

A BIOMARKER FOR BROWN FAT ACTIVITY DETECTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of SG provisional application No. 10202004288W, filed 8 May 2020, the contents of it being hereby incorporated by reference in its entirety for all purposes.

TECHNICAL FIELD

[0002] The present disclosure generally relates to methods for detecting brown adipose tissues (BAT) activation and/or browning of white adipose tissues (WAT) to BAT by measuring the level of methylene tetrahydrofolate dehydrogenase 1-like (MTHFD1L) protein in a blood sample.

BACKGROUND

[0003] It is well established that brown adipose tissue (BAT) activity in adult humans can be demonstrated via positron emission tomography combined with computed tomography (PET-CT) for over a decade. The initial triumphs back then probably contributed to the long-accepted view of this technique being heralded as the 'gold standard' for BAT imaging. While this has sparked a new resurgence of interest to explore the feasibility of BAT for the amelioration of obesity and diabetes mellitus that jointly afflict over a billion people globally, this mission is significantly curtailed by the two major drawbacks of PET-CT, namely its high ionizing radiation dose and its exorbitant cost. Because the investigation and monitoring of the effectiveness of any BAT-based treatment modality would require repeated serial PET-CT scans, there is a quest for an alternative safer and cheaper BAT activity detection methodology that will facilitate and accelerate BAT research. Over time, such efforts have led to the emergence of a slew of newer BAT detection strategies that include fat fraction magnetic resonance imaging or fat fraction MRT (MR FF), infrared thermography (IRT), near infrared spectroscopy (NIRS), indirect calorimetry, and blood exosomal miRNA. MRI measures reduced fat content due to BAT activity by monitoring changes in fat fraction. Infrared thermography measures BAT activity based on amount of infrared released as heat energy.

Indirect calorimetry measure energy expenditure or increase in metabolic rate with BAT activation. Blood exosomal miRNA detects specific miRNA patterns or signatures associated with BAT. All methods above aim to provide a safer and more cost-effective modality with good reliability and precision that can one day replace PET-CT as the gold standard. PET-CT
5 uses positron emission and X-rays absorption to measure glucose uptake by active BAT. Having said that, the above methods can be highly costly, inconvenient and/or exposes the subject to radioactive tracers.

[0004] In view of the above, there is a need to provide alternative means for detecting brown adipose tissues (BAT) activation and/or browning of white adipose tissues (WAT) to BAT, that
10 overcomes, or at least ameliorates, one or more of the disadvantages described in the currently available methods.

SUMMARY

15 [0005] According to a first aspect, there is provided a method for detecting brown adipose tissues (BAT) activation and/or browning of white adipose tissues (WAT) to BAT in a subject, wherein the method comprises:

- a. obtaining a first blood sample from the subject;
- b. determining a baseline level of methylene tetrahydrofolate dehydrogenase 1-like
20 (MTHFD1L) protein in the first blood sample;
- c. exposing the subject to a stimulation;
- d. obtaining a second blood sample from the subject after the stimulation; and
- e. measuring a post-stimulation level of MTHFD1L protein in the second blood sample;
 - e1. if the post-stimulation level of MTHFD1L protein is **higher** than the baseline
25 level of MTHFD1L protein, determining **presence** of BAT activation and/or browning of WAT to BAT in the subject, or
 - e2. if the post-stimulation level of MTHFD1L protein is **the same or lower** than the baseline level of MTHFD1L protein, determining **absence** of BAT activation and/or browning of WAT to BAT in the subject .

30 [0006] According to a second aspect, there is provided a method for detecting brown adipose tissues (BAT) activation and/or browning of white adipose tissues (WAT) to BAT in a subject, wherein the method comprises:

- a. determining a baseline level of methylene tetrahydrofolate dehydrogenase 1-like (MTHFD1L) protein in a first blood sample obtained from the subject;
- b. measuring a post-stimulation level of MTHFD1L protein in a second blood sample obtained from the subject after the subject has been exposed to a stimulation; and
 - 5 b1. if the post-stimulation level of MTHFD1L protein is **higher** than the baseline level of MTHFD1L protein, determining **presence** of BAT activation and/or browning of WAT to BAT in the subject, or
 - 10 b2. if the post-stimulation level of MTHFD1L protein is **the same or lower** than the baseline level of MTHFD1L protein, determining **absence** of BAT activation and/or browning of WAT to BAT in the subject .

[0007] According to a third aspect, there is provided a qualitative BAT activity test kit for detecting brown adipose tissues (BAT) activation and/or browning of white adipose tissues (WAT) to BAT in a subject according to the method as disclosed herein, wherein the qualitative BAT activity test kit comprising:

- 15 - a test strip A made from porous material allowing for capillary flow of a buffer,
 - wherein the test strip A comprises a sample receiving region A and an active reagents region A,
 - wherein the active reagents region A is located downstream of the sample receiving region A,
 - 20 ○ wherein the active reagents region A comprises one or more active reagents capable of a visually detectable change in the presence of MTHFD1L protein, and
 - wherein the sample receiving region A is for application of a blood sample obtained from a subject **prior to** a stimulation,
- 25 and
 - a test strip B made from porous material allowing for capillary flow of a buffer,
 - wherein the test strip B comprises a sample receiving region B and an active reagents region B,
 - wherein the active reagents region B is located downstream of the sample receiving area B,
 - 30 ○ wherein the active reagents region B comprises one or more active reagents capable of a visually detectable change in the presence of MTHFD1L protein,

and

- wherein the sample receiving region B is for application of a blood sample obtained from a subject **after** a stimulation,

wherein when the blood sample obtained from a subject **prior to** a stimulation and/or the

- 5 blood sample obtained from a subject **after** a stimulation comprises MTHFD1L protein, the buffer is capable of dissolving said MTHFD1L protein,

wherein if the buffer comprises dissolved MTHFD1L protein, the dissolved MTHFD1L protein catalyzes a chemical reaction on the one or more active reagents capable of a visually detectable change in the presence of MTHFD1L protein thereby resulting in a visually detectable change

- 10 in the active reagent region A and/or the active reagent region B which indicates the presence of MTHFD1L protein in the blood sample.

