICATION OF PHYSIOLOGICAL IMBALANCE IN AN ANIMAL

Variable	Number of obs.	Mean value	P ₅ – P ₉₅ inter percentile	Free glucose	Glucose 6-P
				Coefficient of correlation, r	Coefficient of correlation, r
Free glucose, mM	3261	0.340	0.128 – 0.567		
Glucose 6-P, mM	3262	0.126	0.105 – 0.286	-0.523**	
Days in milk	3233	112	9 – 309	0.425**	-0.577**
Milk yield per milking, kg	1895	13.3	7.6 – 22.4	-0.005	0.030
Time since last milking, h	1637	10.3	6.6 – 15.5	-0.105**	-0.155**
Fat, %	2822	4.74	3.24 – 6.73	-0.460**	0.227**
Lactose, %	2849	4.63	4.45 – 5.06	0.228**	-0.198**
Protein, %	2982	3.61	2.80 – 4.66	0.031	-0.020
Log SCC ^a	3003	4.88	4.20 – 5.85	-0.089**	0.180**
Citrate, %	535	0.22	0.17 – 0.29	-0.278**	-0.179**
BOHB ^b , µM	3262	95	44 – 195	-0.420**	0.307**

^a SCC = Somatic Cell Counts

^b BOHB = β-hydroxy-butyrate

** P < 0.01

Figure 1

(57) **Abstract:** The present invention relates to methods and systems for predicting the risk of physiological imbalance in an animal, including measuring for specific risk indicators, such as glucose and isocitrate, and how information on physiological imbalance can be used for decision on whether prediction of the risk of the animal entering into a sub-clinical or clinical disease state or reproductive status is relevant. The present invention is giving the basis for future automated proactive feeding management systems for securing optimal performance, reproduction and welfare through maintaining physiological balance that will reduce risk of diseases.

METHOD AND SYSTEM FOR IDENTIFICATION OF PHYSIOLOGICAL IMBALANCE IN AN ANIMAL

5 Technical field of the invention

The present invention relates to methods and systems for predicting the risk of physiological imbalance in an animal. As an example, the animal may be a cow. The methods and systems of the invention rely on a sample of a body fluid of the animal, such as urine, blood or milk.

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Background of the invention

Animal production systems have been steadily increasing production output over the past decades. For example, the amount of milk produced per cow per year has
15 increased continually over recent decades. The increases in animal production systems are primarily due to improvements in genetics, nutrition, and environmental management. However, this increase in production has resulted reduced reproductive performance and increase in e.g. mastitis incidence and the problem of sub-clinical diseases such as e.g. fatty liver and ketosis is probably as
20 big as previously. Although management and treatment for diseases has improved over the past few decades, farmers still lack accurate and reliable methods for identifying risk animals that will allow the farmer to take preventive measures, such as e.g. changing input factors (e.g. feeding) to the animal.

25 A system has been suggested to predict e.g. health state. US2008255763 discloses systems and methods for observing and predicting a physiological state of an animal. The physiological state of an animal, such as the animal's health state, is determined from a comparison of a pattern in measured parameters, i.e. sample values, and a reference pattern (or a reference parameter value) which is
30 typical for healthy animals and a pattern which is typical for animals suffering from a certain disease, respectively. Once an aggregation of data covering all physiological states to be observed or predicted is available, probabilities of a particular animal belonging to the various states is determined.

Summary of the invention

Prior art has focused on systems and methods for observing and predicting a physiological state of an animal. The present invention is focusing on the situation of marked deviations in physiological parameters may lead to a general increased risk of disease and reduced performance or reproduction and that prevention of these deviations will prevent the risk of disease and reduced performance and/or reproduction for that animal.

The inventors of the present invention have realized that early detection for physiological imbalance and subsequent prevention of this physiological imbalance can help prevent an animal from entering into a physiological state that relates to sub-clinical or clinical diseases or suboptimal performance and/or reproduction.

Thus, an object of the present invention relates to a method for predicting the risk of physiological imbalance in an animal.

In particular, it is an object of the present invention to provide a system for predicting the risk of physiological imbalance in an animal.

Thus, one aspect of the invention relates to a method for predicting the risk of physiological imbalance in an animal, said method comprises the steps of:

(i) repetitively providing at least one sample from a body fluid obtained from the animal;

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(ii) determining the concentration or amount of a first risk indicator present in the sample, the first risk indicator may be selected from the group consisting of free glucose, glucose-6-phosphate, glucose-1-phosphate, free galactose, UDP-glucose, UDP-galactose, glycerol 3-phosphate, beta-hydroxybutyric acid (BHBA), isocitrate, citrate, malate, , 2-oxo-glutarate, isocitrate dehydrogenase, malate dehydrogenase, pyruvate carboxylase, glycogen phosphorylase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aspartate aminotransferase, aldehyde dehydrogenase, phosphoglucomutase, triosephosphate isomerase, lactate,

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cholesterol, non-esterified fatty acids (NEFA), milk protein, milk fat, and milk lactose;

5 (iii) determining the rate of change and/or the level of change of the first risk indicator;

(iv) comparing said rate change and/or level change with one or more reference patterns of said first risk indicator, in order to predict the risk of physiological imbalance in the animal.

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Another aspect of the present invention relates to a system for predicting the risk of physiological imbalance in an animal, the system comprising:

- a computer comprising a processor and being operatively connected to a database,
- 15 – at least one sample providing device for repetitively providing at least one sample of a body fluid of the animal,
- an analysis apparatus for:

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(A1) determining the concentration or amount of a first risk indicator present in the sample, the first risk indicator may be selected from the group consisting of free glucose, glucose-6-phosphate, glucose-1-phosphate, free galactose, UDP-glucose, UDP-galactose, glycerol 3-phosphate, beta-hydroxybutyric acid (BHBA), isocitrate, citrate, malate, 2-oxo-glutarate, isocitrate dehydrogenase, malate dehydrogenase, pyruvate carboxylase, glycogen phosphorylase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aspartate aminotransferase, aldehyde dehydrogenase, phosphoglucomutase, triosephosphate isomerase, lactate, cholesterol, non-esterified fatty acids (NEFA), milk protein, milk fat, and milk lactose;

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(A2) optionally, determining the concentration or amount of at least one second risk indicator present in the sample, the at least one second risk indicator, is different from the first risk indicator and may be selected from the group consisting of free glucose, glucose-

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6-phosphate, glucose-1-phosphate, free galactose, UDP-glucose, UDP-galactose, glycerol 3-phosphate, beta-hydroxybutyric acid (BHBA), isocitrate, citrate, malate, 2-oxo-glutarate, isocitrate dehydrogenase, malate dehydrogenase, pyruvate carboxylase, glycogen phosphorylase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aspartate aminotransferase, aldehyde dehydrogenase, phosphoglucomutase, triosephosphate isomerase, lactate, cholesterol, non-esterified fatty acids (NEFA), milk protein, milk fat, and milk lactose;

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- a data interface for repetitively entering the concentration or amount of the first risk indicator and, optionally, the at least one second risk indicator in the database, wherein the database is adapted to store multiple database entries representing the indicator at various points in time, and wherein the processor is programmed to:

15

(P1) determine the rate of change of the first indicator and, optionally, the at least one second indicator;

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(P2) determine the level change of the first indicator and, optionally, the at least one second indicator and,

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(P3) compare said rate change and/or level change with one or more reference patterns of said first risk indicator and, optionally, the at least one second risk indicator, in order to predict the risk of physiological imbalance in the animal.

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Brief description of the figures

Figure 1 shows descriptive statistics for milking data and variables measured in milk,

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Figure 2 shows data for glucose 6-phosphate and free glucose measured in 3232 milk samples from Danish Friesian and Jersey cows. Parity x race significantly affected the glucose 6-phosphate as well as the free glucose content (parity x race; $p < 0.001$),

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Figure 3 shows the comparisons between a colorimetric and a fluorometric analytical method. Mean values (μM) and corresponding 0.05-0.95 inter percentile were 581 (368 – 788) and 552 (363 – 770), for colorimetric and fluorometric methods, respectively. The colorimetric and the fluorometric results were highly correlated ($r = 0.903$),

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Figure 4 shows a graphical representation of the concentration of glucose (a) and glucose 6-phosphate (b) in milk as a function of days in milk (DIM, $n = 3233$),

20 Figure 5 shows blood glucose concentrations at weeks relative to parturition (week = 0) for multiparous (i.e. parity > 1) Danish Red Holstein cows ($n = 192$) including weekly mean and the 70% lower confidence limit,

Figure 6 shows weights for selected metabolites to be included in the model to
25 predict energy balance. Metabolites were weighted at each week via the absolute value of the estimates generated from the regression model in Figure 9. For each metabolite, the weight at each week was adjusted to a % relative to 100,

Figure 7 shows weekly Pearson's correlations between degree of physiological
30 imbalance and calculated energy balance, milk yield, energy intake, or plasma NEFA, BHBA and glucose for cows during the periparturient period,

Figure 8 shows frequency distributions for cows that did or did not develop disease during wk 1 (WEEK1; i.e. 0 – 7 DIM) or during wk 2 through 9 of lactation (EARLYLACT),

- 5 Figure 9 shows regression coefficient estimates, standard error and the probability that regression coefficient deviates from 0 for NEFA, glucose and BHBA as well as information for the regression model selected to predict degree of physiological imbalance based on between-cow variations in calculated EBAL from wk -4 to 9 relative to parturition.

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Figure 10 shows the least square means (LSM), SEM and the standardized differences (SDiff) for estimated between-cow variations in physiological imbalance (PI), calculated energy balance (EBAL), and plasma concentration of NEFA, BHBA and glucose at week -1 relative to development of metritis, retained placenta or milk fever during the first week after parturition.

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Figure 11 shows the least square means (LSM), SEM and the standardized differences (SDiff) for estimated between-cow variations in physiological imbalance (PI), calculated energy balance (EBAL), and plasma concentration of NEFA, BHBA and glucose at week -1 relative to development of all diseases, lameness, or mastitis during either the first week after parturition.

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Figure 12 shows results from Project 3 regarding the differences in plasma concentrations of glucose (A), non-esterified fatty acids (NEFA; B), beta-hydroxybutyrate (BHBA; C), milk BHBA (D), milk glucose (E), daily milk yield (F), liver triacylglycerol (TAG; G), and calculated energy balance (H) at time points relative to dietary-induced physiological imbalance via nutrient restriction (h = 0-96) in 47 Holstein cows in early (●), mid- (■), and late (▲) lactation. *Differences (P < 0.05) between cows in early and mid-lactation at any given time point.

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#Differences (P < 0.05) between cows in early and late lactation at any given time point. @Differences (P < 0.05) between cows in mid- and late lactation at any given time point,

Figure 13 shows the results from Project 3 regarding differences in percent change in concentrations of various milk components at time points relative to

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nutrient restriction (h = 0-96) for 47 Holstein cows in early (A) and later lactation (B),

Figure 14 shows results from Project 3 regarding differences in concentrations of plasma glucose (A), plasma non-esterified fatty acids (NEFA; B), plasma beta-hydroxybutyrate (BHBA; C), daily milk yield (D), milk BHBA (E), milk glucose (F), calculated energy balance ([15]; G), and liver triacylglycerol (TAG; H) at time points relative to nutrient restriction (0 - 96 h) from 12 Holstein cows in early (Early; ●) and mid-(Mid; □) lactation classified as having either the least (Normal; solid lines) or greatest (Severe; dashed lines) degree of physiological imbalance based on an index generated from plasma NEFA, BHBA, and glucose. *Differences ($P < 0.05$) between normal and severe cows in early lactation at any given time point. For cows in mid-lactation, no differences ($P > 0.05$) were observed between normal and severe cows at any given time point,

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Figure 15 shows results from Project 4 regarding the differences in concentration of blood non-esterified fatty acids (NEFA; A), beta-hydroxybutyrate (BHBA; B), glucose (C), and liver triacylglycerol (TAG; D) at weeks relative to parturition (week = 0) for 8 Holstein cows classified as in physiological imbalance based on liver TAG content at 7 days in milk, and

20

Figure 16 shows a flow diagram showing the preferred embodiment of the present invention and the general design.

25 The present invention will now be described in more detail in the following.

Detailed description of the invention

The prior patent (US2008255763) disclosed systems and methods for observing and prediction and physiological state of an animal. However, no clear definition was given for physiological state. The prior art has focused on systems and methods for observing and predicting a physiological state of an animal. Thus, body fluids of animals, in particular milk, urine and blood have been analyzed in order to obtain values of parameters, such as cell count in milk, lactate

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dehydrogenase (LDH), N-Acetyl-[beta]-D-glucosaminidase (NAGase), ketone bodies such as acetoacetate, beta-hydroxybutyrate (BHBA) and acetone, urea content, progesterone, or others, each of which by itself or in combination with others indicates a certain physiological state. For example, a high LDH

5 concentration usually indicates mastitis, whereas the progesterone content is used for estrus detection, pregnancy or cysts depending on concentration and changes over time.

Prior to discussing the present invention in further details, the following terms and
10 conventions will first be defined:

In the present context, the term "animal characteristics" (characteristics that can not be changed with time) should be understood as specific characteristics of the animal, such as e.g. genus, species, breed (e.g. cattle breed), genotype, and sex.

15

In the present context, the term "physiological state" (characteristics that can change with time) should be understood as a broad term covering a state of the body or bodily functions, such as age, and parity, reproductive status (such as non-estrus vs. estrus, non-pregnant (NP) vs. pregnant (P), productive status
20 (such as non-lactating (NL) vs. lactating (L)), and production relative to production capacity (e.g. in relation to maximum genetic potential) and any combinations of the former.

In the present context, the term environmental factors should be understood as a
25 broad term covering e.g. production system, season, diet, regional or national population differences.

In the present context, the term "physiological imbalance" should be understood as situation where one or more parameters of an animal of certain animal
30 characteristics deviate from the normal at a given physiological state, and who consequently have an increased risk of diseases (clinical or subclinical), reduced production and/or reduced reproduction (Ingvarlsen, 2006; Ingvarlsen and Friggens, 2005).

A "deviation from the normal" should be understood as e.g. concentrations of parameters in blood that are above or below the confidence limit (e.g. 70%) at given physiological state in healthy lactating animals of given animal characteristics (see example in Figure 5).

5

The inventors of the present invention have realized that bringing animals in physiological imbalance in balance can prevent them from developing clinical or sub-clinical diseases. Hence, contrary to prior art, the present invention focuses on early identification and prevention of physiological imbalance that can thereby
10 severely reduce the number of animals developing clinical or sub-clinical diseases, rather than detecting clinical disease after it has already happened, and acting subsequently to that.

Furthermore, the inventors of the present invention have identified specific
15 indicators of physiological imbalance, making it possible to estimate the risk of or degree of physiological imbalance.

Thus, one aspect of the invention relates to a method for predicting the risk of physiological imbalance in an animal, said method comprises the steps of:

20

(i) repetitively providing at least one sample from a body fluid obtained from the animal;

25

(ii) determining the concentration or amount of a first risk indicator present in the sample, the first risk indicator may be selected from the group consisting of free glucose, glucose-6-phosphate, glucose-1-phosphate, free galactose, UDP-glucose, UDP-galactose, glycerol 3-phosphate, beta-hydroxybutyric acid (BHBA), isocitrate, citrate, malate, 2-oxo-glutarate, isocitrate dehydrogenase, malate dehydrogenase, pyruvate carboxylase,
30 glycogen phosphorylase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aspartate aminotransferase, aldehyde dehydrogenase, phosphoglucomutase, triosephosphate isomerase, lactate, cholesterol, non-esterified fatty acids (NEFA), milk protein, milk fat, and milk lactose;

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(iii) determining the rate of change and/or the level change of the first risk indicator;

5 (iv) comparing said rate change and/or level change with one or more reference patterns of said first risk indicator, in order to predict the risk of physiological imbalance in the animal.

A risk of physiological imbalance based on combinations of risk indicators may in certain circumstances be parameterized to describe physiological imbalance better
10 than any individual risk indicator.

Hence, in one embodiment of the present invention, the method further comprises the steps of:

15 (v) determining the concentration or amount of at least one second risk indicator present in the sample, the at least one second risk indicator, is different from the first risk indicator and may be selected from the group consisting of free glucose, glucose-6-phosphate, glucose-1-phosphate, free galactose, UDP-glucose, UDP-galactose, glycerol 3-phosphate, beta-
20 hydroxybutyric acid (BHBA), isocitrate, citrate, malate, 2-oxo-glutarate, isocitrate dehydrogenase, malate dehydrogenase, pyruvate carboxylase, glycogen phosphorylase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aspartate aminotransferase, aldehyde dehydrogenase, phosphoglucomutase, triosephosphate isomerase, lactate,
25 cholesterol, non-esterified fatty acids (NEFA), milk protein, milk fat, and milk lactose;

(vi) determining the rate of change and/or the level change of the at least one second risk indicator;

30 (vii) comparing said rate change and/or level change with one or more reference patterns of said first risk indicator and, optionally, the at least one second risk indicator, in order to predict the risk of physiological imbalance in the animal.

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Provided a prediction for the risk of physiological imbalance in the animal, makes it possible to predict the risk of sub-clinical or clinical disease in an animal.

In one embodiment of the present invention, provided that the prediction, based
5 on the one or more risk indicator, indicates a risk of physiological imbalance in the animal, further comprising the step of:

(viii) determining the concentration or amount of at least one disease
indicator present in the sample;

10

(ix) determining the rate of change and/or the level change of the at least
one disease indicator;

(x) comparing said rate change and/or level change with one or more
15 reference patterns of said at least one disease indicator, in order to predict the risk of the animal entering into a sub-clinical or clinical disease state.

The variation in the risk indicator may be influenced by systematic factors such as
e.g. animal characteristics, physiological state, and environmental factors.

20

The risk of physiological imbalance in an animal may be determined from a
comparison of a pattern in measured parameters, i.e. sample values, and a
reference pattern (or a reference parameter value) which is typical for animals
with certain animal characteristics.

25

Hence, in another embodiment, the one or more reference patterns are based on
different physiological states.

In yet another embodiment, the one or more reference patterns are based on the
30 specific animal characteristics.

In a further another embodiment, the one or more reference patterns are based
on environmental factors

The variation in the risk indicators may also be influenced by variation within a particular embodiment listed above (e.g. within breed).

In yet another embodiment, the one or more reference patterns are based on
5 previous data obtained from the same animal.

In another embodiment of the present invention, the animal is selected from the group of animal species consisting of ruminants.

10 In one embodiment of the present invention, the animal is selected from the group of animal species consisting of non-ruminants.

In another embodiment of the present invention, the body fluid is selected from the group consisting of milk, blood and urine.

15

As can be deduced from the example section, free glucose and isocitrate have been identified as risk indicators in milk (Figure 13).

Regardless of stage of lactation, substantial changes (-38%) in milk free glucose
20 concentration and isocitrate (96%) were observed during nutrient restriction (0-96 h). Furthermore, changes in milk free glucose and isocitrate were the most rapid (i.e. significant changes by 24 h after nutrient restriction) when compared to other milk components (i.e. glucose-6-phosphate and BHBA). In addition, free glucose and isocitrate in milk were correlated to plasma glucose NEFA and BHBA
25 that are essentially the most important parameters in the index of physiological imbalance described based on blood parameters.