[0008] According to a fourth aspect, there is provided an enzymatic chemistry based quantitative BAT activity test kit for detecting brown adipose tissues (BAT) activation and/or browning of white adipose tissues (WAT) to BAT in a subject according to the method as

- 15 disclosed herein, wherein the enzymatic chemistry based quantitative BAT activity test kit comprises:

- a. a sample collection stick, wherein the sample collection stick comprises one or more active reagents capable of undergoing an enzymatic reaction in the presence of MTHFD1L protein,
- 20 b. a detector system capable of detecting a product formed by the enzymatic reaction, and
- c. a monitoring device capable of determining the level of MTHFD1L in the blood sample based on the amount of product formed by the enzymatic reaction.

[0009] According to a fifth aspect, there is provided an antibody based quantitative BAT activity test kit for detecting brown adipose tissues (BAT) activation and/or browning of white adipose tissues (WAT) to BAT in a subject according to the method as disclosed herein, wherein the quantitative BAT activity test kit comprises:

- a. a sample collection stick, wherein the sample collection stick comprises a capture antibody capable of binding to MTHFD1L protein and a detection antibody capable of binding to a capture antibody-MTHFD1L protein complex, wherein the detection antibody is connected to a tag, wherein the tag is a fluorescent tag capable of emitting
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fluorescence in the presence of MTHFD1L protein or a tag capable of undergoing a color change in the presence of MTHFD1L protein, wherein when the tag is a tag capable of undergoing a color change in the presence of MTHFD1L protein, the sample collection stick further comprises a substrate capable of reacting with the tag,

- b. a detector system capable of detecting the fluorescence or the color change formed by the tag, and
- c. a monitoring device capable of determining the level of MTHFD1L in the blood sample based on the amount of fluorescence formed by the tag or based on the intensity of color change formed by the tag.

[0010] According to a sixth aspect, there is provided a method for detecting or monitoring a metabolic disorder or metabolic syndrome that affect BAT activity in a subject using the method as disclosed herein, the qualitative BAT activity test kit as disclosed herein, the enzymatic chemistry based quantitative BAT activity test kit as disclosed herein, and/or the antibody based quantitative BAT activity test kit as disclosed herein.

[0011] According to a seventh aspect, there is provided method of discovering a drug, a nutraceutical, and/or a functional food that activate BAT and/or induce browning of WAT to BAT in a subject using the method as disclosed herein, wherein the stimulation is the drug, the nutraceutical, and/or the functional food; and

wherein if the post-stimulation level of MTHFD1L protein is **higher** than the baseline level of MTHFD1L protein, determine that the drug, the nutraceutical, and/or the functional food activates BAT and/or induces browning of WAT to BAT in a subject.

DEFINITION OF TERMS

[0012] The following words and terms used herein shall have the meaning indicated:

[0013] The term “brown adipose tissue” or “BAT” refers to a form or a type of adipose tissue that is found in almost all mammals important for thermoregulation. “Brown adipose tissue” or “BAT” is thermogenic and consumes substantial amounts of glucose and fatty acids as fuel for thermogenesis and energy expenditure. Therefore, it may play a role in combating obesity and/or metabolic disease.

[0014] The term “white adipose tissue” or “WAT” refers to another type of adipose tissue besides brown adipose tissue or BAT that is also found in almost all mammals. “White adipose tissue” or “WAT” is used by the body for energy storage and as a thermal insulator to help the body maintain body temperature. The terms “WAT” and “BAT” are inversely related. In one example, a subject having zero or low level of BAT will have a high level of WAT and vice versa.

[0015] The term “brown adipose tissue activation” or “BAT activation” refers to a metabolic process which results in increase of glucose and fatty acid clearance as well as resting metabolic rate in adult humans. A prolonged elevation of BAT activity may improve insulin sensitivity. BAT activation can be triggered by various stimulations which include but are not limited to cold exposure, capsinoids ingestion, and thyroid hormone excess in humans.

[0016] The term “browning of white adipose tissue to brown adipose tissue” or “browning of WAT to BAT” refers to the emergence of brown adipocytes and or the conversion of white adipocytes into beige adipocytes in white adipose tissue. The process may represent adaptation to increased thermogenic demand, exercise, injury (thermal injury), and disease (cancer).

[0017] The term “subject” refers to human or other mammals. Suitable mammals that fall within the scope of the disclosure include, but are not restricted to, primates, livestock animals (e.g. sheep, cows, horses, donkeys, pigs), laboratory test animals (e.g. rabbits, mice, rats, guinea pigs, hamsters, squirrels), companion animals (e.g. cats, dogs) captive wild animals (e.g. foxes, deer, dingoes), and mammals that hibernate (e.g. bears, monotremes, ground squirrels, hedgehogs and marsupials).