Fat was highly correlated in milk ($r = 0.68$; $P < 0.001$), and changes in milk fat and milk yield have been associated with other diseases, such as mastitis (Moyes
30 et al., 2009).

As a direct consequence of free glucose as being a risk biomarker for physiological imbalance, it is speculated that other parameters (metabolites or enzymes) from the gluconeogenesis/glycolysis pathway may also function as such. Non-limiting
35 examples of parameters from the gluconeogenesis/glycolysis pathway are:

glucose-6-phosphate, glucose-1-phosphate, glycerate 3-phosphate, lactose, UDP-glucose, and UDP-galactose.

In a similar manner, as a direct consequence of isocitrate being a risk indicator for physiological imbalance, it is speculated that other parameters (metabolites or enzymes) from the Citric Acid Cycle (TCA Cycle/Krebs Cycle) or exports from the TCA-cycle (e.g. citrate) may also function as such. Non-limiting examples of parameters from the Citric Acid Cycle are: malate, isocitrate dehydrogenase, malate dehydrogenase, and pyruvate carboxylase.

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Other non-limiting examples of parameters relating to physiological imbalance related to the hydrolysis/synthesis of TAG (glycerol 3-phosphate, milk fat), fatty acid oxidation (NEFA), cholesterol biosynthesis (cholesterol), the protein breakdown/synthesis (glutamate dehydrogenase and milk protein), liver damage (aspartate aminotransferase and aldehyde dehydrogenase), and milk yield.

Risk indicators for physiological imbalance index

Fatty acids (NEFA) are products of lipolysis (i.e. breakdown of fat) and are also substrates for lipogenesis (i.e. synthesis of fat). During periods of imbalance, NEFA are released from fat tissue TAG, transported through the bloodstream, and are used as an energy source by many tissues when glucose is limited. In the liver, NEFA have 3 fates depending on energy needs, hormone balance, and substrate availability and include 1) complete breakdown with the release of energy via the tricarboxylic acid (TCA) cycle, 2) re-synthesized as TAG and stored within the liver or the newly synthesized TAG are exported into blood to other tissues, or 3) during periods of low blood glucose, breakdown of NEFA is incomplete and NEFA are then converted into ketone bodies (i.e. BHBA) which are released from the liver to the blood and can be used as an alternate energy source by many tissues (e.g. kidney, heart, and skeletal muscle) when glucose is low. Concentrations of blood NEFA and BHBA are associated with multiple diseases. Since most diseases in the dairy industry are multifactorial, the use of individual metabolites may not be the most optimal method to predict risk of clinical disease for lactating dairy cows. The use of an index of physiological imbalance based on a couple or several parameters (e.g. plasma NEFA, BHBA,

glucose and cholesterol) and/or ratios between parameters, as well as calculated or predicted energy balance, body weight and weight changes, and body condition score or changes in body condition score may be more useful as indicators for degree of physiological imbalance that will more accurately reflect e.g. the overall
5 metabolic status of cows throughout lactation than using changes in individual parameters alone.

For results relating to the generation of an index for physiological imbalance, its' relationship to risk of disease and risk indicators in milk for physiological
10 imbalance please see the 'Examples' section below.

As can be deducted from the examples section, glucose, BHBA, and NEFA have been identified as risk biomarkers in blood and have been used to generate an index for degree of physiological imbalance. However, e.g. when considering dairy
15 cows, these risk indicators are preferably measured in the milk, since samples can be taken automatically during milking in a non-invasive manner. The inventors have shown that the levels of glucose and BHBA in blood and milk are correlated. We have concluded that free glucose and isocitrate in milk are potential biomarkers for degree of physiological imbalance for cows during lactation.

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Based on biochemical pathways coupled with laboratory capabilities, analyses in blood and milk is expected to be possible for at least 5 proteins for use as potential risk indicators for physiological imbalance (i.e. isocitrate dehydrogenase, glutatmate dehydrogenase, malate dehydrogenase, glyceraldehyde 3-phosphate
25 dehydrogenase, and phosphoglucomutase).

In one embodiment of the present invention, the risk of physiological imbalance is predicted by measuring free glucose as the first risk indicator.

30 In another embodiment of the present invention, the risk of physiological imbalance is further predicted by measuring isocitrate as the second risk indicator.

In yet another embodiment of the present invention, the animal is a cow predicted
35 in physiological imbalance based on a first and/or second indicator, and the health

status such as risk of sub-clinical or clinical disease ketosis is identified by measuring BHBA as a the second or the third risk indicator.

EMBODIMENTS OF THE PRESENT INVENTION

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General Application of the Present Invention

In a preferred embodiment of the present invention the general design are as shown in Figure 16. The models may have 4 or more major outputs: 1) an overall risk of physiological imbalance presented to the user; 2) a calculation or indication
10 of when to take the next sample that feeds back to the analysis apparatus; 3) in the case of high risk of physiological imbalance (PI; i.e. risk of PI > default risk limit), additional analysis of disease indicators to determine the risk of a disease state, e.g. sub-clinical or clinical ketosis; and 4) in the case of low risk of physiological imbalance (i.e. risk of PI < default risk limit), additional analysis for
15 e.g. measurements of reproductive status (i.e. R_n ; i.e. estrus vs. non-estrus).

Two modules generate these outputs: 1) using the information provided by the signal(s), i.e. PI_1 and, optionally, PI_2 , coming from the analysing apparatus and other additional risk factors (e.g. day in milk, body weight, and energy balance)
20 for generation of risk of physiological imbalance (i.e. output 1); and 2) using the risk assessment generated by output 1 coupled with additional factors to determine risk of clinical or sub-clinical disease such as e.g. ketosis (i.e. output 2) or e.g. reproductive status (i.e. output 3). This structural separation is designed to optimize analyses that reduce false positives for disease and improve
25 reproductive conception rates.

Another aspect of the present invention relates to a system for predicting the risk of physiological imbalance in an animal, the system comprising:

- a computer comprising a processor and being operatively connected to a
30 database,
- at least one sample providing device for repetitively providing at least one sample of a body fluid of the animal,
- an analysis apparatus for:

(A1) determining the concentration or amount of a first risk indicator present in the sample, the first risk indicator may be selected from the group consisting of free glucose, glucose-6-phosphate, glucose-1-phosphate, free galactose, UDP-glucose, UDP-galactose, glycerol 3-phosphate, beta-hydroxybutyric acid (BHBA), isocitrate, citrate, malate, lactate, 2-oxo-glutarate, cholesterol, non-esterified fatty acids (NEFA), isocitrate dehydrogenase, malate dehydrogenase, pyruvate carboxylase, glycogen phosphorylase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aspartate aminotransferase, aldehyde dehydrogenase, phosphoglucomutase, triosephosphate isomerase, milk protein, milk fat, and milk lactose;

(A2) optionally, determining the concentration or amount of at least one second risk indicator present in the sample, the at least one second risk indicator, is different from the first risk indicator and may be selected from the group consisting of free glucose, glucose-6-phosphate, glucose-1-phosphate, free galactose, UDP-glucose, UDP-galactose, glycerol 3-phosphate, beta-hydroxybutyric acid (BHBA), isocitrate, citrate, malate, lactate, 2-oxo-glutarate, cholesterol, non-esterified fatty acids (NEFA), isocitrate dehydrogenase, malate dehydrogenase, pyruvate carboxylase, glycogen phosphorylase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aspartate aminotransferase, aldehyde dehydrogenase, phosphoglucomutase, triosephosphate isomerase, milk protein, milk fat, and milk lactose;

- a data interface for repetitively entering the concentration or amount of the first risk indicator and, optionally, the at least one second risk indicator in the database, wherein the database is adapted to store multiple database entries representing the indicator at various points in time, and wherein the processor is programmed to:

(P1) determine the rate of change of the first indicator and, optionally, the at least one second indicator;

(P2) determine the level change of the first indicator and, optionally, the at least one second indicator and,

5 (P3) compare said rate change and/or level change with one or more reference patterns of said first risk indicator and, optionally, the at least one second risk indicator, in order to predict the degree of or risk of physiological imbalance in the animal.

10 In one embodiment of the present invention, provided that the risk of physiological imbalance (PI) is higher than the default limit, the processor is further programmed to:

15 (P4a) predict the risk of the animal entering into a sub-clinical or clinical disease state (Figure 16; output 3, e.g. ketosis.).

In another embodiment of the present invention, provided that the risk of physiological imbalance (PI) is lower than the default limit, the processor is
20 further programmed to:

(P4b) predict the reproductive status (Figure 16; output 4, e.g. estrus detection especially for cows in early lactation).

25 In yet another embodiment of the present invention, the processor is further programmed to:

(P4a) predict the risk of the animal entering into a sub-clinical or clinical disease state.

30

In still another embodiment of the present invention, the processor is further programmed to:

35 (P4b) predict the reproductive status.

In another embodiment of the present invention, the analysis apparatus is further capable of:

5

(A3a) determining the concentration or amount of at least one disease indicator present in the sample.

In yet another embodiment of the present invention, the processor is further
10 programmed to:

(P5a) determine the rate of change of the at least one disease indicator;

15

(P6a) determine the level change of the at least one disease indicator;

20

(P7a) compare the rate change and/or level of change of the at least one disease indicator with one or more reference patterns of said at least one disease indicator, in order to predict the health status, e.g. the risk of the animal entering into a sub-clinical or clinical disease state.

In another embodiment of the present invention, the analysis apparatus is further
25 capable of:

(A3b) determining the concentration or amount of at least one indicator (e.g. progesterone) of reproductive status present in the sample.

30

In yet another embodiment of the present invention, the processor is further programmed to:

(P5b) determine the rate of change of the at least one indicator
35 (e.g. progesterone) of reproductive status;

(P6b) determine the level change of the at least one indicator (e.g. progesterone) of reproductive status;

- 5 (P7b) compare the rate change and/or level of change of the at least one indicator (e.g. progesterone) of reproductive status with one or more reference patterns of said at least one indicator of reproductive status, in order to predict the reproductive status.

- 10 The term "univariate data analysis" refers to data analysis in which data relating to a single variable are analysed. The univariate data analysis may comprise analysis of correlated univariate variables.

- The term "multivariate data analysis" refers to data analysis in which data relating
15 to at least two variables are analysed.

- It should be understood that a result from the univariate or multivariate analysis may be used as an input for further analysis. The further analysis may be univariate or multivariate. For example, the output from a Principal Component
20 Analysis (PCA) may be used as an input for a State Space Model (SSM) or vice versa or any other multivariate model approach.

- In one embodiment of the present invention, the processor is programmed to: perform at least one mathematical analysis of the at least one sample value, and
25 compare the at least one mathematical analysis with a pattern in measured parameters in order to select, the point in time for providing a subsequent sample and performing a subsequent analysis of said subsequent sample for at least one of the parameters.

- 30 In another embodiment of the present invention, the mathematical analysis is a statistical analysis.

In yet another embodiment of the present invention, the statistical analysis is a univariate analysis of the database entries to obtain a first set of data

representing expected sample values of at least one of the parameters at future points in time.

In still another embodiment of the present invention, the statistical analysis is a
5 multivariate analysis of the database entries to produce a second set of data derived from combined analysis of sample values of at least two parameters.

A non-limiting example on how to process the data is as follows:

1. For each risk indicator, outliers are excluded;
- 10 2. Repetitive data are smoothed to exclude random variation;
3. Change in slope and/or level is determined;
4. Change in slope is converted into a SlopeRisk;
5. Change in level is converted into a LevelRisk;
6. Univariate risk is calculated based on SlopeRisk and LevelRisk.

15

If the prediction of risk of physiological imbalance is based on more than one risk indicator (1-n) then:

Overall risk of physiological imbalance = $f(\text{univariate risk } 1, \dots, \text{univariate risk } n)$.

20

The inventors have realized that the concentration (molar) in milk of free glucose and glucose-6-phosphate can be important markers for physiological imbalance in a mammal. However, the inventors have found that the concentrations in milk of free glucose and glucose-6-phosphate are not linearly related both under normal
25 conditions and under physiological imbalance. Hence, it is important to test the two substrates independently of each other to obtain as much information as possible.

In one embodiment, the method further comprises the step of combining the
30 indicator based risk with one or more additional risk factors in order to predict the risk of physiological imbalance. Such combination may be performed as the sum of indicator based risk of physiological imbalance and additional risk factor based risk. Alternatively, multivariate determined risk of physiological imbalance based on measured indicators and additional risk factors can be performed (see Figure

16). Additional risk factor based risk may be predicted using univariate biomodels or multivariate approaches.

Hence, in one embodiment, the processor in the system is programmed to
5 combine the indicator based risk with one or more additional risk factors in order to predict the risk of physiological imbalance.

The Additional Risk Factor (ARF)

The crucial point about the Additional Risk Factor is that the risk of physiological
10 imbalance included here are not already included in the Indicator Based Risk, i.e. any factor which can be measured in the body fluid of the animal should not be included here. The elements that make up the ARF are described below, they combine to give ARF.

15 Milk Yield (MY) (kg/day).

Acceleration in Milk Yield (MYAcc)

This is used as an index of the degree of physiological stress that the cow is experiencing. MYAcc is a way of combining milk yield and days from calving which
20 we believe crystallises the components of these two factors which are relevant to the physiological stress being experienced by the cow. MYAcc is highest immediately after calving and is higher for higher yielding cows.

Given that there is a biometric model for milk yield then, in principle, the slope of
25 the smoothed milk yield curve i.e., acceleration in milk yield, is readily available. Additionally, MaxAccM is a scaling constant is needed to give the level of acceleration which will return a risk of 1.

Current Lactation Disease History (CLDHRiskM)

30 Here the distinction is made between diseases that indicate increased infection burden (metritis, teat tramp, acidosis) and those which just add to the general stress the cow is experiencing e.g. ketosis, milk fever, retained placenta and other. For each of these, there is an increased risk of physiological imbalance on the day of occurrence that then decays to zero over time.

35

At any one time, there may be more than 1 disease risk in operation, these are combined in the following way. Within each class of disease (infection vs general) the greatest DisRiskM is chosen i.e. it is assumed that within class risks are not additive. The overall current lactation disease history risk (CLDHRiskM) is the sum of two DisRiskMs, the highest within each class.

Energy Status (EnStat)

In the situation where the nutritional environment is limiting, excessive mobilisation of body energy reserves may occur putting pressure on the cow's physiological balance. Energy Status is a measure of this, it is also the output of the Energy Status Model. It is expected that poor energy status will increase the risk of mastitis physiological imbalance (among others). Thus, Energy Status is included as an ARF.

In one aspect, the aim of the present invention is to provide a method for determining the concentration of free glucose in a liquid comprising glucose and glucose-6-phosphate, the method comprising the steps of:

- 1) bringing into contact with at least one liquid sample A_n from a liquid sample M at least one type of glucose-6-phosphate-dehydrogenase and NAD(P), thereby producing 6-phosphogluconate and NAD(P)H₂, wherein n is a natural integer of at least 1;
- 2) bringing into contact with the at least one liquid sample A_n at least one type of NAD(P)H dehydrogenase and a fluorophore/chromophore precursor, thereby producing a fluorophore/chromophore;
- 3) determining the concentration of glucose-6-phosphate in the at least one liquid sample A_n by detecting the amount produced of fluorophore/chromophore and comparing it to a reference;
- 4) bringing into contact with at least one liquid sample B_n from the liquid sample M at least one type of hexokinase and adenosine triphosphate (ATP), thereby converting free glucose into glucose-6-phosphate, wherein n is a natural integer of at least 1;

- 5) bringing into contact with at least one liquid sample B_n from a liquid sample M at least one type of glucose-6-phosphate-dehydrogenase and NAD(P), thereby producing 6-phosphogluconate and NAD(P)H₂;
- 5 6) bringing into contact with the at least one liquid sample B_n at least one type of NAD(P)H dehydrogenase and a fluorophore/chromophore precursor, thereby producing a fluorophore/chromophore;
- 10 7) determining the concentration of glucose-6-phosphate in the at least one liquid sample B_n by detecting the amount produced of fluorophore/chromophore and comparing it to a reference;
- 15 8) indirectly determining the concentration of free glucose in the liquid sample M by subtracting the concentration in 6) with the concentration in 3).

In another aspect, the aim of the present invention is to provide a dry stick test device for the determination of free glucose in a liquid sample comprising free
20 glucose and glucose-6-phosphate (such as a milk sample) by means of a chemical assay, wherein said dry stick device is constructed in such a manner so as to determine the amount of both glucose-6-phosphate and free glucose in a liquid sample. The dry stick test device comprises:

- 25 (i) optionally a solid support,
- (ii) at least one reagent pad A comprising at least one type of glucose-6-phosphate-dehydrogenase, NAD(P), at least one type of NAD(P)H dehydrogenase and a fluorophore/chromophore precursor,
- 30 (iii) at least one reagent pad B comprising at least one type of glucose-6-phosphate-dehydrogenase, NAD(P), at least one type of NAD(P)H dehydrogenase, at least one type of hexokinase, adenosine triphosphate (ATP) and a fluorophore/chromophore precursor.

35

Nicotinamide adenine dinucleotide, abbreviated NAD, is a coenzyme. The compound is a dinucleotide, since it consists of two nucleotides joined through their phosphate groups. One nucleotide contains an adenine base and the other nicotinamide.

5

Nicotinamide adenine dinucleotide phosphate, abbreviated NADP, is a coenzyme. NADP differs from NAD in the presence of an additional phosphate group on the 2' position of the ribose ring that carries the adenine moiety. In the present context the term "NAD(P)" means NAD and/or NADP.

10

In the present context the term "NAD(P)H dehydrogenase" means NADH dehydrogenase (EC 1.6.99.3) and/or NADPH dehydrogenase (EC 1.6.99.1).

The glucose-6-phosphate-dehydrogenase according to the present invention is
15 classified in IUBMB Enzyme Nomenclature as 1.1.1.49.

The hexokinase according to the present invention is classified in IUBMB Enzyme Nomenclature as 2.7.1.1.

20 A fluorophore (or fluorochrome, similarly to a chromophore) is a fluorescent chemical compound that can re-emit light upon light excitation.

A chromophore is the part of a molecule responsible for its color. The color arises when a molecule absorbs certain wavelengths of visible light and transmits or
25 reflects others.

In the present context, a fluorophore/chromophore precursor is to be understood as a molecule that upon reduction with at least one type of NAD(P)H produces a fluorophore/chromophore.

30

Examples of fluorophore/chromophore precursors suitable in each specific assay may be easily recognised by the person skilled in the art and may be introduced into a dry stick test device according to the present invention.