[0018] The term “methylene tetrahydrofolate dehydrogenase 1-like protein” or “MTHFD1L protein” refers to a biomarker that is useful for detecting brown adipose tissue activation (BAT activation) and/or browning of white adipose tissue to brown adipose tissues (browning of WAT to BAT) in a subject. It is an exosomal protein that is over-expressed and detectable in plasma after BAT activation in subjects. This mitochondrial protein is packaged as a cargo within multivesicular bodies of the endosomal compartment and secreted as exosomes via exocytosis from activated brown adipocytes into the circulation. In the mass spectrometry database, the molecular identity of this protein is denoted by B7Z809.

[0019] The term “stimulation” refers to action of various agents or stimulants on a subject which results in brown adipose tissue activation (BAT activation) and/or browning of white adipose tissue to brown adipose tissues (browning of WAT to BAT).

[0020] The terms “basal BAT”, “basal BAT inactive state”, and “classical BAT” are used throughout the specification. More specifically, BAT can be present from birth in regions of the body known to harbor brown fat and is then termed “classical BAT”. However, there is also another form of BAT called “beige fat” derived from the browning of white fat. This latter
5 form of BAT is inducible from white adipose tissue, and is therefore usually termed inducible BAT as opposed to classical BAT. Both forms of BAT can be quiescent/inactive in a basal state (i.e. “basal BAT”) and then stimulated (or activated) by appropriate stimuli to activated BAT. In a subject who possesses BAT, the preferred term is inactive BAT and activated BAT (covering both classical brown and inducible beige fat). In other words, the term “basal BAT”
10 is the broadest term among the terms mentioned in this paragraph. The terms “classical BAT” and “inducible BAT” are subsets of the term “basal BAT”. Both “classical BAT” and “inducible BAT” can be active or inactive.

[0021] The term “baseline level of MTHFD1L protein” refers to the level of MTHFD1L protein that is already present in a subject prior to a stimulation which results in brown adipose
15 tissue activation (BAT activation) and/or browning of white adipose tissue to brown adipose tissues (browning of WAT to BAT).

[0022] The term “higher” when used in relation to the term “baseline level of MTHFD1L protein” refers to a level of MTHFD1L protein measured after a stimulation and said level is at least 1.1 times, or at least 1.2 times, or at least 1.3 times, or at least 1.4 times, or at least 1.5
20 times, or at least 1.6 times, or at least 1.7 times, or at least 1.8 times, or at least 1.9 times, or at least 2 times, or at least 3 times, or at least 4 times, or at least 5 times, or at least 6 times, or at least 7 times, or at least 8 times, or at least 9 times, or at least 10 times the baseline level of MTHFD1L protein.

[0023] The term “the same” when used in relation to the term “baseline level of MTHFD1L protein” refers to a level of MTHFD1L protein measured after a stimulation and said level is identical to the baseline level of MTHFD1L protein. The term “the same” when used in relation
25 to the term “baseline level of MTHFD1L protein” may also refer to an amount between 0.99 to 1.01 times the baseline level of MTHFD1L protein.

[0024] The term “lower” when used in relation to the term “baseline level of MTHFD1L protein” refers to a level of MTHFD1L protein measured after a stimulation and said level is
30 at least 0.9 times, or at least 0.8 times, or at least 0.7 times, or at least 0.6 times, or at least 0.5

times, or at least 0.4 times, or at least 0.3 times, or at least 0.2 times, or at least 0.1 times, or at least 0.05 times, or at least 0.01 times the baseline level of MTHFD1L protein.

[0025] The term “metabolic disorder” refers to any of the diseases or disorders that disrupt normal metabolism, the process of converting food to energy on a cellular level. The term
5 “metabolic syndrome” refers to group of risk factors that increases risk for heart disease and other health problems, such as diabetes and stroke or a combination of diabetes, high blood pressure (hypertension) and obesity that puts a subject at greater risk of getting coronary heart disease, stroke and other conditions that affect the blood vessels.

[0026] The term “nutraceutical” refers to any substance that is a food or part of a food and
10 provides medical or health benefits, including the prevention and treatment of disease. The term “functional food” refers to food comprising health-giving additives.

[0027] The term “exosomes” refers to small microvesicles 30-100nm in size found in nearly all eukaryotic cells. They are released from the interior of cells to the exterior environment and transfer DNA, RNA, and proteins to other cells, thereby altering the function of the target cells.

15 [0028] Unless specified otherwise, the terms “comprising” and “comprise”, and grammatical variants thereof, are intended to represent “open” or “inclusive” language such that they include recited elements but also permit inclusion of additional, unrecited elements.

[0029] Throughout this disclosure, certain examples may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity
20 and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6
25 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[0030] Certain examples may also be described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the disclosure. This includes the generic description of the examples with a proviso or
30 negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

DETAILED DISCLOSURE OF THE EMBODIMENT

- [0031] One of the most convenient and readily acceptable form of clinical evaluation in medical practice as well as research is a blood test via venipuncture or finger prick. This motivates the inventors to consider elucidating a circulating biomarker that can reliably indicate BAT activation. As one of the more recent paradigms of inter-organ crosstalk involves exosomes (membrane bound extracellular vesicles 30-100 nm in size, produced in the endosomal compartment of most eukaryotic cells), the inventors decided to profile the exosomal proteomics of active BAT. The aim of this present disclosure was to explore the hypothetical assumption that such a unique circulating protein biomarker which accurately reflects BAT activation actually exist. The inventors therefore examined whether such an exosomal protein would be found to be elevated when BAT is activated by known classical stimuli including, but not limited to, cold exposure, capsinoids ingestion and thyroid hormone excess in people with hyperthyroidism.
- [0032] The inventors present the protein called MTHFD1L (otherwise termed as formyltetrahydrofolate synthetase), a mitochondrial monofunctional enzyme with formyltetrahydrofolate synthetase activity, which the inventors discovered for the very first time that this protein is elevated following the activation of brown adipose tissues (BAT) in mammals including, but not limited to humans and rats. This is as yet totally novel and never been published or described by anyone else in the scientific literature. This implies that BAT detection can conceivably be achieved in the near future just by using small amount of blood samples without resorting to the use of radioactive tracers or expensive imaging technology such as PET-CT, MRI or highly costly and inconvenient whole body indirect calorimetry. Unlike in the currently available technology for detecting BAT activation such as PET-CT, fat fraction MRI, infrared thermography (IRT), indirect calorimetry, and blood exosomal miRNA, the method of the present disclosure is advantageous because:
1. unlike PET-CT, the method of the present disclosure is cheaper and does not require exposing subject to high amount of radiation because it detects blood biomarker MTHD1L that reflect BAT activation rather than glucose uptake;
 2. unlike fat fraction MRI, the method of the present disclosure is cheaper and does not require complicated fat fraction segmentation because it measures MTHD1L level in blood rather than detecting fat content changes in BAT;

3. unlike IRT, the method of the present disclosure has a more streamlined protocol because it measures MTHD1L level in blood rather than measuring temperature and heat;

4. unlike indirect calorimetry, detection of MTHD1L level in blood according to the method of the present disclosure does not require cumbersome calorimetry; and

5 5. unlike blood exosomal miRNA, the method of the present disclosure only detects one specific enzyme (i.e. MTHD1L) which is technically easier than detecting one or many miRNAs.

[0033] BAT activity detection using a blood based biomarker has been described mainly for microRNAs. This has not been developed into a test kit as yet. That is because miRNA is
10 very unstable compared with a protein/enzyme such as MTHFD1L and thus it is difficult to isolate from blood. A test based on miRNA is likely to be research based, or else must be analyzed in the lab. On the other hand, a test based on protein/enzyme can potentially be miniaturized and designed as a point-of-care test. Other than the present disclosure, a method based on a specific protein or enzyme in exosomes circulating in blood following BAT
15 activation has not been described or published. It is not trivial and not obvious to a skilled person to actually attempt to isolate exosomes and then run sophisticated mass spectrometry to elucidate the proteomics for all multiple categories (models) of BAT activation.

[0034] Further to the above, it is quite unlikely for a skilled person to suspect that only one key protein/enzyme would show up as the dominant biomarker of BAT activation out of a
20 possible range of thousands of proteins elaborated. On the contrary, a skilled person would instead expect to encounter a number of small protein peaks representing a signature that must be collectively analyzed and quantified before it can be confidently claimed that BAT is activated. Hence, it would not be obvious to the skilled person that a single protein would be found that can be capitalized upon to develop a BAT activation detection kit.

25 [0035] Thus, according to a first aspect, there is provided a method for detecting brown adipose tissues (BAT) activation and/or browning of white adipose tissues (WAT) to BAT in a subject, wherein the method comprises:

- a. obtaining a first blood sample from the subject;
- b. determining a baseline level of methylene tetrahydrofolate dehydrogenase 1-like
30 (MTHFD1L) protein in the first blood sample;
- c. exposing the subject to a stimulation;
- d. obtaining a second blood sample from the subject after the stimulation; and

- e. measuring a post-stimulation level of MTHFD1L protein in the second blood sample;
 - e1. if the post-stimulation level of MTHFD1L protein is **higher** than the baseline level of MTHFD1L protein, determining **presence** of BAT activation and/or browning of WAT to BAT in the subject, or

- 5 e2. if the post-stimulation level of MTHFD1L protein is **the same or lower** than the baseline level of MTHFD1L protein, determining **absence** of BAT activation and/or browning of WAT to BAT in the subject.

[0036] According to a second aspect, there is provided a method for detecting brown adipose tissues (BAT) activation and/or browning of white adipose tissues (WAT) to BAT in
 10 a subject, wherein the method comprises:

- a. determining a baseline level of methylene tetrahydrofolate dehydrogenase 1-like (MTHFD1L) protein in a first blood sample obtained from the subject;
- b. measuring a post-stimulation level of MTHFD1L protein in a second blood sample obtained from the subject after the subject has been exposed to a stimulation; and
- 15 b1. if the post-stimulation level of MTHFD1L protein is **higher** than the baseline level of MTHFD1L protein, determining **presence** of BAT activation and/or browning of WAT to BAT in the subject, or
- b2. if the post-stimulation level of MTHFD1L protein is **the same or lower** than the baseline level of MTHFD1L protein, determining **absence** of BAT activation
 20 and/or browning of WAT to BAT in the subject.