In an embodiment of the present invention the fluorophore/chromophore precursor is selected from the group consisting of a tetrazolium salt; 4-aminoantipyrine/3,5-dimethoxy-N-ethyl-N-(2-hydroxy-3-sulfopropyl)-aniline sodium salt; 4-aminoantipyrine/1-naphthol-3,6-disulfonic acid-2-sodium salt; 4-aminoantipyrine/N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine sodium salt; 4-aminoantipyrine/1,7-dihydroxynaphthalene; 4-aminoantipyrine/3,5-dichloro-2-hydroxybenzene sulfonate; Tetrazolium violet; 3,5-dinitrobenzoic acid; Copper sulfate; N-1-naphthyl-N'-diethylenediamine-oxalic acid; Fast Red TR salt; Bromocresol green; Bromophenol blue; Arsenazo III; 2-(3,5-dimethoxy-4-hydroxyphenyl)-4,5-bis(4-dimethylaminophenyl)-imidazole; Pyridylazo dye; Magenta coupler dye; 1,5-bis(2-hydroxy-3,5-dichlorophenyl)-3-cyano formazan; Copper tartrate; 3-methyl-2-benzothiazolinone hydrazone; N-propyl-4-(2,6-dinitro-4-chlorobenzyl)quinolinium ethane sulfonate; Hydroxydiaryl imidazole; 2-methoxy-4-morpholinophenyl diazonium tetrachlorozincate; 3,3',5,5'-tetramethylbenzidine; 4-aminophenazone/3,5-dichloro-2-hydroxybenzene sulfonate; Primaquine diphosphate/3-methyl-2-benzothiazoline hydrazone; 2,5-dinitrobenzoic acid; 2-(p-indophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride; 3-hydroxy-1,2,3,4-tetrahydrobenzo-(h)-quinoline; or any derivative thereof.

20

The samples to be analysed

In the present context, the term "a sample" relates to any sample found in the form of liquid, solid or gas and which may be liquefied at the time of assaying. In order to wet the porous material used in the development pad and/or in the at least one reagent pad to permit migration, a liquid sample may be applied. Furthermore, it is preferred that a minimum number of handling steps, of the liquid sample is necessary before applying it to the dry stick test device. In the present context, the term "handling steps" relates to any kind of pre-treatment of the liquid sample before or after it has been applied to the assay device. This pre-treatment comprises separation, filtration, dilution, distillation, concentration, inactivation of interfering compounds, centrifugation, heating, fixation, addition of reagents, or chemical treatment.

In an embodiment of the present invention the sample may be collected from a mammal, preferably the mammal is selected from the group consisting of herd

animals, cows, camels, buffaloes, pigs, horses, deer, sheep, goats, pets, dogs, cats and humans.

In a preferred embodiment of the present invention, the sample can be derived
5 from any desirable source, however, it is preferred that the sample is selected from the group consisting of milk, blood, serum, plasma, saliva, urine, sweat, ocular lens fluid, cerebral spinal fluid, ascites fluid, mucous fluid, synovial fluid, peritoneal fluid, amniotic fluid or the like.

10 Besides physiological fluids, other liquid samples such as various water samples, food products and the like can be used. In addition, a solid test sample can be used once it is modified to form a liquid sample, for instance in the form of a solution, a suspension or an emulsion.

15 The inventors of the present invention have shown that oxamate inhibited unspecific production of NAD(P)H most likely originating from the action of lactate dehydrogenase (LDH, EC 1.1.1.27). Hence, in one embodiment the reagent pad A and/or B further comprise an inhibitor of lactate dehydrogenase.

20 Oxamate is a well-known competitive inhibitor of LDH (Larsen, 2005) and it has been used formerly in comparable assays to suppress LDH activity (Larsen & Nielsen, 2005). Hence, in one embodiment the reagent pad A and/or B further comprise oxamate.

25 In one embodiment, the dry stick test device further comprises a development pad.

In another embodiment, the at least one reagent pad A and/or B provide a first environment for said reagent(s), said first environment permitting an improved
30 storage stability of the reagent(s) and dry stick device when in a non-moistened state, the dry stick device further comprising a regulating pad being in contact with the at least one reagent pad A and/or B, wherein the regulating pad creating a second environment for said reagent(s) when in a moistened state, said second environment permitting an increased rate of reaction between the analyte and the
35 reagent(s), and wherein the condition in the first environment is provided by

adjusting the pH-value to a value that deviates from the optimal pH-value of the enzyme(s) and wherein the condition in the second environment is provided by regulating the pH-value to a value that approaches the optimal pH-value of the enzyme(s) and wherein the different environments have different pH-values.

5

One aspect relates to the use of a dry stick device according to the present invention for the determination of free glucose and glucose-6-phosphate in a milk sample.

10 Preparation of the dry stick

The dry stick device according to the present invention may be prepared by any conventional methods provided for the preparation of dry stick devices. In a preferred embodiment the method for providing a dry stick device according to the present invention comprises the steps of:

15

(i) providing a reagent pad A by impregnating a first porous material with an aqueous solution comprising at least one type of glucose-6-phosphate-dehydrogenase, NAD(P), at least one type of NAD(P)H dehydrogenase and a fluorophore/chromophore precursor, the at
20 least one reagent pad providing a first environment for said reagent(s),

20

(ii) thereafter drying the reagent pad A,

25

(iii) providing a reagent pad B by impregnating a first porous material with an aqueous solution comprising at least one type of glucose-6-phosphate-dehydrogenase, NAD(P), at least one type of NAD(P)H dehydrogenase, at least one type of hexokinase, adenosine triphosphate (ATP) and a fluorophore/chromophore precursor, the at
30 least one reagent pad providing a first environment for said reagent(s),

30

(iv) thereafter drying the reagent pad B,

(v) providing a regulating pad by impregnating a second porous material with an aqueous solution comprising a second environment for said reagent, when in a moistened state, permitting an increased rate of reaction between the analyte and the reagent,

5

(vi) thereafter drying the impregnated second porous material, and

(vii) contacting the reagent pad with the regulating pad, optionally on a solid support, to obtain the dry stick device.

10

The at least one reagent pad and the regulating pad may be contacted by substantially fully overlapping the pads, by partial overlap of the pads or by laying the regulating pad adjacent to at least one reagent pad. In an embodiment of the present invention the arrangement of the pads may be selected in such a manner to avoid precipitation of a sample component on the top face of the device. The sample components that may precipitate can be selected from the group consisting of proteins, carbohydrate, fat, cells, or other component present in the sample.

15

20 In a preferred embodiment of the present invention the first environment may be selected in such a manner as to favour the storage of the reagent(s) capable of reacting with the analyte and providing a detectable signal – as described earlier. Furthermore, the second environment may be selected in such a manner as to favour the performance of the reagent(s) capable of reacting with the analyte and providing a detectable signal – also as described earlier. Alternatively or additionally the second environment may be selected in such a manner as to favour the rate of reaction between the analyte and the reagent(s) capable of reacting with the analyte providing a detectable signal - as described earlier.

25

30

The solid support

The device according to the present invention may be supported by a solid support. In the present context, the term "solid support" refers to a material, which has no influence on the migration or on the reaction of the liquid sample or on reagent(s) or the agents capable of increasing the rate of the reaction. The

35

solid support provides a stabilising basis for the assay device and provides sufficient strength to maintain the desired physical shape and has substantially no interference with the production of a detectable signal.

- 5 In one embodiment of the present invention, the material for the solid support is selected from the group consisting of tubes, polymeric beads, nitrocellulose strips, membranes, filters, plastic sheets and the like.

- Naturally, synthetic and natural occurring materials that are synthetically modified
10 can be used as the material of the solid phase. Such materials include polysaccharides, for instance cellulosic materials such as paper and cellulosic derivatives, such as cellulose acetate and nitrocellulose, silica- or inorganic materials, such as, for example, deactivated alumina, diatomaceous earth, MgSO_4 or other inorganic finely divided material uniformly dispersed in a porous
15 polymeric matrix, wherein the matrix may comprise one or more polymers such as homopolymers and copolymers of vinyl chloride, for instance, polyvinyl chloride, vinyl chloride-propylene copolymer, and vinyl chloride-vinyl acetate copolymer, cloth, both naturally occurring (for instance, cotton) and synthetic (for instance, nylon), porous gels, such as silica gel, agarose, dextran, and gelatin,
20 polymeric films, such as polyacrylamide, and the like.

- In an embodiment of the present invention, the solid support may be omitted from the dry stick test device. In this case the dry stick test device comprises at least one reagent pad and, optionally, a development pad. When performing a
25 determination of an analyte using a dry stick test device without a solid support, the sample may be applied to the dry stick test device on one surface and the detectable signal may be detected on the same or another surface, thus it is preferred that any possible precipitation of sample components on the surface where the detectable signal are to be detected may be limited or avoided.

30

The reagent pad

In the present context the term "reagent pad" relates to one or more pads comprising a reagent or a combination of reagents. The reagent or the combination of reagents may preferably be impregnated into the reagent pad in

such a manner that the reagent or the combination of reagents is/are immobilised when in dry state and mobile when in moistened state.

In the present context of the present invention the term "reagent" relates to the chemical substance that reacts with or participate in or is necessary for the determination of an analyte, a derivative of said analyte or an indicator compound for said analyte to provide a detectable signal. A similar definition of the combination of reagents may be provided which relates more specifically to 2 or more reagents, such as 3 or more reagents, e.g. 4 or more reagents, such as 5 or more reagents, e.g. 6 or more reagents.

In an embodiment of the present invention the dry stick test device comprises at least 2 reagent pads, such as at least 3 reagent pads, e.g. at least 4 reagent pads, such as at least 5 reagent pads, e.g. at least 6 reagent pads. In this embodiment the reagents that reacts with or participate in or is necessary for the determination of an analyte, a derivative of said analyte or an indicator compound for said analyte to provide a detectable signal may be introduced into different reagent pads. This may improve stability, storage properties and applicability of the dry stick device because non-compatible compounds can be included in different reagent pads of the dry stick device.

The development pad

In the present context, the term "development pad" relates to a pad capable of regulating the environment and the conditions for the sample comprising the analyte to an environment that facilitates the determination of the analyte, a derivative of said analyte or an indicator compound for said analyte.

In an embodiment of the present invention, the development pad may comprise one or more controlling compounds capable of increasing the rate of the reaction between the analyte, a derivative of said analyte or an indicator compound for said analyte present in the sample and the reagent(s). In an embodiment of the present invention the controlling agent may be an acid or a base.

In yet another embodiment of the present invention, the development pad is in contact with at least one reagent pad by substantially fully overlapping, by partial

overlap or by laying adjacent to at least one reagent pad. In an embodiment of the present invention the development pad is overlapping the at least one reagent pad by at least 5%, such as at least 10%, e.g. at least 25%, such as at least 50%, e.g. at least 75%, such as at least 80%, e.g. at least 90%, such as at least 5 95%. In the present context the term "substantially fully overlapping" relates to two separate pads (the regulating pad and the at least one reagent pad) being placed on top of one another. In the present context the term "partial overlap" relates to two separate pads (the regulating pad and the at least one reagent pad) being overlapping with only part of the pad(s). A partial overlap of 100% relates 10 to a full overlap and a deviation of 5% from the 100% full overlap relates to a substantially full overlap.

In an embodiment of the present invention the development pad and the at least one reagent pad(s) are laying adjacent to one another. This means that the pads 15 are placed in contact with each other (touching each other). An overlap of 0% (but in contact) relates to the term "laying adjacent", furthermore, an overlap of less than 5% is considered being within the term of "laying adjacent", such as an overlap of at the most 4%, e.g. an overlap of the most 3%, such as an overlap of the most 2% or e.g. an overlap of the most 1%.

20

Controlling compound

In the development pad a controlling compound is immobilised. In the present context the term "controlling compound" relates to a substance that has the function as a propellant or a fuel in the specific assay for the determination of the 25 analyte (free glucose and/or glucose-6-phosphate), a derivative of said analyte or an indicator compound for said analyte. The controlling compound may also be the chemical substance responsible for the precipitation of sample components or the chemical compound causes the sample components not to precipitate. In an embodiment of the present invention the controlling compound may be separated 30 from at least one of the reagents in order to improve the stability of the dry stick test device.

In yet another embodiment of the present invention the controlling compound may be an acidic or an alkaline compound. Preferably, the controlling compound is 35 an acidic compound capable of providing a pH-value of the sample in the dry stick

test device, when in a moistened state, below pH 6, such as below pH 5, e.g. below pH 4, such as below pH 3, e.g. below pH 2, such as below pH 1, e.g. below pH 0, such as in the range of pH 0-6, e.g. in the range of pH 0-5, such as in the range of pH 0-4, e.g. in the range of pH 0-3, such as in the range of pH 0-2, e.g. in the range of pH 0-1, such as in the range of pH 1-6, e.g. in the range of pH 2-6, such as in the range of pH 3-6, e.g. in the range of pH 4-6, such as in the range of pH 5-6.

In another embodiment of the present invention the controlling compound may be an alkaline compound capable of providing a pH-value of the sample in the dry stick test device, when in a moistened state, of pH 8 or above, such as in the range of pH 8-14, e.g. in the range of pH 8-13, such as in the range of pH 8-12, e.g. in the range of pH 8-11, such as in the range of pH 8-10, e.g. in the range of pH 8-9, such as in the range of pH 9-12, e.g. in the range of pH 10-13, such as in the range of pH 10-11.

It should be noted that embodiments and features described in the context of one of the aspects of the present invention also apply to the other aspects of the invention.

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.

The invention will now be described in further details in the following non-limiting examples.

Examples

Project 1: Fluorometric determination of free glucose and glucose 6-phosphate in cow milk and other opaque matrices

SUMMARY: Analyses of free glucose and glucose 6-phosphate in milk has until now been dependent upon a time consuming and troublesome procedure which has limited investigations in this area. The present article presents a reliable, new analytical procedure, based on enzymatic degradation and fluorometric detection, which enables a laboratory to analyse hundreds of samples daily, without tedious

pre-treatment of the sample. More than 3,200 milk samples were analyzed in order to validate the analytical procedure and to demonstrate associations to other variables and factors that affected free milk glucose and glucose 6-phosphate. Glucose and glucose 6-phosphate is mutually strongly associated, however inversely. The concentration of glucose and glucose 6-P in milk is only marginally correlated to milk yield and time since last milking, strongly suggesting that these monosaccharides are not just products of lactose hydrolysis in the milk post secretion, but merely secreted from the producing cells together with lactose and other constituents. Glucose increases notably the first (roughly) fifty days in milk, whereas glucose 6-phosphate decreases correspondingly. Danish Friesian cows produce milk with a much higher concentrations of glucose 6-phosphate than Danish Jerseys, whereas this is not the matter for glucose. Parity of the cow, in all instances seems to influence for both breeds. Glucose and glucose 6-phosphate seems to be negatively and positively correlated, respectively, to the well established indicator of ketosis, beta-hydroxy butyrate in milk, indicating an auspicious future for milk glucose and glucose 6-phosphate as indicators of animal physiological status. The present analytical procedure facilitates further investigations in milk glucose and glucose 6-phosphate and the significance of this in connection with established physiological factors used for description of cow status *et cetera*.

Project 2: Development of an index for physiological imbalance and its use as an indicator of risk of disease during early lactation

SUMMARY: From examples below, an index to predict degree of physiological imbalance was developed (Project 2). Each week from -3 to 9 weeks relative to calving, all plasma metabolites were individually adjusted to an overall mean = 0 and variance = 1. The normalized variables were included in regression analyses by week of lactation to identify metabolites that explain the variation in calculated energy balance (EBAL), as a reflection of degree of physiological imbalance. NEFA, BHBA and glucose were weighted within each week based on regression coefficients (i.e. x_1 - x_3) generated from a model to predict EBAL. The weekly physiological imbalance index was defined as $\text{index} = (x_1 \times [\text{NEFA}]) + x_2 \times [\text{BHBA}] - x_3 \times [\text{glucose}]/3$. The index was compared to calculated EBAL and individual metabolites (i.e. NEFA, BHBA, and glucose) for use as an early indicator for risk of disease. For diseases that developed ≥ 2 weeks after calving, no

variables were associated with risk of diseases. Prepartal physiological imbalance and plasma NEFA were better predictors of diseases, i.e. metritis, retained placenta and milk fever, at week 1 than EBAL and plasma BHBA and glucose.

5 **Project 3: Indicators of dietary-induced physiological imbalance in milk throughout lactation**

SUMMARY: From examples below, we observed that nutrient restriction to experimentally increase physiological imbalance resulted in marked changes in plasma NEFA, BHBA, and glucose, liver TAG, milk yield, and energy balance and
10 that stage of lactation plays a pivotal role with regard to the degree of change in individual parameters. We observed the greatest and most rapid changes in free glucose and isocitrate in milk after restriction, regardless of stage of lactation. Therefore, automated sampling on-farm for free glucose and isocitrate in milk may be potential indicators of degree of physiological imbalance.

15

Projects 4 and 5: Identification of risk indicator for physiological imbalance by advanced biotechnology

SUMMARY: Results from Project 3 clearly show that multiple parameters are affected in physiological imbalance. Therefore, we generated an index for
20 physiological imbalance similar to that in Project 2. The index was positively correlated to plasma NEFA, daily milk yield, liver TAG and negatively correlated to EBAL and plasma glucose. Based on the index generated from blood metabolites in Project 3, liver samples from a subset of cows in early and mid-lactation with the greatest (i.e. physiological imbalance cows) and least (i.e. healthy) degree of
25 physiological imbalance were selected for iTRAQ-based proteomic profiling (Project 4) and results identified isocitrate dehydrogenase as a potential indicator for physiological imbalance. Since liver TAG is highly correlated to the development of several metabolic diseases (e.g. fatty liver and ketosis), liver samples from a subset of cows at approximately 7 days in milk with the highest (n
30 = 4) and lowest content of liver TAG (n = 4) were analyzed using iTRAQ-based proteomic profiling (Project 5). Results identified 4 proteins as potential indicators of physiological imbalance (glutamate dehydrogenase, malate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, and phosphoglucomutase).

35 **Project 6: Biomarkers in milk of physiological imbalance.**

SUMMARY: A meta-analysis was conducted using 3 separate studies consisting of 265 cows of two breeds (Holstein and Jersey) ranging from parities 1 to 5 extending over an entire lactation period (i.e. 0-69 weeks in milk). An index for physiological imbalance was generated as described in Project 2 based on plasma glucose, NEFA and BHBA. Free glucose in milk explained most of the variation in physiological imbalance for cows in early lactation whereas isocitrate in milk explained most of the variation in physiological imbalance for cows in later lactation (i.e. > 13 weeks in milk). We identified free glucose and isocitrate in milk as potential biomarkers for degree of physiological imbalance for cows throughout lactation. Cows with a greater degree of physiological imbalance experienced higher isocitrate and lower free glucose in milk when compared to cows with lower degree of physiological imbalance. Breed and stage of lactation altered concentrations of free glucose and isocitrate; and free glucose in milk was affected by parity. Results will be implemented in future biomodels for in-line and real-time measurement of degree of physiological imbalance on-farm.

Project 1 objective

The objective is to describe a new enzymatic-fluorometric method for determination of glucose and glucose 6-phosphate.

- a) The basic principles are enzymatic oxidation of glucose and glucose 6-phosphate with concomitant production of NAD(P)H₂ and a subsequent reaction where enzymatic coupling of NAD(P)H₂ to a fluorophore precursor develops a fluorescent product.
- b) Document its' usefulness in opaque matrices like milk without pre-treatment e.g. high speed centrifugation and acid precipitation of proteins.