[0037] Without wishing to be bound by theory, MTHFD1L is likely to be overexpressed when BAT is activated, and then its level will return back to baseline after some hours after the activation is stopped. In view thereof, the methods as described in the first and second aspect above require obtaining a first and a second blood sample. That is because two samples (which
 25 are obtained before and after stimulation) showing a significant rise in level post-stimulation is an advantageous proof to determine activatable BAT is present.

[0038] In one example, the subject of the method of the present disclosure is a mammal. In another example, the mammal may include, but is not limited to a human or a rat. Without wishing to be bound by theory, it is highly likely that other mammals or mammalian vertebrates
 30 that possess BAT will also express MTHFD1L, especially when the BAT is activated. Notably, mammals are warm-blooded homeothermic animals that thermoregulate so as to defend their body temperatures from falling towards the temperature of the surrounding environment.

Hence, if any mammal is exposed to the cold, it will at first shiver to generate heat from contracting muscles, and then try to conserve more heat by activating BAT and browning WAT. Mammals that hibernate in the winter naturally possess large quantities of BAT as well to survive very low temperatures.

5 [0039] MTHFD1L is found in humans and in rats, and indeed in all mammals. MTHFD1L is a mono-functional enzyme found in the mitochondria of cells of both humans and other mammalian vertebrates. In terms of enzymatic function, MTHFD1L possesses formyl C1-tetrafolate synthetase activity. MTHFD1L appears to be preferentially expressed in both rats and humans during BAT activation.

10 [0040] In one example, the subject of the method of the present disclosure does not have cancer. That is because in certain cancers, the cancer cells actually rely on MTHFD1L to generate tetrahydrofolate (THF) which is crucial as a source of 1-carbon group to be transferred to uridine to form thymidylate, a critical nucleic acid base for DNA synthesis required by rapidly dividing cancer cells. As such, it is expected that MTHFD1L to be constantly elevated
15 in people or animals with aggressive malignancies. On the other hand, in a healthy subject or a subject that does not have cancer the MTHFD1L protein level would be very low in basal BAT-inactive states. It will only escalate upwards when BAT is activated or following BAT stimulation of classical BAT or after browning of WAT.

[0041] In the present application, only BAT-positive subjects were tested because the
20 inventors want to evaluate the presence of a specific protein that is produced during the phase of BAT activation, and to be able to link/associate this protein to BAT. An example of the strongest evidence that support that this protein is a BAT secreted product comes from the rat data in Example 2 which showed the presence of MTHFD1L within the mitochondria of BAT. Since the expression of MTHFD1L protein is increased by BAT activation, without wishing to
25 be bound by theory, it is expected that the level of MTHFD1L should be negligible or present in very low subthreshold amounts in subjects without BAT or BAT-negative subjects. There should not be any changes in the levels after subjecting such individuals to a known BAT activating stimulus. From a scientific perspective, the main reason for evaluating MTHFD1L in BAT-negative subjects to use data obtained from those subjects as a control. In other words,
30 the main reason for evaluating MTHFD1L in BAT-negative subjects is to show that the level of MTHFD1L at baseline will be very low or undetectable, and the level after a classical BAT activating stimulus will hardly change, as opposed to people who are BAT-positive who should

show higher baseline/basal values and significantly increased levels compared to baseline/basal levels post-stimulation. Having said the above, in subjects who are shown to be BAT-negative, under conditions of chronic cold stimulation or ingestion of browning nutraceuticals, they may gradually become converted to BAT-positive after a period of weeks to months. This example indicates that a subject can switch from being BAT-negative to being BAT-positive under suitable stimuli. This phenomenon can be useful in testing the browning properties of certain drugs, foods ingredients or nutraceuticals.

[0042] In one example, the blood sample of the method of the present disclosure is blood plasma. In another example, the blood sample of the method of the present disclosure is blood serum. In yet another example, the blood sample of the method of the present disclosure is whole blood. Without being bound by theory, the sample obtained for the method of the present disclosure is blood sample because the MTHFD1L protein is secreted directly from the BAT into adjacent capillaries and then to the blood. The blood sample may be obtained using any techniques commonly used in the art which include, but are not limited to, finger prick, arterial sampling, venipuncture sampling, fingerstick sampling. A person skilled in the art is aware that the blood sample may require further processing. For example, if the blood sample is whole blood or venous or even arterial blood samples, the blood can be spun by centrifuge to yield plasma or clotted to yield serum from which MTHFD1L can leaked out of exosomes to become detectable.

[0043] In one example, the stimulation of the method of the present disclosure is a stimulation that results in BAT activation and/or browning of WAT to BAT. In another example, the stimulation that results in BAT activation and/or browning of WAT to BAT may include, but is not limited to, capsinoid stimulation, cold stimulation, or hyperthyroid treatment. Other forms of stimulation may include, but are not limited to other drugs and nutrients are also known to stimulate BAT, such as adrenergic agents (e.g. norepinephrine, isoprenaline, etc), spices (e.g. capsaicin, menthol, etc), and nutraceuticals (e.g. catecholamine, gingerol, curcumin, capsaicin, menthol, etc). Endogenous peptides and hormones can also stimulate BAT. As thyroid hormone is a well-established hormone required as an obligatory factor for the differentiation of adipocyte precursors along a BAT cell fate as well as a hormone that can activate existing mature BAT tissue, the inventors decided to study the degree of BAT activation in patients suffering from hyperthyroidism as a model of thyroid hormone excess. There are certainly many other peptide hormones that activate BAT, such as natriuretic factors

(e.g. ANP and BNP), fibroblast growth factor-21 (FGF-21) and bone morphogenetic proteins (eg. BNP7 and BNP8). Such stimulation methods are known in the art, and exemplary protocols for carrying out such stimulation methods are described in Example 1. The subject may be exposed to one type of stimulation at a time or the subject may be exposed to a combination of two or more stimulation at a time.

[0044] The methods as described in the first and second aspects above are useful for developing various applications including, but are not limited to, qualitative test kits, microfluidic devices, quantitative or semi-quantitative test kits, lab on chips devices, antibody tests, ELISA, and others. In one example, there is provided a method of BAT activation detection using a Lab-on-Chip approach that is based on the inventors' discovery of MTHFD1L which is expressed and secreted by BAT in the form of exosomes released into the circulation.

[0045] In another example, there is also provided a lab-on-chip microfluidic platform comprising a membrane for isolating exosomes from blood sample via filtration method.

[0046] In yet another example, there is also provided a test kit comprising a specific antibody of MTHFD1L protein. Such test kit can be used as a bedside test by researchers and clinicians. Such test kit also has the potential to be a relatively low cost and rapid test that can accelerate BAT research by biomedical scientists, pharmaceutical companies, and food companies investigating and developing drugs, nutraceuticals, and functional foods to activate BAT and/or induce browning of WAT. When more potent BAT stimulants are found, the demand and use of this detection method can also increase substantially in an effort to find BAT-based treatment for obesity, metabolic syndrome, and type 2 diabetes. Diseases that are associated with BAT activation may also be monitored using the method and/or test kit of the present disclosure. In one example, using the method and/or rest kit disclosed herein may be useful in confirming euthyroidism at a personalized level better than using a population-based normal range of plasma free thyroxine (FT4) and thyroid stimulating hormone (TSH). At present, there are potential inherent inaccuracies of confirming a euthyroid state based on free thyroxine and TSH levels. Since the level of BAT activity correlates with circulating levels of thyroid hormones (T3/T4), it can potentially be used as an independent marker of thyroid status, based on the assumption that a specific normal range of plasma MTHFD1L can be established that indicates euthyroidism in a healthy population.

[0047] Thus, according to a third aspect, there is provided a qualitative BAT activity test kit for detecting brown adipose tissues (BAT) activation and/or browning of white adipose

tissues (WAT) to BAT in a subject according to the method as disclosed herein, wherein the qualitative BAT activity test kit comprising:

- a test strip A made from porous material allowing for capillary flow of a buffer,
 - o wherein the test strip A comprises a sample receiving region A and an active reagents region A,
 - o wherein the active reagents region A is located downstream of the sample receiving region A,
 - o wherein the active reagents region A comprises one or more active reagents capable of a visually detectable change in the presence of MTHFD1L protein, and
 - o wherein the sample receiving region A is for application of a blood sample obtained from a subject **prior to** a stimulation,
- and
- a test strip B made from porous material allowing for capillary flow of a buffer,
 - o wherein the test strip B comprises a sample receiving region B and an active reagents region B,
 - o wherein the active reagents region B is located downstream of the sample receiving area B,
 - o wherein the active reagents region B comprises one or more active reagents capable of a visually detectable change in the presence of MTHFD1L protein, and
 - o wherein the sample receiving region B is for application of a blood sample obtained from a subject **after** a stimulation,

wherein when the blood sample obtained from a subject **prior to** a stimulation and/or the blood sample obtained from a subject **after** a stimulation comprises MTHFD1L protein, the buffer is capable of dissolving said MTHFD1L protein, wherein if the buffer comprises dissolved MTHFD1L protein, the dissolved MTHFD1L protein catalyzes a chemical reaction on the one or more active reagents capable of a visually detectable change in presence of MTHFD1L protein thereby resulting in a visually detectable change in the active reagent region A and/or the active reagent region B which indicates the presence of MTHFD1L protein in the blood sample. In one example, the buffer capable dissolving the MTHFD1L protein is composed of normal saline water comprising alkali in a concentration that is sufficient to produce a pH that

optimizes the enzymatic catalysis performed by MTHFD1L and FDH. In one example, the visually detectable change is a color change. In one example, the active reagents capable of a visually detectable change in the presence of MTHFD1L protein include, but are not limited to, tetrahydrofolate (THF), adenosine triphosphate (ATP), formate, formyltetrahydrofolate dehydrogenase (FDH), and ultrasensitive pH colorimetric indicator. In one example, the active reagents region A and/or the active reagents region B comprises tetrahydrofolate (THF), adenosine triphosphate (ATP), formate, formyltetrahydrofolate dehydrogenase (FDH), and ultrasensitive pH colorimetric indicator. In one example, wherein when the active reagents capable of a visually detectable change in the presence of MTHFD1L protein are tetrahydrofolate, adenosine triphosphate (ATP), formate, formyltetrahydrofolate dehydrogenase (FDH), and ultrasensitive pH colorimetric indicator, the visually detectable change is a change from a blue band to a red band. The color change as exemplified above is not limited to a color change from blue to red. For example, the kit can be fabricated with reagents so that the baseline level of MTHFD1L below a certain threshold will be intentionally left colorless. A person skilled in the art is aware that the color change will depend on the choice of the colorimetric indicator used.