Project 2 objective

The objective for Project 2 was to generate an index for PI based on several plasma metabolites and to compare the use of this index with calculated energy balance (EBAL) and individual plasma metabolites in relation to risk of disease during early lactation. The project comprises the following main activities:

- a) develop an index of PI based on blood metabolites (i.e. NEFA, BHBA and glucose) for cows in early lactation
- c) determine the usefulness of the PI index as an early indicator for risk of

diseases during early lactation

Project 3 objective

The objective for Project 3 was to identify and validate risk indicators for
5 monitoring physiological imbalance and individual differences in the response of
cows to changes in the nutrient supply. The project comprises the following main
activities:

- a) characterizing the changes in milk components during nutrient restriction for
10 identification of potential risk indicators for physiological imbalance;

Project 4 objective

The aim of Project 4 was to describe the liver proteome in early and mid-lactation
for cows at different degrees of physiological imbalance with a special focus on
15 biomarkers and pathways involved in periparturient disease complexes. The
project comprises the following main activities:

- a) identification of risk biomarkers for physiological imbalance in liver biopsies by
means of quantitative proteome analysis (LC-MS/MS);
20
b) applied screening for relevant liver risk indicators in more accessible samples
(milk/blood/urine) via combined mass spectrometry and clinical-chemical analyses
with a view to describing the practical application and value of the risk indicators.

25 Project 5 objective

The objective for Project 5 was to identify and validate risk indicators for
monitoring physiological imbalance in liver samples from cows 1 week after
parturition (i.e. high risk period for physiological imbalance) by means of
quantitative proteome analysis (LC-MS/MS). The project comprises the following
30 main activities:

- a) identification of risk biomarkers for physiological imbalance in liver biopsies
by means of quantitative proteome analysis (LC-MS/MS);

35 Project 6 objective

The objective of was to identify potential biomarkers in milk that relate to degree of PI as an early warning system for risk of disease on-farm and to quantify the variation in the biomarkers of interest with regard to systemic effects i.e. breed, parity and stage of lactation and across different production systems. The project
5 comprises the following main activities:

- a) identify potential biomarkers in milk that relate to degree of PI as an early warning system for risk of disease on-farm;
- b) quantify the variation in the biomarkers of interest with regard to systemic effects and across different production systems;

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Materials and Methods

Project 1:

Glucose 6-phosphate was determined separately by enzymatic oxidation by
15 glucose 6-P dehydrogenase using NADP^+ dependent enzyme from *Saccharomyces* sp. (EC 1.1.1.49; Roche 10 127 655 001). The sum of free glucose and glucose 6-phosphate (henceforth denoted total glucose) was determined by enzymatic oxidation by hexokinase (EC 2.7.1.1; Roche 11 426 362 001) and glucose 6-phosphate dehydrogenase from *Leuconostoc* sp. (cofactor NAD^+ and NADP^+ ; EC
20 1.1.1.49; Roche 10 165 875 001). Free glucose was consequently estimated as the difference between the two results.

The enzymatic-fluorometric method of total glucose determination is a three step enzymatic procedure to obtain the fluorescent product equivalent to the glucose content. The two first steps are identical to the widely used
25 hexokinase and glucose-6-phosphatase mediated conversion of glucose to gluconate-6-phosphatase and NAD(P)H (Bergmeyer et al. 1974). The last step is an enzymatic coupling of the reducing equivalents from NAD(P)H to the non-fluorescent compound resazurin mediated by the enzyme diaphorase (EC 1.6.99.-
_). Resazurin is reduced by NAD(P)H and the highly fluorescent substance
30 resorufin is developed and measured fluorometrically (Larsen and Nielsen, 2005). Reducing equivalents (NADPH) from the separate glucose 6-phosphate oxidation is in the same way coupled to resazurin in order to quantify glucose 6-phosphate fluorometrically.

35 Reagents, combined glucose and glucose 6-phosphate analyses:

Reagent 1. Tris-buffer, 100 mM, pH 7.6 with 10 mM Mg^{++} , 3.6 mM Na-oxamate (MW 111.03), 1.9 mM ATP (Na_2 -salt, MW 551.2) and 1.9 mM NAD (Na_2 -salt, MW 717.5). Immediately before use the solution was supplied with hexokinase enzyme and glucose-6-phosphatase (Roche 10 737 275 001), 3.1 U and 1.6 U/ml, respectively.

Reagent 2. Tris-buffer, 60 mM, pH 7.2, with 1.28 mM resazurin (Sigma R-2127, MW 251.2), 0.01% Triton X-100, and 9.8 U/ml diaphorase (Toyobo Enzymes DAD-301). 10 drops of Tween 80 were added per 15 ml.

Reagents, glucose 6-P analyses:

10 Reagent 1. Tris-buffer, 60 mM, pH 7.2 with 3.6 mM Na-oxamate and 1.9 mM $NADP^+$ (di- Na -salt, MW 787.4). Immediately before use the solution was supplied with glucose 6-phosphate dehydrogenase enzyme 1.6 U/ml.

Reagent 2. Similar to the combined glucose and glucose 6-P analyses.

15 Procedure, both analyses

Dosage and dilution of sample and addition of reagents were performed in a robotic system (Biomek[®] 2000; Beckman Coulter). Milk samples (standards and control samples) distributed in a 96-well micro plate were initially diluted 1:2 with distilled water. 45 μ l diluted samples were transferred to a daughter plate where
20 50 μ l reagent 1 was provided ($t = 0$; 1. incubation). After 3 minutes, 80 μ l of reagent 2 was added, and the plate transferred to the fluorometer (Fluostar, Galaxy, BMG Labtechnologies; 2. incubation). At $t = 6$ min, the samples were excited with 544 nm monochromatic light and read at 590 nm light. Each plate contained 2 * 8 standard solutions (glucose) and 2 * 4 control solutions. The
25 samples were read against a standard curve; control samples worked as internal control and day to day check. Standards and control samples were prepared (independently) from glucose monohydrate (MW 198.2) and glucose 6-P (MW 304.2), respectively (stored in excicator), and water. Standard concentrations used were 0; 0.24; 0.48; 0.72; 0.96; 1.20; 1.80; and 2.40 mM (combined
30 glucose and glucose 6-phosphate) and 0; 0.08; 0.16; 0.24; 0.32; 0.48; 0.64; and 0.80 mM (glucose 6-phosphate). Control samples were 0.5; 1.0; 1.5; and 2.0 mM (combined glucose and glucose 6-phosphate) and 0.15; 0.30; 0.45; and 0.75 mM (glucose 6-phosphate), respectively.

35 Validation of the method

Standards and control material

Measurements of standard curves and control samples from 30 micro plates analysed different days were gathered. Intra- and inter-assay precision and accuracy (% bias) was calculated from the control samples; average slope and correlation coefficient were calculated from the standards.

Intra- and inter assay precision, samples

- Total glucose: 144 milk samples were replicated three times within the same plate and 72 samples were replicated three times between plates.
- 10 Glucose 6-phosphate: 72 milk samples were replicated three times within the same plate and 72 samples were replicated three times between plates.

Total glucose, comparison between two analytical methods

- Two hundred and twenty seven fresh milk samples, preserved with 100 mg Bronopol ® /kg, were analysed for the sum of glucose and glucose 6-phosphate by two different methods, 1) a reference method, i.e. an enzymatic spectrophotometric analysis in an autoanalyzer according to standard procedures (glucose hexokinase, Siemens Diagnostics® Clinical Methods for ADVIA 1650). However, the procedure was optimised (greater fraction of sample) due to the expected low concentration of glucose and the analytical window restrained (standards from 0 – 2.4 mM). Intra- and inter-assay precision as well as accuracy for the procedure was within 3 (CV)%. The milk samples were initially precipitated and filtered for protein and fat with Carrez'-solution as recommended by Boehringer Mannheim (1995); the samples were by this procedure diluted 5 times compared to the original milk, 2) by the present enzymatic-fluorometric method with no preceding precipitation of protein and fat.

Glucose 6-phosphate, comparison between two analytical methods

- An equivalent comparison was established for glucose 6-P. A spectrophotometric endpoint analysis was established for glucose 6-phosphate: Reagent 1 (R₁): Tris-buffer, 60 mM, with 1.9 mM NADP⁺, pH 7.2; reagent 2 (R₂): Tris-buffer, 60 mM, with 1.9 mM NADP⁺ and 6.7 U Glucose 6-phosphate dehydrogenase per ml (Roche 10 127 655 001), pH 7.2. Procedure: 25 µl undiluted sample was incubated with 55 µl R₁ for 5 min. Baseline was read at 340 nm (unspecific reaction). 10 µl of R₂ was added and the absorbance was read at t

= 10 min (specific reaction). The absorbance was compared with a standard curve (0 – 0.8 mM). The spectrophotometric analyses were performed using an autoanalyzer (ADVIA 1650, Siemens Diagnostics®). Intra-assay precision and accuracy (bias %) were both within 2% for samples and control samples, respectively (0.15; 0.30; 0.45; and 0.75 mM). One hundred and six samples treated by the Carrez-procedure were analysed by this spectrophotometric method and compared with the fluorometric method.

Total glucose and glucose 6-phosphate, the effect of addition of Na-oxamate

One hundred and seven milk samples were analysed by the fluorometric method for both total glucose and glucose 6-phosphate with and without Na-oxamate in the reaction medium (1.9 mM during 1. incubation and 1.0 mM during 2. incubation).

Spiking of milk samples with glucose and glucose 6-phosphate

Ninety six samples were analysed (in duplicate) for total glucose and glucose 6-phosphate according to the described procedure. Furthermore, the samples were spiked with 0.48; 0.72; 0.96; or 1.20 mM glucose and 0.32; 0.48; or 0.64 mM glucose 6-phosphate. The recovery of the glucose and the glucose 6-P addition was calculated.

Total glucose and glucose 6-phosphate, effect of storage of samples

To investigate the effect of storage, one hundred and nine milk samples were analysed for total glucose and glucose 6-phosphate three times separated by 24 h at 4 °C and 4 h at room temperature.

The association between milk glucose, milking data and other milk variables

More than 3,200 milk samples from a local herd were used to investigate the relationship between selected milking data, basic milk parameters and udder health indicated by milk somatic cell counts (SCC). The milking system is a robotic system, where representative milk samples are taken in a 10 ml tube, pre-dosed with the preservative Bronopol ® to obtain 100 mg/kg sample. The samples were stored at 4 °C and brought to the laboratory every morning. The milk samples were analysed immediately upon arrival for a period of 2 months.

Milk citrate, lactose, fat and protein were determined by IR-spectroscopy (CombiFoss 4000, Foss Electric Ltd., Hillerød, DK). Determination of somatic cell counts (SCC) was performed at a commercial laboratory (Eurofins, Holstebro, DK) using standard Fossomatic cell counter (EN ISO 13366-3; Foss Electric Ltd., Hillerød, DK). β -hydroxybutyrate, BOHB, was determined by an endpoint fluorometric method according to Larsen and Nielsen (2005).

Project 2:

A total of 634 lactations from 317 cows consisting of 3 breeds ranging from parity 1 to 4 were used. Weekly blood samples were analyzed for selected metabolites i.e. urea nitrogen, albumin, cholesterol, NEFA, glucose, and BHBA. Energy intake and energy balance (EBAL) were calculated. Veterinary treatment records and daily composite milk somatic cell counts were used to determine incidence of disease. Data were adjusted for numerous fixed effects (e.g., parity, breed and week around calving) before further statistical analysis. The time of disease (TOD) was recorded as the day in which the signs of disease were observed (TOD = 0). Week prior to and after TOD was $\pm n$ week relative to TOD = 0. Each week, all plasma metabolites were individually adjusted to an overall mean = 0 and variance = 1. The normalized variables were included in regression analyses by week of lactation to identify metabolites that explain the variation in calculated EBAL, as a reflection of degree of physiological imbalance. NEFA, BHBA and glucose were weighted within each wk based on regression coefficients (i.e. x_1 - x_3) generated from a model to predict EBAL. Data from week -1 relative to TOM were analyzed using a mixed linear model to relate degree of physiological imbalance and metabolites in blood to risk of disease. The weekly physiological imbalance index was defined as $\text{physiological imbalance} = (x_1 \times [\ln(\text{NEFA})] + x_2 \times [\ln(\text{BHBA})] - x_3 \times [\text{glucose}])/3$.

Project 3:

Forty-seven healthy Holstein dairy cows in early (E; $n=14$; 22–86 days in milk), mid (M; $n=15$; 100–217 days in milk) and late (L; $n=18$; 235–355 days in milk) lactation were used. Of these, 26 cows were primiparous and 21 cows were multiparous (≥ 2 nd lactation). At the beginning of the study, all cows were fed a standard total mixed ration for ad libitum intake. After 5-d (five days), all cows were nutrient restricted to induce physiological imbalance by providing about 40%

of net energy for lactation requirements based on body weight, milk production and composition by supplementing the standard total mixed ration with 60% wheat straw. After 4-d of nutrient restriction, cows returned to full feed. Clinical-biochemical analyses were performed on blood (i.e. glucose, insulin, NEFA, and BHBA), liver (i.e. glycogen and TAG) and milk samples (i.e. fat, protein, lactose, glucose, BHBA, and citrate). Energy balance was calculated for all cows throughout the study period based on National Research Council requirements (2001). For each cow, the change in each variable before and during nutrient restriction was calculated.

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Project 4:

A subset of cows from Project 3 in early and mid-lactation were used to calculate a physiological imbalance index using average plasma NEFA, BHBA and glucose concentrations from the period prior to nutrient restriction.

15 The index for physiological imbalance was generated similar to Project 2: $\text{index} = [\ln(\text{NEFA})] + [\ln(\text{BHBA})] - [\text{glucose}]$. Within stage of lactation, a subset of 6 cows classified as having either the greatest ($n = 3$; severe) or least ($n = 3$; normal) degree of physiological imbalance were analyzed using iTRAQ based proteomic profiling for cows in both early ($n = 6$) and mid- ($n = 6$) lactation. The inventors have suggested including cholesterol as it is correlated with milk glucose as well as including calculated energy balance, body weight, and/or body condition score. In addition, the value of ratios to predict physiological imbalance and the identification of indicators from body fluids will also be assessed.

25 **Project 5:**

A subset of liver samples ($n = 8$) collected 1 week after parturition from cows with the highest (22.9%) and lowest (7.1%) liver TAG content were analyzed using iTRAQ based proteomic profiling to identify potential indicators in liver for physiological imbalance.

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Project 6:

Two hundred and sixty-five cows consisting of two breeds (202 Holstein and 63 Jersey), with 85 primi- and 180 multiparous cows ranging from 0 to 69 weeks in milk were used. Blood samples ($n = 534$) were collected weekly via coccygeal veni-puncture. Plasma was collected and analyzed for NEFA, BHBA,

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- glucose, albumin, cholesterol, triglycerides, total protein and plasma urea nitrogen. Daily milk yield and feed intake was recorded. An index for physiological imbalance was generated as described in Project 2. Composite milk samples were collected (n = 2,308) periodically for all cows and analyzed for % fat,
- 5 protein, lactose and concentration of free glucose, glucose-6-phosphate, BHBA and isocitrate.

Results and Discussion

Project 1: Determination of free glucose and glucose-6-phosphate in milk

10 *Standards and control material*

The total glucose analysis: The intra-assay precisions were 2.6; 2.0; 2.1; and 2.9 CV% respectively for the 0.50; 1.00; 1.50; 2.00 mM control samples (n = 30).

The corresponding figures for the inter-assay precision were 5.9; 3.3; 4.0; and 4.0 CV% (n = 30). Bias were equivalently -6.7; -3.7; -0.5; and +4.8 % (n = 30).

- 15 The glucose 6-phosphate analysis: The intra assay precisions were 2.3; 2.3; 2.7; and 2.6 CV% respectively for the 0.15; 0.30; 0.45; 0.75 mM control samples (n = 30). The corresponding figures for the *inter* assay precision were 6.2; 5.1; 4.7; and 4.9 CV% (n = 30). Bias were equivalently +4.6; +4.7; +4.7; and +7.2 % (n = 30).
- 20 The average slope and correlation coefficient (r) of the standard curves was 1.000 and 0.998, respectively (n = 30), both for total glucose and for glucose 6-phosphate analysis.

Intra- and inter assay precision, milk samples

- 25 Intra and inter plate precision was respectively 3% (CV; n = 3, N = 144) and 3.4% (CV; n = 3, N = 72) for total glucose and 3.3% and 4.4% respectively (CV; n = 3, N = 72) for glucose 6-phosphate.

Total glucose, comparison between two analytical methods

- 30 Mean values (μ M) and corresponding 0.05-0.95 inter percentile were 581 (368 – 788) and 552 (363 – 770), for colorimetric and fluorometric methods, respectively. The colorimetric and the fluorometric results were highly correlated (r = 0.903), fig. 3.

- 35 *Glucose 6-phosphate,, comparison between two analytical methods*

Mean values (μM) and corresponding 0.05-0.95 inter percentile were 151 (5 – 410) and 75 (24 – 164), for colorimetric and fluorometric methods, respectively. The colorimetric and the fluorometric results were highly correlated ($r = 0.978$). The straight line regression between the two set of observations was: Glu 6-phosphate, mM (fluorometric) = 0.32 mM (spectrophotometric) + 0.03 mM.

Total glucose and glucose 6-phosphate, the effect of addition of Na-oxamate

Total glucose in the 107 milk samples was on average 540 μM without addition of oxamate (0.05 – 0.95 inter percentile 384 – 675) and 521 μM (0.05 – 0.95 inter percentile 398 – 653) with addition of oxamate in the reaction medium, i.e. on average 3.7 % less with oxamate ("oxamate" = $0.94 \times \text{"without oxamate"} + 0.01$; $r = 0.962$). Correspondingly, glucose 6-phosphate concentration was on average 74 μM (0.05 – 0.95 inter percentile 24 – 164) without oxamate in the reaction medium, while 69 μM (0.05 – 0.95 inter percentile (19 – 158) was obtained with oxamate, i.e. 14.6 % less with oxamate ("oxamate" = $1.00 \times \text{"without oxamate"} - 0.01$; $r = 0.998$).

Spiking of milk samples with glucose and glucose 6-phosphate

The obtained results for samples spiked with 0.48; 0.72; 0.96; or 1.20 mM glucose were on average 1.37 mM whereas the expected outcome was calculated to 1.41 mM ($n = 96$), i.e. 2.0% less than expected. The association between measured values and expected value is by correlation: measured value = $0.90 \times \text{expected} + 0.10$; $r = 0.984$, $p < 0.001$). The corresponding results for the 0.32; 0.48; or 0.64 mM glucose 6-phosphate spiking was on average 0.51 mM ($n = 96$) versus the expected value, 0.58 mM, i.e. 13.4 % less than expected. The association between measured values and expected value is by correlation: measured value = $1.14 \times \text{expected} - 0.15$; $r = 0.965$, $p < 0.001$).