[0048] An exemplary embodiment of the qualitative BAT activity test kit above is provided in Fig. 9A as test kit 100. The test kit comprised two filter paper strips or test strip (depicted as test strip A (110) and test strip B (120) in Fig. 9A) in which reagents such as formate, ATP, tetrahydrofolate (THF), formyltetrahydrofolate dehydrogenase (FDH), and an ultrasensitive pH colorimetric indicator are embedded at the active reagents regions (depicted as active reagents region A (112) and active reagents region B (122) in Fig. 9A; denoted as “result well” in Fig. 9B). To use the test kit 100, blood sample obtained from a subject **prior to** a stimulation is added to sample receiving region A (111) and blood sample obtained from a subject **after** a stimulation is added to sample receiving region B (121). If the blood sample comprised MTHFD1L, when the MTHFD1L dissolved in the buffer reaches the active reagents regions, MTHFD1L will catalyze conversion of formate, ATP, and THF into 10-formyltetrahydrofolate (10-CHO-THF) as the reaction product. The 10-CHO-THF produced can in turn be acted on by a second enzyme, formyltetrahydrofolate dehydrogenase (FDH), which is embedded into the filter paper as well. The end result is production of THF and CO₂. The CO₂ produced can be detected by an ultrasensitive pH colorimetric indicator to show a red color band which confirms the presence of MTHFD1L enzyme in the blood sample. In the exemplary

embodiment provided in Fig. 9B, a BAT-positive outcome will appear as a red band in the results well of the post-stimulated lane at the bottom (i.e. which corresponds to active reagents region B (122) in Fig. 9A) and a blue band in the pre-stimulated well (i.e. which corresponds to active reagents region A (112) in Fig. 9A). However, if the blood sample came from a BAT-negative individual, then both the pre-stimulated (or baseline) and post-stimulated wells which will only show a blue band.

[0049] According to a fourth aspect, there is provided a enzymatic chemistry based quantitative BAT activity test kit for detecting brown adipose tissues (BAT) activation and/or browning of white adipose tissues (WAT) to BAT in a subject according to the method as disclosed herein wherein the enzymatic chemistry based quantitative BAT activity test kit comprises: a. a sample collection stick, wherein the sample collection stick comprises one or more active reagents capable of undergoing an enzymatic reaction in the presence of MTHFD1L protein, b. a detector system capable of detecting a product formed by the enzymatic reaction, and c. a monitoring device capable of determining the level of MTHFD1L in the blood sample based on the amount of product formed by the enzymatic reaction. In one example, the active reagents capable of undergoing enzymatic reaction in the presence of MTHFD1L protein may include, but are not limited to, tetrahydrofolate (THF), adenosine triphosphate (ATP), and formate. In another example, the active reagents capable of undergoing enzymatic reaction in the presence of MTHFD1L protein are tetrahydrofolate (THF), adenosine triphosphate (ATP), and formate. In one example, the detector system capable of detecting a product formed by the enzymatic reaction may include, but it not limited to, a detector capable of detecting formyl-THF, a colorimetric detector system capable of detecting CO₂, and a sensitive microchip of semi-conductor device capable of detecting a change of electrical current and voltage generated due to electron transfers of a redox reaction. In one example, the change of electrical current and voltage detected by the sensitive microchip of the semi-conductor device is generated due to electron transfers of the redox reactions. Similar to the qualitative BAT activity test kit disclosed above, the quantitative BAT activity test is also based on the known catalytic reaction of MTHFD1L, which leads to the generation of 10-CHO-THF, following which this molecule can undergo oxidation by formyltetrahydrofolate dehydrogenase (FDH) into THF and carbon dioxide (CO₂).

[0050] According to a fifth aspect, there is provided an antibody based quantitative BAT activity test kit for detecting brown adipose tissues (BAT) activation and/or browning of white

adipose tissues (WAT) to BAT in a subject according to the method as disclosed herein, wherein the quantitative BAT activity test kit comprises: a. a sample collection stick, wherein the sample collection stick comprises a capture antibody capable of binding to MTHFD1L protein and a detection antibody capable of binding to a capture antibody-MTHFD1L protein complex, wherein the detection antibody is connected to a tag, wherein the tag is a fluorescent tag capable of emitting fluorescence in the presence of MTHFD1L protein or a tag capable of undergoing a color change in the presence of MTHFD1L protein, wherein when the tag is a tag capable of undergoing a color change in the presence of MTHFD1L protein, the sample collection stick further comprises a substrate capable of reacting with the tag, b. a detector system capable of detecting the fluorescence or the color change formed by the tag, and c. a monitoring device capable of determining the level of MTHFD1L in the blood sample based on the amount of fluorescence formed by the tag or based on the intensity of color change formed by the tag. In one example, there is provided an antibody based quantitative BAT activity test kit for detecting brown adipose tissues (BAT) activation and/or browning of white adipose tissues (WAT) to BAT in a subject according to the method as disclosed herein, wherein the quantitative BAT activity test kit comprises: a. a sample collection stick, wherein the sample collection stick comprises a capture antibody capable of binding to MTHFD1L protein and a detection antibody capable of binding to a capture antibody-MTHFD1L protein complex, wherein the detection antibody is connected to a tag, wherein the tag is a fluorescent tag capable of emitting fluorescence in the presence of MTHFD1L protein, b. a detector system capable of detecting the fluorescence formed by the tag, and c. a monitoring device capable of determining the level of MTHFD1L in the blood sample based on the amount of fluorescence formed by the tag. In other words, if the system used is based on detection antibody connected to a fluorescent tag (i.e., based on immunofluorescence in which secondary antibodies can be tagged with a fluorescent label), then the fluorescence intensity reflects concentration of the MTHFD1L. In another example, there is provided an antibody based quantitative BAT activity test kit for detecting brown adipose tissues (BAT) activation and/or browning of white adipose tissues (WAT) to BAT in a subject according to the method as disclosed herein, wherein the quantitative BAT activity test kit comprises: a. a sample collection stick, wherein the sample collection stick comprises a capture antibody capable of binding to MTHFD1L protein, a detection antibody capable of binding to a capture antibody-MTHFD1L protein complex, wherein the detection antibody is connected to a tag, wherein the tag is a tag capable of