Total glucose and glucose 6-phosphate, effect of storage of samples

The durability of total glucose and glucose 6-phosphate in milk was tested in 109 non-preserved random samples. The samples were analysed just after arrival from the farm (A), after 24 h storage at 4 °C (B) and after incubation for 4 h at room temperature (C). Storage for 24 h at 4 °C only marginally changed the obtained results, i.e. average of total glucose decreased 5 %, and average of glucose 6-phosphate increased 16 %, whereas 4 h of incubation at room

temperature reduced the mean total glucose by 14 % and increased the glucose 6-phosphate by 11 %. All comparable correlations were highly significant.

The association between milk glucose, milking data and other milk variables

- 5 Simple correlations between the concentration of milk glucose and glucose 6-phosphate and other observations are given in Figure 1. Days in milk (DIM) is significantly and negatively associated with glucose 6-phosphate content in milk and positively associated with glucose content in milk, Figure 4. Milk yield is not correlated with glucose 6-phosphate and glucose; time since last milking is
- 10 inversely correlated with glucose and glucose 6-phosphate. The concentration of fat correlates negatively with free glucose, while it correlates positively to glucose 6-phosphate, the reverse situation, is valid for milk lactose. The association between glucose and glucose 6-phosphate to milk protein is insignificant. Both glucose and glucose 6-phosphate correlate negatively with milk citrate, for milk
- 15 BHBA the correlation was positive with glucose 6-phosphate and negative with glucose. A strong negative correlation was observed between glucose 6-phosphate and glucose.

- The concentration of glucose 6-phosphate in milk was affected by breed of the animal (Danish Friesian vs. Jersey), parity and the interaction between these
- 20 variables ($p < 0.001$), Figure 2. Free glucose was significantly affected by parity and the interaction between breed and parity ($p < 0.001$).

Discussion

- The present enzymatic-fluorometric methods for determination of free glucose and
- 25 glucose 6-phosphate in milk are reliable analytical methods. The determination of free glucose and glucose 6-phosphate responds linearly to standards, to the indigenous monosaccharide content of milk, and to spiking of milk samples with the respective monosaccharides. The precision of the assay is acceptable for analytic as well as descriptive use.

- 30 The assays work without time or material consuming pre-treatment of the samples, and it is in this context a "high throughput" assay, i.e. hundreds of samples may be analysed daily in automated laboratories.

- In the present study, 1.0-1.9 mM of oxamate inhibited unspecific production of NAD(P)H most likely originating from the action of lactate dehydrogenase (LDH,
- 35 EC 1.1.1.27), however, other NAD(P)H producing enzymes may be present.

Oxamate is a well-known competitive inhibitor of LDH (Larsen, 2005) and it has been used formerly in comparable assays to suppress LDH activity (Larsen & Nielsen, 2005).

LDH is a well-documented indigenous enzyme in milk. The activity originates
5 mainly from somatic cells, leucocytes and invading microorganisms, and therefore, mastitic milk is associated with higher LDH activities (e.g. Chagunda et al, 2006 a; Chagunda et al, 2006 b; Friggens et al. 2007) just as endotoxins like *E. coli* lipopolysaccharides (LPS) infused in milk quarters increase the activity (Larsen et al, 2010). LDH will mediate the conversion of lactate to pyruvate
10 (oxidative direction) with a concomitant production of NAD(P)H from NAD(P)⁺. Thus a basic level of lactate and the presence of LDH are likely to interfere with the present assay for glucose and glucose 6-phosphate. L-lactate is present in non-mastitic milk (approx. 0.1 – 0.2 mM) and increases up to 30-fold during mastitis (SCC; Davis et al, 2004) or experimental inflammations (Silanikove et al,
15 2011). However, the pH in the present reaction mixture (pH 7.2-7.6) does not favour the lactate to pyruvate direction mediated by LDH and subsequent production of NADH – on the contrary (Vassault, 1995; Wahlefeld, 1995). However, less favourable pH conditions may serve when basic ingredients are available. Increased lactate concentration and higher activity of LDH during
20 mastitis, should be – with all things being equal – background for a positive correlation between log SCC and NADH production in a reaction mixture of a non-inhibited system. The association between log SCC and glucose was however weak and negative ($\log \text{SCC} = -0.024 \times \text{total glucose} + 0.47$; $r = 0.09$, $n = 3003$) indicating that unspecific reactions by LDH are not strongly interfering the present
25 assay of glucose in milk. The negative correlation between glucose and SCC has previously been described by Marschke and Kitchen (1984), who included NAGase activity in milk (also a mastitis indicators).

The free glucose level in milk obtained in the present study, i.e. mean 340 μM , P_5 – P_{95} inter percentile 128-567 μM ($n = 3261$) is in good accordance with
30 investigations carried out within the last decades. Marschke and Kitchen (1984) reported a range from 20 to 570 μM (mean 220 μM ; $n = 188$) of glucose after centrifugation and precipitation of samples. Lemosquet et al (2004) reported on average between 480 – 580 μM ; Rigout et al (2002) between 430 – 570 μM ; Hurtaud et al. (1998 and 2000) between 720 – 830 μM and 510 – 650 μM ,
35 respectively; and Faulkner and Pollack (1989) between 350 – 580 μM . Most of

these studies are based on a relatively limited number of analyses and animals; and most of the studies use both centrifugation and precipitation as pre-treatment before analyses (enzymatic determination of glucose, colorimetry), although in some instances, it is difficult to tell if precipitation of protein has been conducted
5 before colorimetry or not or if the glucose 6-phosphate fraction of the total glucose has been considered separate when the "hexokinase assay" has been used.

Most estimations of glucose 6-phosphate in milk are based on precipitation of protein and fat (perchloric acid precipitation) and subsequent colorimetric
10 determination; and most studies find a concentration between 20 and 140 μM (e.g. Hurtaud et al. 1998 and 2000; Rigout et al. 2002 and 2003; Faulkner 1980; Faulkner and Pollock, 1989) that are marginally lower than the present enzymatic-fluorometric determination but within the same range for a milk metabolite.

The fact that milk yield and hours since last milking are weakly correlated to free
15 glucose and glucose 6-phosphate (hours since last milking even inversely correlated), is from an analytical and physiological point of view very important. If free glucose in milk was a consequence of hydrolysis of lactose *post* secretion, longer deposition in the udder would increase hydrolysis resulting in higher concentrations of free glucose. However, that is not the case suggesting the free
20 glucose in milk originated prior to secretion by epithelial cells.

The positive correlation between free glucose and lactose may reflect intra-cellular conditions; i.e. uptake of glucose from circulation, the utilization of glucose in lactose synthesis (various steps) and the secretion of lactose and glucose to the milk. The inverse correlation between glucose 6-phosphate and lactose may be
25 related to lactose synthesis and milk production because glucose 6-phosphate is an intermediate in the pentose phosphate cycle, developing reducing equivalents (NADPH) used for reductive biosynthesis of milk fat especially.

The measured milk variables in the present study show several interesting connections to glucose 6-phosphate and free glucose, e.g. positive correlations
30 between the well-established indicator of ketosis, BOHB and glucose 6-phosphate and a negative correlation between BOHB and glucose. The correlations reveal a connection between the constituents and indicate that milk glucose and glucose 6-P could be important markers of physiological status together with other indicators in milk, although additional statistical analyses based on physiological
35 considerations are needed.

The average concentration free glucose in milk is not significantly different between Danish Holstein and Jersey breeds, 0.343 mM vs. 0.339 mM ($n = 2717$ and 515, respectively), whereas the concentration of glucose 6-phosphate is significantly different (0.130 vs. 0.090 mM). However, parity seems to affect the
5 glucose level and the glucose 6-phosphate level, i.e. older Danish Friesian cows have lower concentrations of glucose and glucose 6-phosphate, whereas the opposite was observed for Jersey cows. These results are un-explainable and warrants further investigation into the relationship between glucose in milk and parity for dairy cows during lactation. In addition, the higher concentration of
10 glucose 6-phosphate and a lower concentration of glucose in colostrum are, to our knowledge, unknown.

A relatively limited number of studies have focused on free glucose in milk in association with metabolism and health of the animal and the impact of external factors. Faulkner et al. (1981) showed that milk glucose concentration in goats fell
15 considerably during starvation. Faulkner and Pollock (1989) observed an increase in milk glucose when cows were fed unprotected soybean free fatty acids.

Furthermore, Faulkner (1999) reported that milk glucose concentration increased considerably due to sub-cutaneous injections of growth hormone in lactating goats. A number of studies have investigated the effect of duodenal infusion of
20 glucose on milk glucose concentration, i.e. Hurtaud et al (1998, 2000), Rigout et al. (2002), Lemosquet et al. (2004) in order to establish a relationship between energy status and milk glucose.

Future experiments may elaborate on factors affecting milk glucose and glucose 6-phosphate and elucidate mechanisms behind these monosaccharides in milk.
25 The presented enzymatic-fluorometric determination of both monosaccharides, avoiding pre treatment of samples, might facilitate future research in this area.

Project 2: Index for physiological imbalance and risk of disease

Adjusting for fixed effects eliminates known factors that cause variation in the
30 parameters of interest (i.e. blood NEFA, BHBA and glucose). This, in turn, reduces the correlation between EBAL and blood metabolites. For example, the correlation between raw data for EBAL and NEFA is $r = -0.50$ ($P < 0.001$) whereas after adjustments for fixed effects the correlation is reduced to $r = -0.35$ ($P < 0.001$).

In this study we generated an index for physiological imbalance based on normalized (average=0; variance=1) concentrations of between-cow variations in NEFA, BHBA and glucose in blood during the periparturient period. The regression model selected to predict degree of physiological imbalance based on between-

5 cow variations in calculated EBAL from wk -4 to 9 relative to parturition revealed that plasma NEFA, BHBA and glucose explained the majority of the variation in EBAL. Therefore, plasma NEFA, BHBA and glucose were weighted (Figure 6) based on regression coefficients and used to generate an index for physiological imbalance where index = $(x_1 \times [\ln(\text{NEFA})] + x_2 \times [\ln(\text{BHBA})] - x_3 \times$

10 $[\text{glucose}])/3$ (Figure 9). After parturition, negative correlations were observed between degree of physiological imbalance and EBAL and plasma concentration of glucose whereas energy intake was not significantly correlated with degree of physiological imbalance indicating that degree of naturally occurring physiological imbalance is independent from energy intake (Figure 7). Milk yield as well as

15 plasma concentration of NEFA, BHBA were positively correlated with physiological imbalance during early lactation. Degree of physiological imbalance was significantly correlated with total lipid ($r = 0.39$; $P < 0.001$), TAG ($r = 0.40$; $P < 0.001$) and glycogen ($r = -0.38$; $P < 0.001$) content in liver at 1 week postpartum with similar patterns observed at week 4 postpartum. This provides support for

20 the use of the physiological imbalance index as a predictor of metabolic function of the liver associated with the uptake, utilization and secretion of lipids by the liver.

An index for PI, based on plasma NEFA, BHBA and glucose, related to risk for most diseases, i.e. metritis, milk fever, retained placenta, lameness and mastitis

25 (Figures 8, 10 and 11), and is therefore a better predictor for risk of disease during early lactation than EBAL, glucose and BHBA. The study showed that prepartal degree of PI was a good predictor of disease after calving and may potentially be related to health problems that occurred in the previous lactation (Ingvarsen, 2006). Calculated EBAL failed to predict risk of all diseases during

30 early lactation, except milk fever. However, higher prepartum NEFA was also associated with incidence of diseases such as metritis, mastitis and RP.

Interestingly, prepartal BHBA was not associated with the incidence of any disease after parturition. Our results indicate that prepartal NEFA are a better indicator of risk for disease than BHBA. NEFA explained the majority of the variation in

physiological imbalance and suggests that changes in NEFA more accurately reflect metabolic status and risk of disease than BHBA.

Project 3: Risk indicators in milk for dietary-induced physiological imbalance

5 throughout lactation

Nutrient restriction increased degree of physiological imbalance via increased plasma BHBA and NEFA and liver TAG and decreased glucose and liver glycogen content (Figure 12). Regardless of stage of lactation, greater changes in isocitrate (96%) and free glucose (-23%) concentration in milk were observed during
10 nutrient restriction (0-96 h) when compared BHBA (8%) and glucose-6-phosphate (1%; Figure 13). In addition, greater changes in fat (36%) and milk yield (-32%) were observed during restriction. However, milk fat and milk yield have been associated with other diseases, such as mastitis (Moyes et al., 2009), and therefore, may lack specificity as general risk indicators for physiological
15 imbalance. Furthermore, changes in free glucose and isocitrate in milk were the most rapid (i.e. significant changes by 24 h after nutrient restriction) when compared to other milk components (i.e. fat, glucose-6-phosphate and BHBA; Figure 13). Stage of lactation did not effect changes in isocitrate during nutrient restriction but free glucose and glucose-6-phosphate in milk were lower for cows
20 in early than cows in later lactation. In addition, free glucose in milk was positively correlated to plasma glucose ($r = 0.38$) and liver glycogen content ($r = 0.60$) and negatively correlated to plasma NEFA ($r = -0.17$), BHBA ($r = -0.38$). Interestingly, glucose-6-phosphate in milk was negatively correlated with free glucose in milk ($r = -0.45$). Our results indicate that free glucose and isocitrate in
25 milk as potential general risk indicators for physiological imbalance for cows during lactation.

Since plasma glucose and free glucose in milk were lower for cows in early lactation whereas no differences in lactose or milk yield were observed when
30 compared to cows in mid- and late lactation, the inventors hypothesize that cows in early lactation had increased uptake of blood glucose by the mammary gland for lactose synthesis.

Project 4: Risk indicators in liver for dietary-induced physiological imbalance

35 throughout lactation

Similar to Project 3, results suggest an increased mammary uptake and conversion of plasma glucose to lactose in milk for severe compared to normal cows in early lactation. Cows in physiological imbalance had higher plasma BHBA, NEFA, and liver TAG and lower plasma glucose than cows with a lower degree of physiological imbalance (Figure 14). Based on biochemical pathways coupled with laboratory capabilities, analyses in blood and milk is expected to be possible for at least 1 enzyme (i.e. isocitrate dehydrogenase –TCA cycle) for use as potential risk biomarkers for physiological imbalance.

10 Project 5: Risk indicators for naturally-occurring physiological imbalance in liver after parturition

Cows with higher liver TAG had higher plasma BHBA than cows with lower liver TAG (Figure 15). Based on biochemical pathways coupled with laboratory capabilities, analyses in blood and milk is expected to be possible for at least 4 enzymes (i.e. glutamate dehydrogenase, malate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, and phosphoglucomutase) for use as potential risk biomarkers for physiological imbalance. These enzymes are involved in the conversion of amino acids to energy (glutamate dehydrogenase), glycolysis/gluconeogenesis (glyceraldehyde 3-phosphate dehydrogenase), glycogen synthesis/degradation (phosphoglucomutase), and the TCA cycle (malate dehydrogenase).

Results from all projects identify a potential coping mechanisms utilized by cows in severe physiological imbalance and identified pathways and specific parameters altered by physiological imbalance that provides information into new avenues linking physiological imbalance and risk of disease thereby improving animal health and productivity during lactation.

Project 6: Identification of biomarkers in milk for physiological imbalance.

30 Regression analyses showed that free glucose in milk explained most of the variation ($R^2 = 0.24$) in degree of physiological imbalance for cows in early lactation when compared to other metabolites in milk. For cows > 13 weeks in milk, isocitrate in milk explained most of the variation ($R^2 = 0.22$) in degree of physiological imbalance. Regardless of stage of lactation, both free glucose and isocitrate in milk explained most of the variation ($R^2 = 0.24$) in degree of

physiological imbalance throughout lactation. The R^2 values are based solely on metabolites in milk and therefore exclude other parameters known to cause variations in milk components i.e. feed intake, milk yield and changes in hormones and metabolites in blood.

- 5 Analysis of variance showed that breed, parity and stage of lactation altered concentrations of isocitrate, free glucose and glucose-6-phosphate in milk. For isocitrate, parity did not alter concentration of isocitrate in milk whereas Jersey cows had higher (0.22 ± 0.01 ; $P < 0.001$) concentrations than Holsteins (0.16 ± 0.01) and cows in early lactation had higher (0.20 ± 0.01 ; $P < 0.001$) concentrations when compared to cows in later lactation (0.18 ± 0.01). For free glucose, multiparous cows had higher (0.37 ± 0.01 ; $P < 0.001$) concentrations when compared to primiparous cows (0.32 ± 0.01), Jerseys had lower (0.33 ± 0.01 ; $P < 0.001$) concentrations when compared to Holstein cows (0.36 ± 0.01 ; $P < 0.001$) and cows in early lactation had lower (0.28 ± 0.01 ; $P < 0.001$) concentrations when compared to cows in later lactation (0.41 ± 0.01). Cows with the greatest degree of physiological imbalance, based on $> 75\%$ quartile, had higher isocitrate and lower free glucose than cows with a lower degree of physiological imbalance, based on $< 25\%$ quartile. This study provided evidence for the use of free glucose and isocitrate in milk as biomarkers for degree of physiological imbalance. Results will be implemented in future biomodels for in-line and real-time measurement of degree of physiological imbalance on-farm.

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Claims

1. A method for predicting the risk of physiological imbalance in an animal, said method comprises the steps of:

5 (i) repetitively providing at least one sample from a body fluid obtained from the animal;

(ii) determining the concentration or amount of a first risk indicator present in the sample, the first risk indicator may be selected from the group
10 consisting of free glucose, glucose-6-phosphate, glucose-1-phosphate, free galactose, UDP-glucose, UDP-galactose, glycerol 3-phosphate, beta-hydroxybutyric acid (BHBA), isocitrate, citrate, malate, 2-oxo-glutarate, isocitrate dehydrogenase, malate dehydrogenase, pyruvate carboxylase, glycogen phosphorylase, glutamate dehydrogenase, glyceraldehyde 3-
15 phosphate dehydrogenase, aspartate aminotransferase, aldehyde dehydrogenase, phosphoglucomutase, triosephosphate isomerase, lactate, cholesterol, non-esterified fatty acids (NEFA), milk protein, milk fat, and milk lactose;

20 (iii) determining the rate of change and/or the level change of the first risk indicator;

(iv) comparing said rate change and/or level change with one or more reference patterns of said first risk indicator, in order to predict the risk of
25 physiological imbalance in the animal.

2. A method according to claim 1, further comprising the steps of:

(v) determining the concentration or amount of at least one second risk
30 indicator present in the sample, the at least one second risk indicator, is different from the first risk indicator and may be selected from the group consisting of free glucose, glucose-6-phosphate, glucose-1-phosphate, free galactose, UDP-glucose, UDP-galactose, glycerol 3-phosphate, beta-hydroxybutyric acid (BHBA), isocitrate, citrate, malate, 2-oxo-glutarate,

isocitrate dehydrogenase, malate dehydrogenase, pyruvate carboxylase, glycogen phosphorylase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aspartate aminotransferase, aldehyde dehydrogenase, phosphoglucomutase, triosephosphate isomerase, lactate, cholesterol, non-esterified fatty acids (NEFA), milk protein, milk fat, and milk lactose;

(vi) determining the rate of change and/or the level change of the at least one second risk indicator;

(vii) comparing said rate change and/or level change with one or more reference patterns of said first risk indicator and, optionally, the at least one second risk indicator, in order to predict the risk of physiological imbalance in the animal.

3. A method according to any one of claims 1-2, provided that the prediction, based on the one or more risk indicator, indicates a risk of physiological imbalance in the animal, further comprising the step of:

(viii) determining the concentration or amount of at least one disease indicator present in the sample;

(ix) determining the rate of change and/or the level change of the at least one disease indicator;

(x) comparing said rate change and/or level change with one or more reference patterns of said at least one disease indicator, in order to predict the risk of the animal entering into a sub-clinical or clinical disease state.

4. A method according to any one of claims 1-3, wherein the one or more reference patterns are based on different animal characteristics and/or their physiological states.

5. A method according to any one of claims 1-4, wherein the one or more reference patterns are based on previous data obtained from the same animal.

6. A method according to any one of claims 1-5 wherein the animal is selected from the species consisting of ruminants.
- 5 7. A method according to any one of claims 1-6, wherein the body fluid is selected from the group consisting of milk, blood and urine.
8. A method according to any one of claims 1-6, wherein the risk of physiological imbalance is predicted by measuring free glucose as the first risk indicator.
- 10 9. A method according to claim 8, wherein the risk of physiological imbalance is further predicted by measuring isocitrate as the second risk indicator.
10. A method according to any one of claims 3-9, wherein the sub-clinical or
15 clinical disease state is ketosis, and the disease indicator is beta-hydroxybutyric acid (BHBA).
11. A system for predicting the risk of physiological imbalance in an animal, the system comprising:
- 20 – a computer comprising a processor and being operatively connected to a database,
– at least one sample providing device for repetitively providing at least one sample of a body fluid of the animal,
– an analysis apparatus for:
- 25 (A1) determining the concentration or amount of a first risk indicator present in the sample, the first risk indicator may be selected from the group consisting of free glucose, glucose-6-phosphate, glucose-1-phosphate, free galactose, UDP-glucose, UDP-galactose, glycerol 3-phosphate, beta-hydroxybutyric acid (BHBA),
30 isocitrate, citrate, malate, 2-oxo-glutarate, isocitrate dehydrogenase, malate dehydrogenase, pyruvate carboxylase, glycogen phosphorylase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aspartate aminotransferase, aldehyde
35 dehydrogenase, phosphoglucomutase, triosephosphate isomerase,

lactate, cholesterol, non-esterified fatty acids (NEFA), milk protein, milk fat, and milk lactose;

5 (A2) optionally, determining the concentration or amount of at least one second risk indicator present in the sample, the at least one second risk indicator, is different from the first risk indicator and may be selected from the group consisting of free glucose, glucose-6-phosphate, glucose-1-phosphate, free galactose, UDP-glucose, UDP-galactose, glycerol 3-phosphate, beta-hydroxybutyric acid
10 (BHBA), isocitrate, citrate, malate, 2-oxo-glutarate, isocitrate dehydrogenase, malate dehydrogenase, pyruvate carboxylase, glycogen phosphorylase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aspartate aminotransferase, aldehyde dehydrogenase, phosphoglucomutase, triosephosphate isomerase,
15 lactate, cholesterol, non-esterified fatty acids (NEFA), milk protein, milk fat, and milk lactose;

- a data interface for repetitively entering the concentration or amount of the first risk indicator and, optionally, the at least one second risk indicator in the
20 database, wherein the database is adapted to store multiple database entries representing the indicator at various points in time, and wherein the processor is programmed to:

25 (P1) determine the rate of change of the first indicator and, optionally, the at least one second indicator;

(P2) determine the level change of the first indicator and, optionally, the at least one second indicator and,

30 (P3) compare said rate change and/or level change with one or more reference patterns of said first risk indicator and, optionally, the at least one second risk indicator, in order to predict the risk of physiological imbalance in the animal.

12. A system according to claim 11, wherein the processor is further programmed to:

5 (P4a) predict the risk of the animal entering into a sub-clinical or clinical disease state;

optionally, wherein the analysis apparatus is further capable of:

10 (A3a) determining the concentration or amount of at least one disease indicator present in the sample.

13. A system according to claim 12, wherein the processor is further programmed to:

15 (P5a) determine the rate of change of the at least one disease indicator;

(P6a) determine the level change of the at least one disease indicator;

20 (P7a) compare the rate change and/or level of change of the at least one disease indicator with one or more reference patterns of said at least one disease indicator, in order to predict the health status, e.g. the risk of the animal entering into a sub-clinical or clinical disease state.

25

14. A system according to claim 11, wherein the processor is further programmed to:

30 (P4b) predict the reproductive status;

optionally, wherein the analysis apparatus is further capable of:

35 (A3b) determining the concentration or amount of at least one indicator of reproductive status present in the sample.

15. A system according to claim 14, wherein the processor is further programmed to:

5 (P5b) determine the rate of change of the at least one indicator of reproductive status;

(P6b) determine the level change of the at least one indicator of reproductive status;

10

(P7b) compare the rate change and/or level of change of the at least one indicator (e.g. progesterone) of reproductive status with one or more reference patterns of said at least one indicator of reproductive status, in order to predict the reproductive status.

15

16. A method for determining the concentration of free glucose in a liquid comprising glucose and glucose-6-phosphate, the method comprising the steps of:

20 1) bringing into contact with at least one liquid sample A_n from a liquid sample M at least one type of glucose-6-phosphate-dehydrogenase and NAD(P), thereby producing 6-phosphogluconate and NAD(P)H₂, wherein n is a natural integer of at least 1;

25 2) bringing into contact with the at least one liquid sample A_n at least one type of NAD(P)H dehydrogenase and a fluorophore/chromophore precursor, thereby producing a fluorophore/chromophore;

30 3) determining the concentration of glucose-6-phosphate in the at least one liquid sample A_n by detecting the amount produced of fluorophore/chromophore and comparing it to a reference;

4) bringing into contact with at least one liquid sample B_n from the liquid sample M at least one type of hexokinase and adenosine triphosphate

(ATP), thereby converting free glucose into glucose-6-phosphate, wherein n is a natural integer of at least 1;

5 5) bringing into contact with at least one liquid sample B_n from a liquid sample M at least one type of glucose-6-phosphate-dehydrogenase and NAD(P), thereby producing 6-phosphogluconate and NAD(P)H₂;

10 6) bringing into contact with the at least one liquid sample B_n at least one type of NAD(P)H dehydrogenase and a fluorophore/chromophore precursor, thereby producing a fluorophore/chromophore;

15 7) determining the concentration of glucose-6-phosphate in the at least one liquid sample B_n by detecting the amount produced of fluorophore/chromophore and comparing it to a reference;

8) indirectly determining the concentration of free glucose in the liquid sample M by subtracting the concentration in 6) with the concentration in 3).

20 17. A dry stick device for the determination of free glucose and glucose-6-phosphate in a milk sample comprising:

(i) optionally a solid support,

25 (ii) at least one reagent pad A comprising at least one type of glucose-6-phosphate-dehydrogenase, NAD(P), at least one type of NAD(P)H dehydrogenase and a fluorophore/chromophore precursor,

30 (iii) at least one reagent pad B comprising at least one type of glucose-6-phosphate-dehydrogenase, NAD(P), at least one type of NAD(P)H dehydrogenase, at least one type of hexokinase, adenosine triphosphate (ATP) and a fluorophore/chromophore precursor.

35 18. A dry stick device according to claim 17, wherein reagent pad A and/or B further comprise an inhibitor of lactate dehydrogenase.

19. A dry stick device according to any one of claims 17-18, wherein the at least one reagent pad A and/or B provide a first environment for said reagent(s), said first environment permitting an improved storage stability of the reagent(s) and
5 dry stick device when in a non-moistened state, the dry stick device further comprising a regulating pad being in contact with the at least one reagent pad A and/or B, wherein the regulating pad creating a second environment for said reagent(s) when in a moistened state, said second environment permitting an increased rate of reaction between the analyte and the reagent(s), and wherein
10 the condition in the first environment is provided by adjusting the pH-value to a value that deviates from the optimal pH-value of the enzyme(s) and wherein the condition in the second environment is provided by regulating the pH-value to a value that approaches the optimal pH-value of the enzyme(s) and wherein the different environments have different pH-values.

15

20. Use of a dry stick device according to any one of claims 17-19 for the determination of free glucose and glucose-6-phosphate in a milk sample.

Variable	Number of obs.	Mean value	P ₅ - P ₉₅ inter percentile	Free glucose	Glucose 6-P
				Coefficient of correlation, r	Coefficient of correlation, r
Free glucose, mM	3261	0.340	0.128 - 0.567	-0.523**	
Glucose 6-P, mM	3262	0.120	0.105 - 0.286		-0.577**
Days in milk	3233	112	9 - 309	0.425**	0.039
Milk yield per milking, kg.	1895	13.3	7.6 - 22.4	-0.005	-0.155**
Time since last milking, h	1837	10.3	6.6 - 15.5	-0.105**	-0.155**
Fat, %	2822	4.74	3.24 - 6.73	-0.466**	0.227**
Lactose, %	2849	4.83	4.45 - 5.08	0.228**	-0.198**
Protein, %	2992	3.61	2.80 - 4.66	0.031	-0.020
Log SCC ^a	3003	4.86	4.20 - 5.85	-0.089**	0.180**
Citrate, %	535	0.22	0.17 - 0.29	-0.278**	-0.179**
BOHB ^b , µM	3262	95	44 - 195	-0.420**	0.307**

^a SCC = Somatic Cell Counts

^b BOHB = β-hydroxy-butyrate

** P<0.01

Figure 1

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Party	Number of observations		Glu 6-P, mM		Free Glu, mM	
	Danish Friesian	Jersey	Danish Friesian	Jersey	Danish Friesian	Jersey
1	637	144	0.079	0.099	0.450	0.331
2	1183	212	0.152	0.089	0.309	0.361
3	476	105	0.132	0.082	0.322	0.341
4	295	44	0.132	0.090	0.335	0.273
5	80	10	0.166	0.059	0.262	0.285
6	30		0.176		0.160	
7	16		0.231		0.150	

Figure 2

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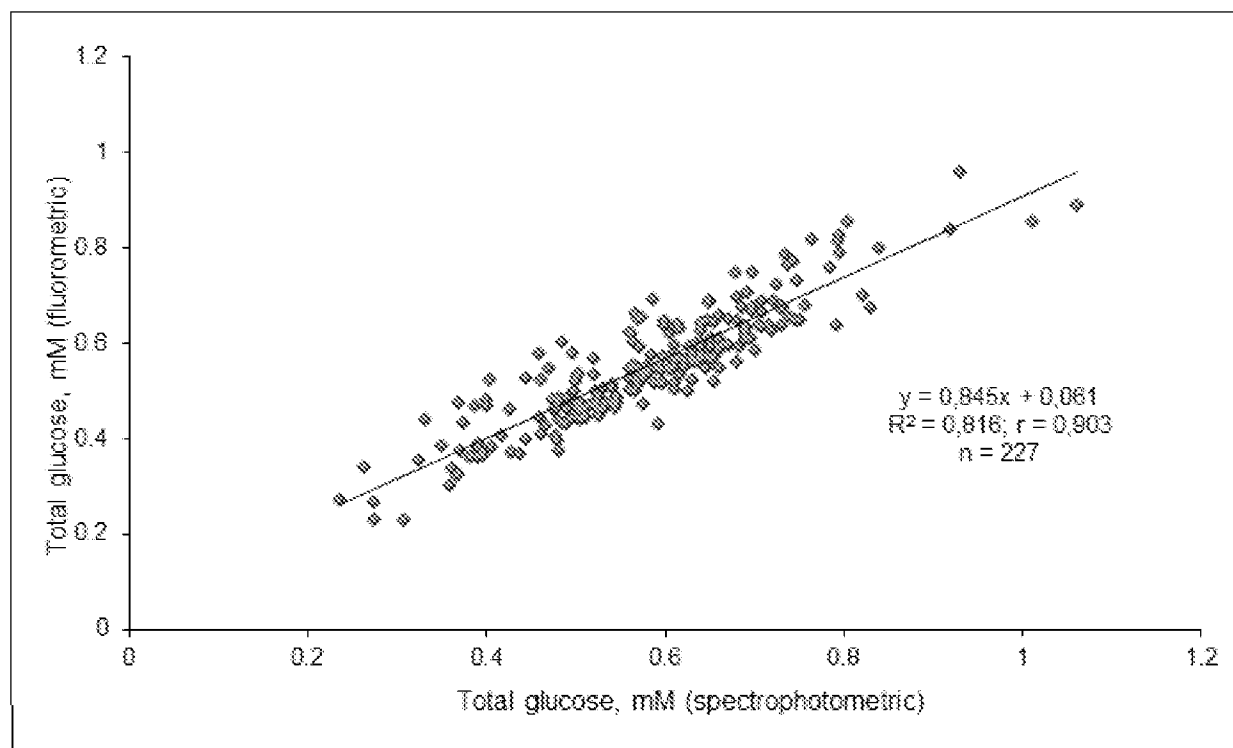


Figure 3

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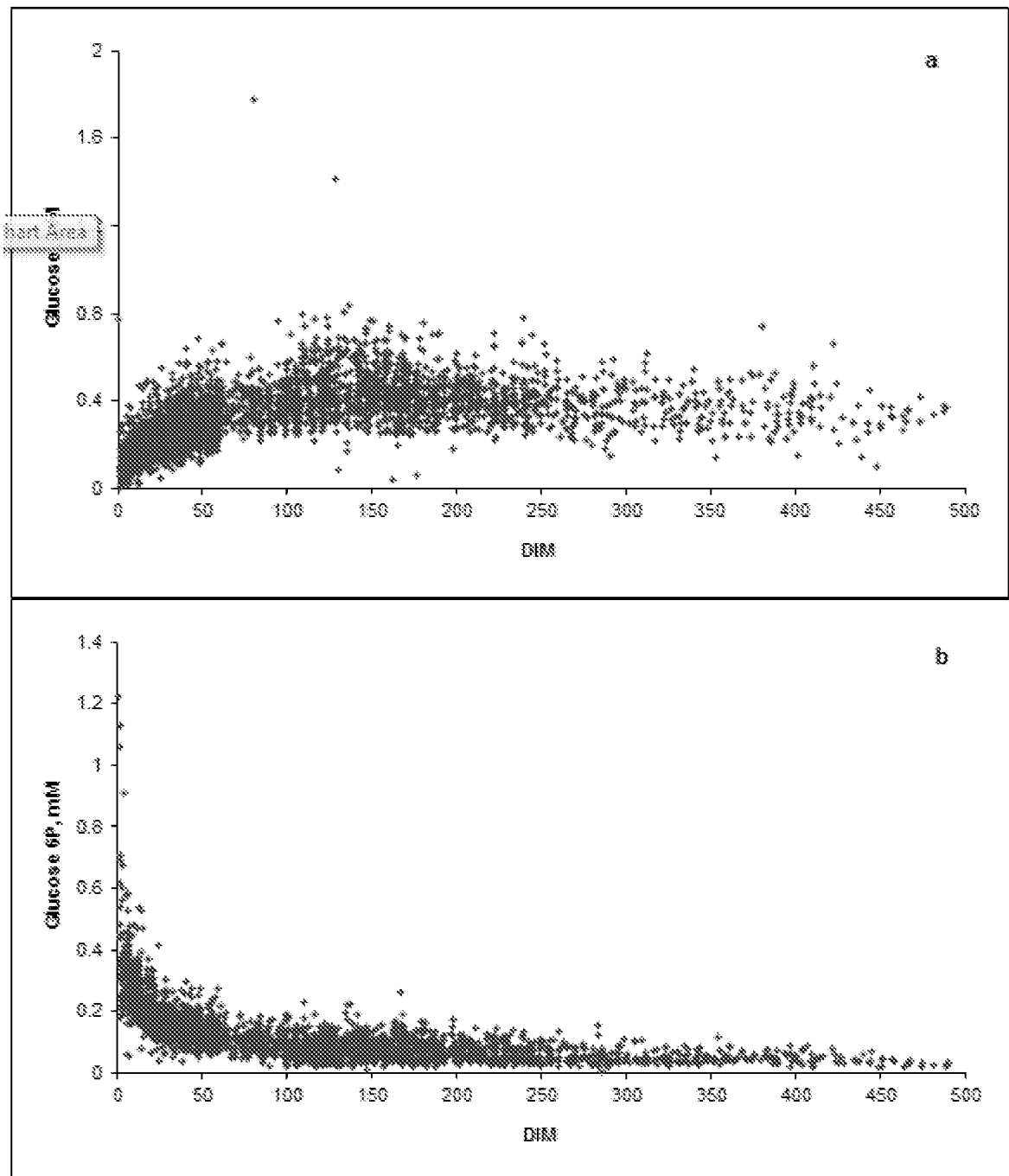


Figure 4

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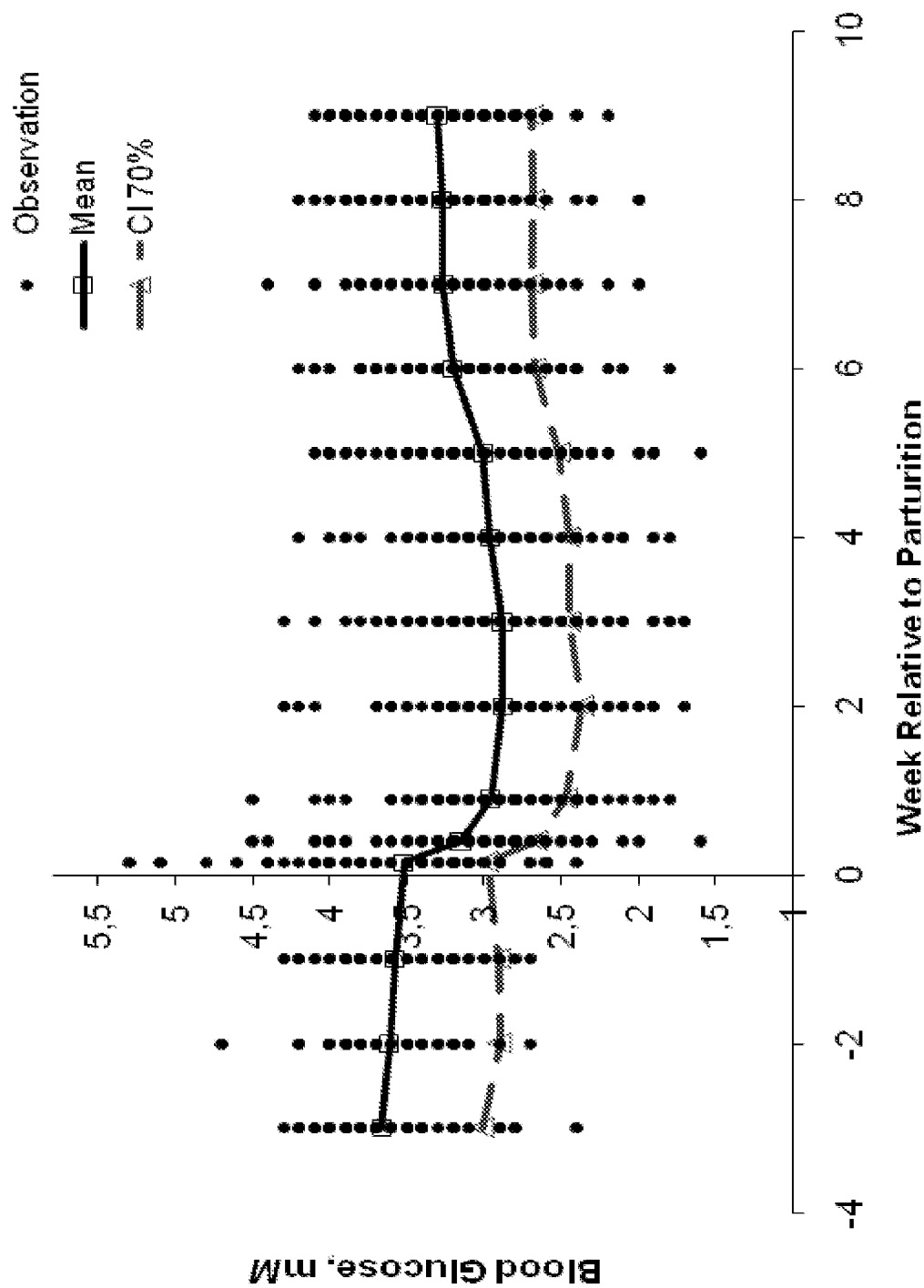


Figure 5

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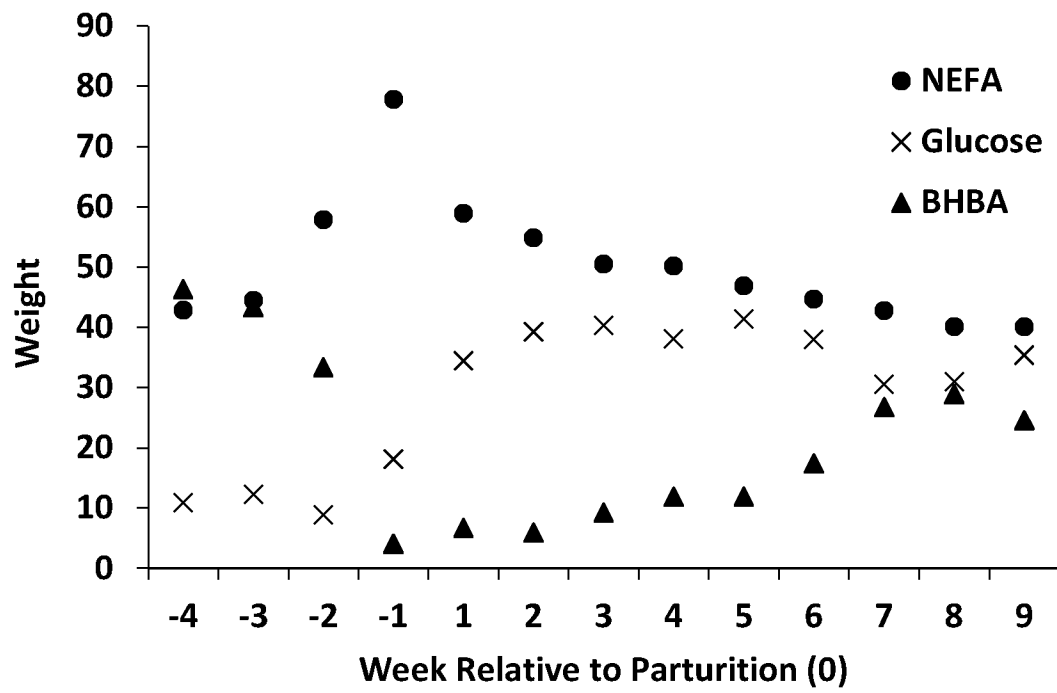


Figure 6

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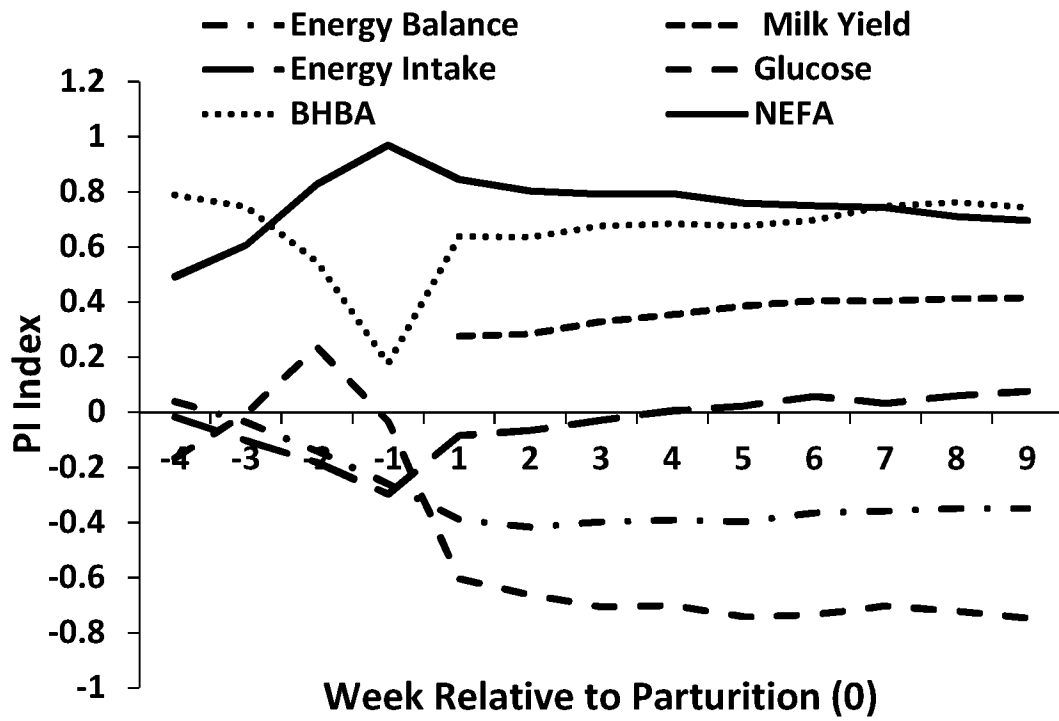


Figure 7

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DISEASE	WEEK1 ¹	EARLYLACT
Retained Placenta (RP)	59(62)	-
Milk Fever	33(36)	-
Dystocia	7(7)	-
Left Displaced Abomasum (LDA)	3(3)	-
<u>Metritis</u>	10(10)	-
Lameness	6(6)	13(13)
Ketosis	-	2(2)
Other ²	16(16)	10(10)
Clinical mastitis ³	69 (69)	24 (24)
Healthy ⁴	113 (145)	113 (145)
Total ⁵	279 (391)	160 (206)

¹Cows (lactations).

²Other = rumen acidosis, uterine prolapse and pneumonia.

³Clinical mastitis = For both WEEK1 and EARLYLACT, cows treated for clinical mastitis and had daily composite milk SCC $\geq 800,000$ cells/mL.

⁴Healthy = Cows that were not treated for and did not show any clinical signs of disease at any time during the early lactation, had daily composite milk SCC $\leq 500,000$ cells/mL within the first week of lactation and daily composite milk SCC did not rise above 100,000 cells/mL for ≥ 3 consecutive days during wk 2 through 9 of lactation.

⁵Total = total number of cows that were classified into WEEK1 and EARLYLACT. Total does not reflect the sum of individual diseases since e.g. several cows may have been classified as healthy in one lactation and diseased in a subsequent lactation.

Figure 8

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Week	n	NEFA, ln(mEq/L)			Glucose, mM			BHBA, ln(mM)			R ²	Pr> F	Root MSE
		Estimate	SE	Pr> t	Estimate	SE	Pr> t	Estimate	SE	Pr> t			
-4	183	-0.09	0.05	0.07	-0.02	0.06	0.66	0.11	0.06	0.08	0.03	0.09	0.79
-3	224	-0.14	0.06	0.02	0.04	0.06	0.50	0.14	0.05	0.01	0.04	0.02	0.79
-2	229	-0.18	0.07	0.003	0.03	0.06	0.66	0.10	0.06	0.06	0.04	0.01	0.79
-1	283	-0.23	0.06	<0.001	0.05	0.05	0.31	0.01	0.06	0.83	0.06	<0.001	0.90
1	500	-0.29	0.05	<0.001	0.17	0.05	<0.001	-0.03	0.05	0.53	0.14	<0.001	0.92
2	423	-0.31	0.05	<0.001	0.22	0.05	<0.001	-0.03	0.06	0.57	0.17	<0.001	0.92
3	427	-0.26	0.05	<0.001	0.21	0.05	<0.001	-0.05	0.06	0.40	0.14	<0.001	0.93
4	493	-0.26	0.05	<0.001	0.20	0.05	<0.001	-0.06	0.06	0.28	0.15	<0.001	0.92
5	497	-0.25	0.04	<0.001	0.22	0.05	<0.001	-0.06	0.06	0.27	0.16	<0.001	0.92
6	492	-0.22	0.04	<0.001	0.18	0.05	<0.001	-0.08	0.06	0.13	0.14	<0.001	0.92
7	486	-0.21	0.05	<0.001	0.15	0.06	0.006	-0.13	0.06	0.02	0.14	<0.001	0.95
8	491	-0.19	0.05	<0.001	0.15	0.05	0.006	-0.14	0.06	0.01	0.13	<0.001	0.93
9	470	-0.18	0.05	<0.001	0.16	0.06	0.004	-0.11	0.06	0.05	0.12	<0.001	0.94

Figure 9

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Least square means, SEM¹ and the standardized differences (**SDiff**)² for estimated between-cow variations in physiological imbalance (**PI**)³, calculated energy balance (**EBAL**)⁴, and plasma concentration of NEFA, BHBA and glucose at week -1 relative to development of metritis, retained placenta or milk fever during the first week after parturition (**WEEK1**).

Variable	Disease														
	Metritis				Retained Placenta				Milk Fever						
	Yes	No	SEM	P	SDiff	Yes	No	SEM	P	SDiff	Yes	No	SEM	P	SDiff
PI	18.3	-3.1	5.1	<0.00	4.2	2.7	-3.1	2.4	0.04	2.4	3.7	-3.1	3.0	0.04	2.3
EBAL	-7.6	-1.1	6.9	0.36	-	-2.4	-1.1	2.8	0.68	-	7.5	-1.1	3.7	0.03	2.3
NEFA	0.17	-	0.05	<0.00	3.8	0.0	-	0.03	<0.	2.7	0.06	-	0.03	0.02	2.7
BHBA	0.03	-	0.03	0.20	1.3	-	-	0.01	0.36	-1.0	-	-	0.02	0.22	-
	-	-	0.03	0.03	-2.3	-	-	0.01	0.48	0.0	0.04	-	0.02	<0.0	2.5

¹SEM = the largest SEM within a disease is shown.

²Standardized differences = (Yes – No)/SEM.

³PI = (x₁ × [NEFA] + x₂ × [BHBA] - x₃ × [glucose])/3 where x₁₋₃ = coefficients generated by regression analysis.

⁴EBAL was calculated using equations from Friggens et al. (2007).

Figure 10

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Least square means, SEM¹ and the standardized differences (**SDiff**)² for estimated between-cow variations in physiological imbalance (**PI**)³, calculated energy balance (**EBAL**)⁴, and plasma concentration of NEFA, BHBA and glucose at week -1 relative to development of all diseases⁵, lameness, or mastitis during either the first week after parturition (**WEEK1**).

Variable	Disease											
	All Diseases ⁵						Lameness			Mastitis		
	Y	N	SEM	P	SDiff		Y	N	SEM	P	SDif	
PI	2.6	-	1.5		3.8		1.3	-3.1	6.8	0.05	0.65	
EBAL		-	1.6	0.38	1.6		-7.2	-1.1	8.8	0.49	-	
NEFA		-	0.01	<0.00	7.0		0.08	-	0.07	0.17	1.4	
BHBA	0.00	-	0.01	0.47	1.1		0.00	-	0.03	0.75		
Glucose		-	0.01		2.0		0.09	-	0.04	0.01	2.5	

¹SEM = the largest SEM within a disease is shown.
²Standardized difference = (Yes – No)/SEM.
³PI = (x₁ × [NEFA] + x₂ × [BHBA] - x₃ × [glucose])/3 where x₁₋₃ = coefficients generated by regression analysis.
⁴EBAL was calculated using equations from Friggens et al. (2007).
⁵All diseases = the total number of diseases reported in **Table 1** for cows in WEEK1.

Figure 11

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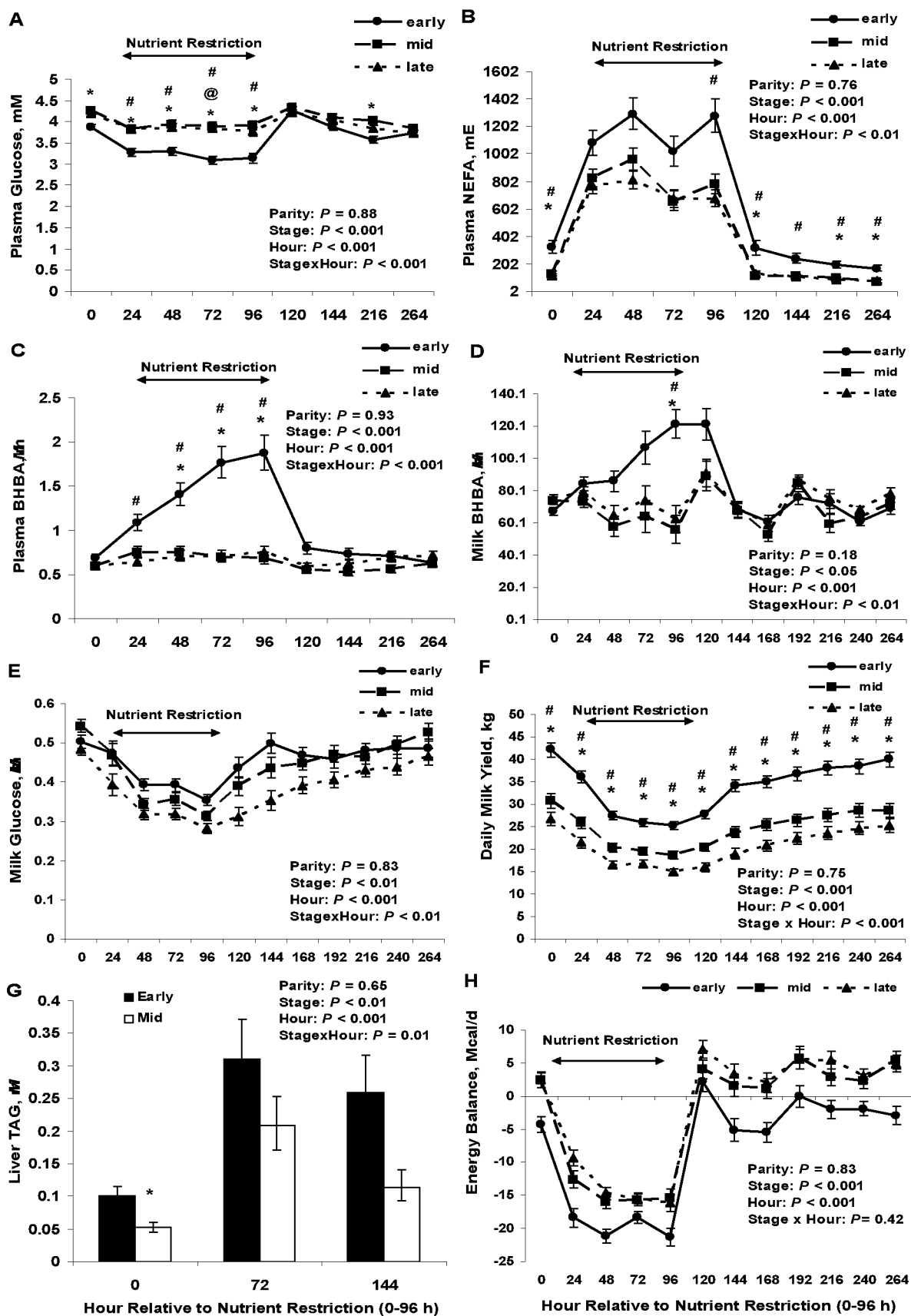


Figure 12

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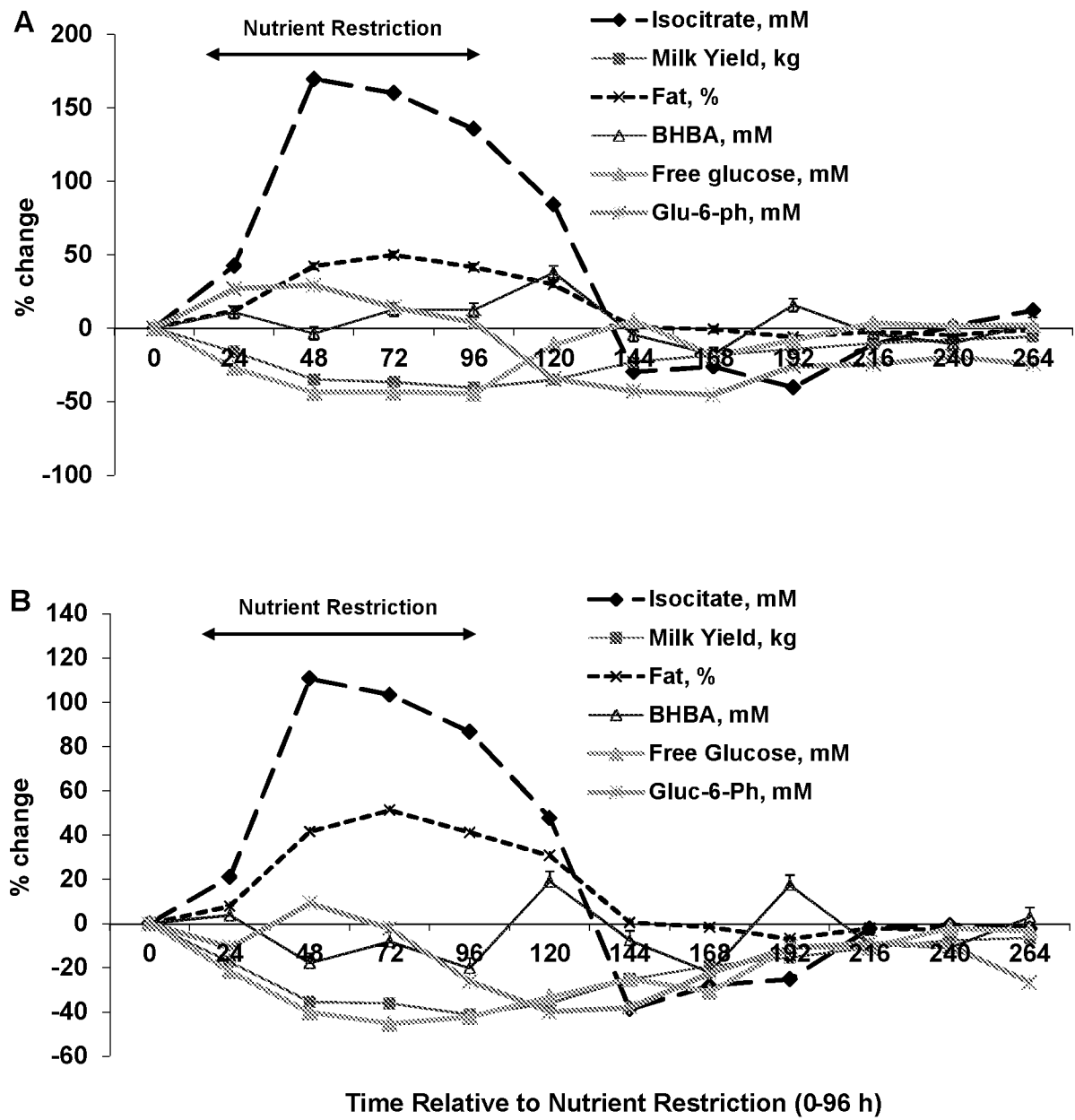


Figure 13

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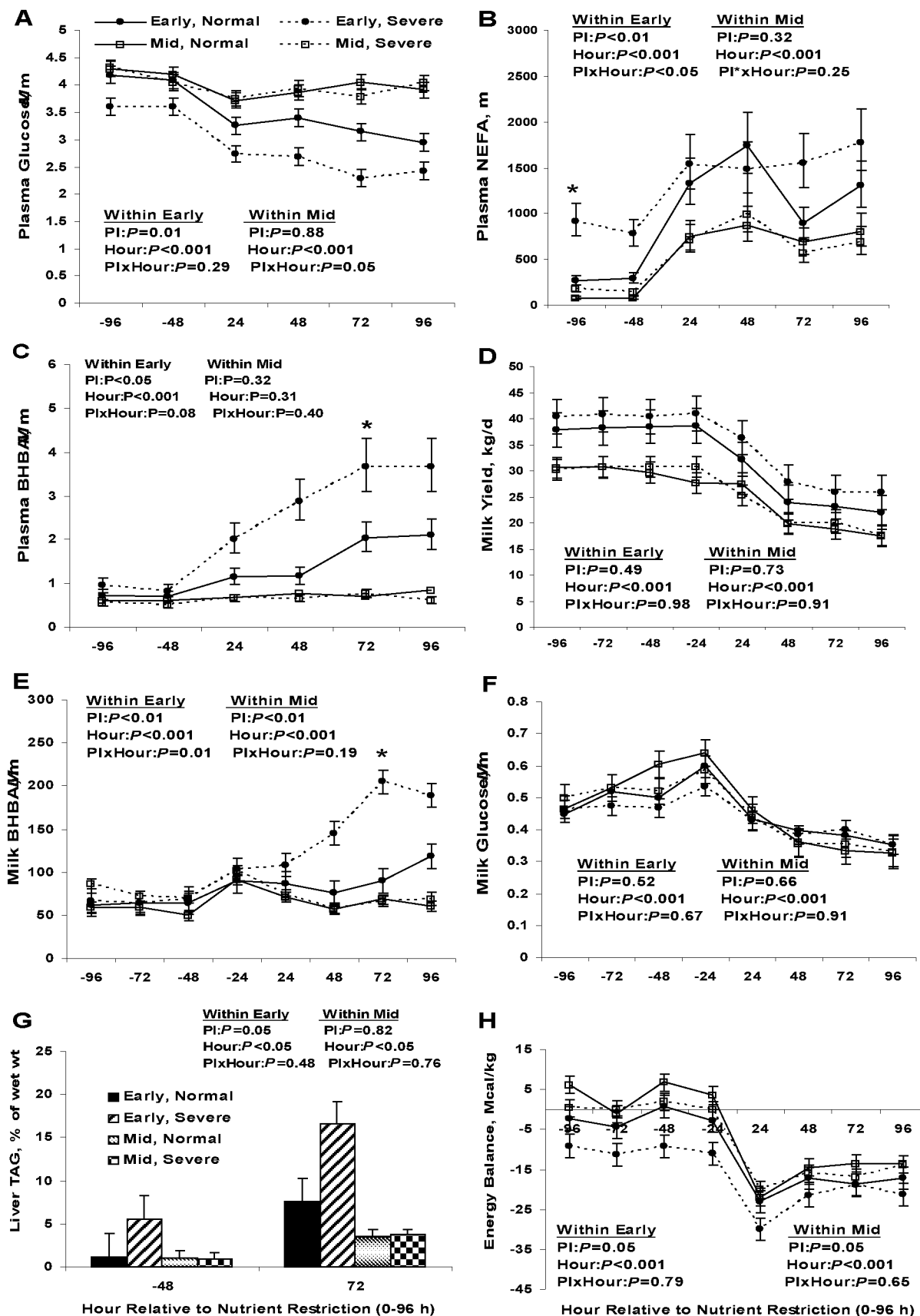


Figure 14

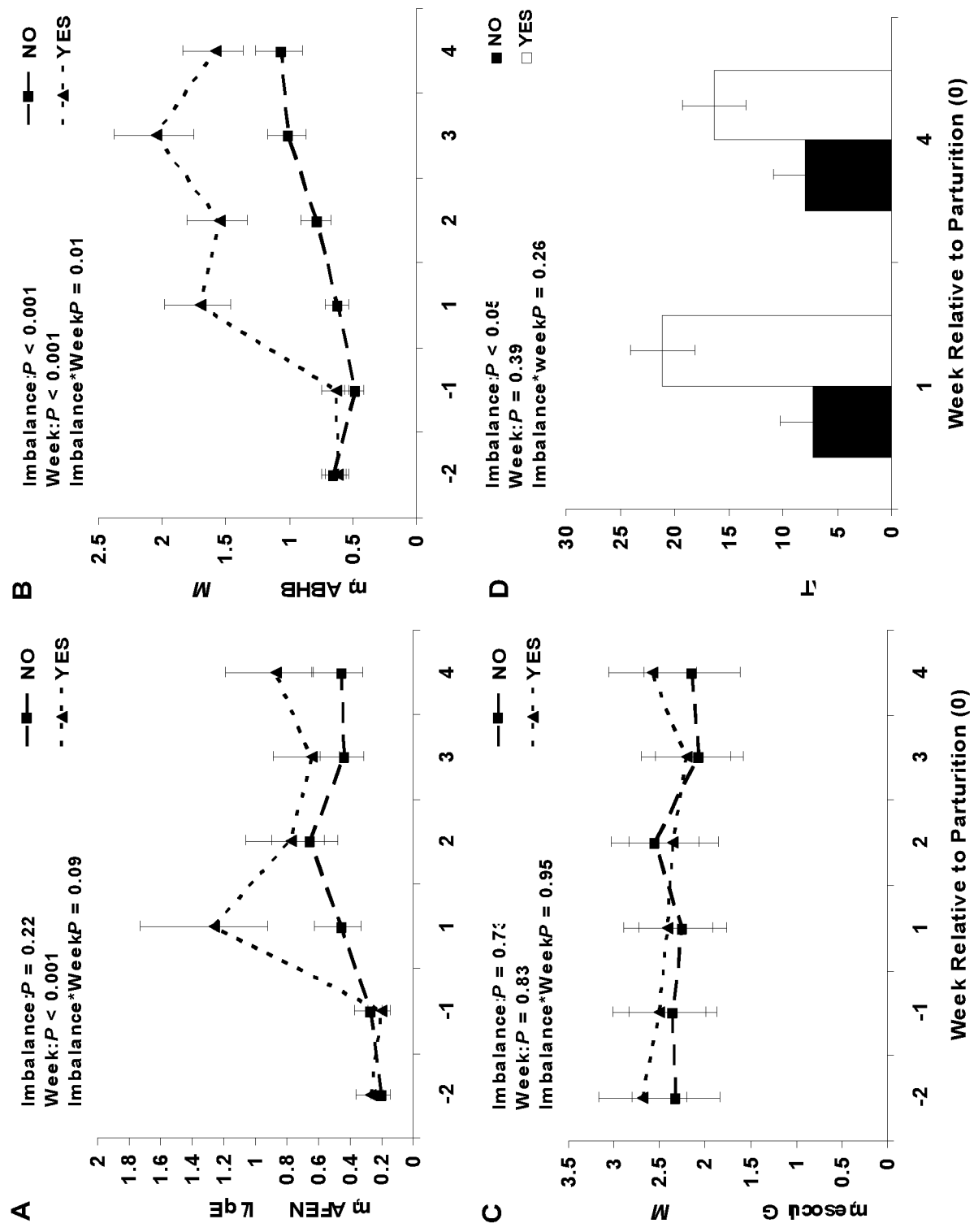


Figure 15

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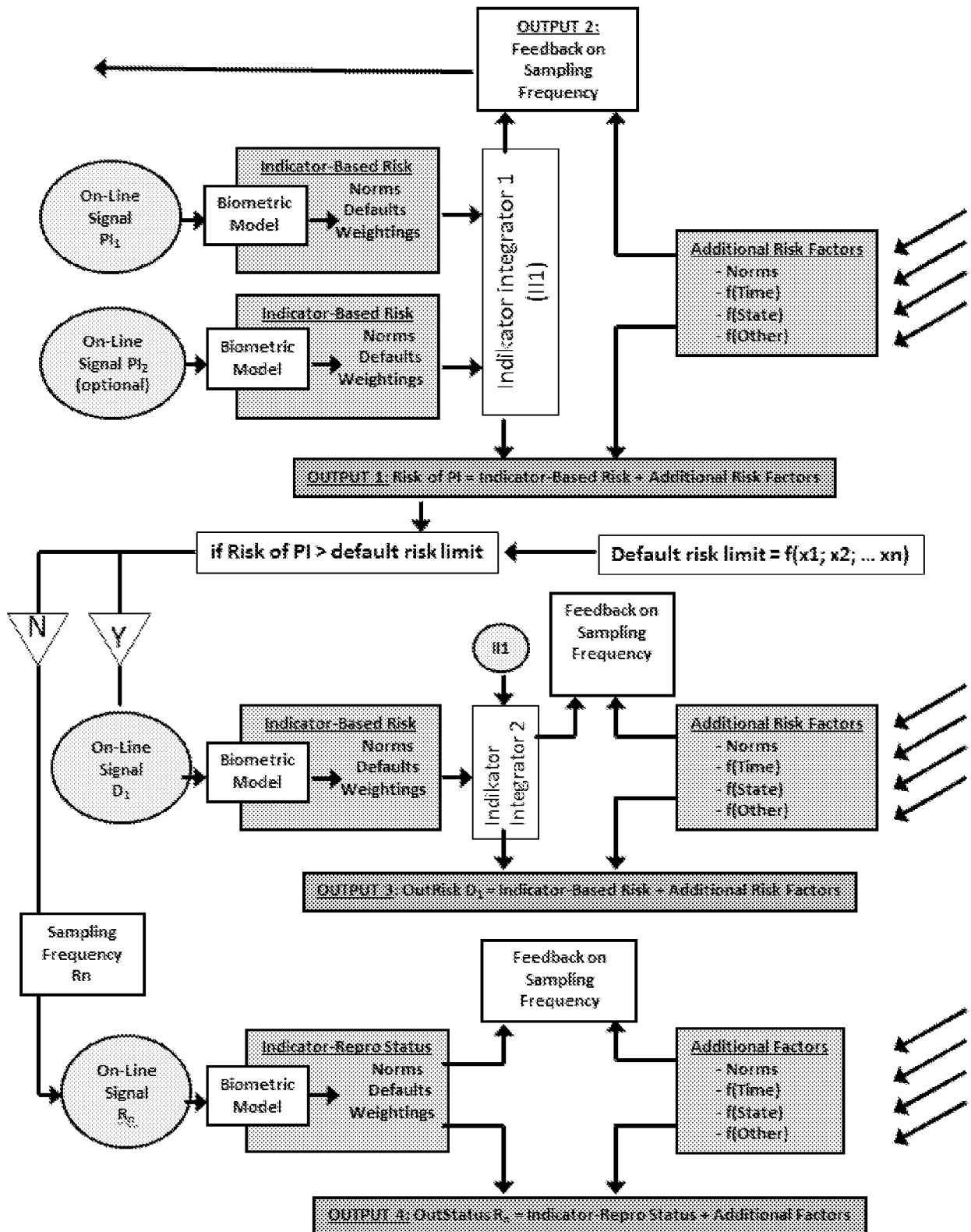


Figure 16

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2012/050186

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/66
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DOUGLAS G N ET AL: "Prepartal Plane of Nutrition, Regardless of Dietary Energy Source, Affects Periparturient Metabolism and Dry Matter Intake in Holstein Cows", JOURNAL OF DAIRY SCIENCE, AMERICAN DAIRY SCIENCE ASSOCIATION, US, vol. 89, no. 6, 1 June 2006 (2006-06-01), pages 2141-2157, XP026957044, ISSN: 0022-0302 [retrieved on 2006-06-01] abstract page 2149, right-hand column, paragraph 3 ----- -/--	1-10

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

1 August 2012

Date of mailing of the international search report

23/10/2012

Name and mailing address of the ISA/

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Authorized officer

Wiesner, Martina

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2012/050186

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	REIST MARTIN ET AL: "Use of threshold serum and milk ketone concentrations to identify risk for ketosis and endometritis in high-yielding dairy cows", AMERICAN JOURNAL OF VETERINARY RESEARCH, AMERICAN VETERINARY MEDICINE ASSOCIATION, US, vol. 64, no. 2, 1 February 2003 (2003-02-01), pages 188-194, XP008154012, ISSN: 0002-9645 abstract; table 4	1-10
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X	----- WO 00/65366 A1 (LIPOMED INC [US]; UNIV NORTH CAROLINA STATE [US]; OTVOS JAMES D [US];) 2 November 2000 (2000-11-02) claims 1-2	1-10
Y	----- US 2004/098207 A1 (FRIGGENS NIC C [DK] ET AL) 20 May 2004 (2004-05-20) abstract	11-15
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INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2012/050186

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NIELSEN N I ET AL: "Predicting Risk of Ketosis in Dairy Cows Using In-Line Measurements of sz-Hydroxybutyrate: A Biological Model", JOURNAL OF DAIRY SCIENCE, AMERICAN DAIRY SCIENCE ASSOCIATION, US, vol. 88, no. 7, 1 July 2005 (2005-07-01), pages 2441-2453, XP026954582, ISSN: 0022-0302 [retrieved on 2005-07-01]	11-15
A	page 2444, column 2, paragraph 2 page 2445, right-hand column, paragraph 2 -----	1-10
Y	YONGJUN ZHAO ET AL: "A MEMS viscometric sensor for continuous glucose monitoring", JOURNAL OF MICROMECHANICS & MICROENGINEERING, INSTITUTE OF PHYSICS PUBLISHING, BRISTOL, GB, vol. 17, no. 12, 1 December 2007 (2007-12-01), pages 2528-2537, XP020129924, ISSN: 0960-1317 claim 1 -----	11-15
Y	PICKUP J C ET AL: "Fluorescence-based glucose sensors", BIOSENSORS AND BIOELECTRONICS, ELSEVIER BV, NL, vol. 20, no. 12, 15 June 2005 (2005-06-15), pages 2555-2565, XP004861166, ISSN: 0956-5663, DOI: 10.1016/J.BIOS.2004.10.002 the whole document -----	11-15
A	LEBLANC S J ET AL: "Metabolic Predictors of Displaced Abomasum in Dairy Cattle", JOURNAL OF DAIRY SCIENCE, AMERICAN DAIRY SCIENCE ASSOCIATION, US, vol. 88, no. 1, 1 January 2005 (2005-01-01), pages 159-170, XP026970280, ISSN: 0022-0302 [retrieved on 2005-01-01] abstract page 169, left-hand column, paragraph 2 ----- -/--	1-15

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2012/050186

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M CHASSAGNE ET AL: "Predictive markers in the late gestation period for retained placenta in black-pied dairy cows under field conditions in France", THERIOGENOLOGY, vol. 49, no. 3, 1 February 1998 (1998-02-01), pages 645-656, XP055034360, ISSN: 0093-691X, DOI: 10.1016/S0093-691X(98)00015-6 abstract	1-10
A	----- MOYES K M ET AL: "Identification of potential markers in blood for the development of subclinical and clinical mastitis in dairy cattle at parturition and during early lactation", JOURNAL OF DAIRY SCIENCE, AMERICAN DAIRY SCIENCE ASSOCIATION, US, vol. 92, no. 11, 1 November 2009 (2009-11-01), pages 5419-5428, XP026954786, ISSN: 0022-0302 [retrieved on 2009-11-01] cited in the application abstract; tables 2,4	1-10
A	----- J. DUBUC ET AL: "Risk factors for postpartum uterine diseases in dairy cows", JOURNAL OF DAIRY SCIENCE, vol. 93, no. 12, 1 December 2010 (2010-12-01), pages 5764-5771, XP055034371, ISSN: 0022-0302, DOI: 10.3168/jds.2010-3429 abstract	1-15
A	----- REZAMAND P ET AL: "Energy Status, Lipid-Soluble Vitamins, and Acute Phase Proteins in Periparturient Holstein and Jersey Dairy Cows With or Without Subclinical Mastitis", JOURNAL OF DAIRY SCIENCE, AMERICAN DAIRY SCIENCE ASSOCIATION, US, vol. 90, no. 11, 1 November 2007 (2007-11-01), pages 5097-5107, XP026956260, ISSN: 0022-0302 [retrieved on 2007-11-01] abstract ----- -/--	1-15

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2012/050186

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	INGVARTSEN ET AL: "Feeding- and management-related diseases in the transition cow", ANIMAL FEED SCIENCE AND TECHNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 126, no. 3-4, 9 March 2006 (2006-03-09), pages 175-213, XP024934225, ISSN: 0377-8401, DOI: 10.1016/J.ANIFEEDSCI.2005.08.003 [retrieved on 2006-03-09] cited in the application the whole document -----	1-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2012/050186

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-15(partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-15(partially)

Method and system for predicting the risk of physiological imbalance by determining the concentration of free glucose.

2. claims: 16-20

A method for measuring free glucose in a liquid comprising glucose and glucose-6-phosphate, a dry stick for measuring free glucose and the use of the dry stick for measuring free glucose in milk.

3-29. claims: 1-15(partially)

Method and system for predicting the risk of physiological imbalance by determining the concentration of a risk indicator selected from the group consisting of glucose-6-phosphate, glucose-1-phosphate, free galactose, UDP-glucose, UDP-galactose, glycerol 3-phosphate, beta-hydroxybutyric acid (BHBA), isocitrate, citrate, malate, 2-oxo-glutarate, isocitrate dehydrogenase, malate dehydrogenase, pyruvate carboxylase, glycogen phosphorylase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aspartate aminotransferase, aldehyde dehydrogenase, phosphoglucomutase, triosephosphate isomerase, lactate, cholesterol, non-esterified fatty acids (NEFA), milk protein, milk fat, and milk lactose.

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2012/050186

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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			US 2008255763 A1 16-10-2008