undergoing a color change in the presence of MTHFD1L protein, and a substrate capable of reacting with the tag, b. a detector system capable of detecting the color change formed by the tag, and c. a monitoring device capable of determining the level of MTHFD1L in the blood sample based on the intensity of color change formed by the tag. In one example, the tag
5 capable of undergoing a color change in the presence of MTHFD1L protein may include but is not limited to a horseradish peroxidase or an alkaline phosphatase. In a one example, the substrate capable of reacting with the tag may include but is not limited to tetramethylbenzidine (TMB). In other words, if the horseradish peroxidase-tetramethylbenzidine system (i.e., HRP-color substrate system) is used, then the absorbance of the color which corresponds to the
10 concentration of MTHFD1L is one way of quantification. In both methods described above, a special reader must be built into the device to read off the absorbance or the fluorescence intensity, and then convert this into a concentration value of MTHFD1L.

[0051] According to an sixth aspect, there is provided a method for detecting or monitoring a metabolic disorder or metabolic syndrome that affect BAT activity in a subject using the
15 method as disclosed herein, the qualitative BAT activity test kit as disclosed herein, the enzymatic chemistry based quantitative BAT activity test kit as disclosed herein, and/or the antibody based quantitative BAT activity test kit. In one example, the metabolic disorder or metabolic syndrome that affect BAT activity may include, but is not limited to thyroid dysfunction, obesity, diabetes, or pheochromocytoma. Obesity, metabolic syndrome, and
20 diabetes are presently pandemic in many countries including Singapore. In certain circumstances, people who are affected by metabolic disorder or metabolic syndrome may lack brown adipose tissue which burns fat. Pheochromocytoma or tumour of the adrenal medulla is a rare but deadly endocrine disorder. Pheochromocytoma leads to excessive catecholamines production. This in turn triggers browning of WAT and increases the formation of BAT, as
25 well as activates BAT. If pheochromocytoma is not diagnosed or if it is diagnosed too late, the affected patients may die prematurely. However, the diagnosis of pheochromocytoma has traditionally been based on the detection of excessive catecholamines and metanephrines in blood and/or urine samples as often exploited by endocrinologists. This diagnosis method is a relatively lengthier and more difficult test to do, and not every hospital laboratory can assay
30 plasma catecholamines and metanephrines. Therefore, the BAT activity test kits disclosed herein is advantageous in detecting pheochromocytoma because they can rapidly confirm the presence of active BAT in a patient suspected of having pheochromocytoma.

[0052] According to an seventh aspect, there is provided a method of discovering a drug, a nutraceutical, and/or a functional food that activate BAT and/or induce browning of WAT to BAT in a subject using the method as disclosed herein, wherein the stimulation is the drug, the nutraceutical, and/or the functional food; and wherein if the post-stimulation level of MTHFD1L protein is **higher** than the baseline level of MTHFD1L protein, determine that the drug, the nutraceutical, and/or the functional food activates BAT and/or induces browning of WAT to BAT in a subject. Without wishing to be bound by theory, certain drugs, nutraceuticals, and/or functional foods may be able to stimulate BAT activity and trigger browning of white adipose tissue (WAT) to BAT. Natural and food-based treatments may be useful for treating metabolic disorders such as obesity, metabolic syndrome and diabetes. Therefore, there is a need for finding functional foods and nutraceuticals that can possess BAT-activating and browning properties. Hence, the availability of a bedside rapid test-kit for BAT activity will accelerate the discovery of many other nutrient and foods that can naturally activate BAT or increase the formation of BAT from unhealthy white fat. This use of change in MTHFD1L levels as a marker of BAT stimulation by a nutraceutical or functional food is a game changer and can lead to the recognition of many more food compounds and nutraceuticals that can activate BAT and increase beige fat.

BRIEF DESCRIPTION OF THE DRAWINGS

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[0053] The disclosure will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0054] **Fig. 1.** depicts a volcano plot showing protein expression levels in pooled plasma of human subjects under acute cold stimulation with resultant BAT activation. Among the upregulated proteins, 'highly similar to C-1-tetrahydrofolate synthase (B7Z809)' was significantly increased. $FDR < 0.05$ and $FC \text{ cutoff} = \log_2(1.2)$.

[0055] **Fig. 2.** depicts a volcano plot showing protein expression levels in pooled plasma of human subjects under acute capsinoids stimulation with resultant BAT activation. Among the upregulated proteins, 'highly similar to C-1-tetrahydrofolate synthase (B7Z809)' was significantly increased. $FDR < 0.05$ and $FC \text{ cutoff} = \log_2(1.2)$.

[0056] **Fig. 3.** depicts a volcano plot showing protein expression levels in pooled plasma of hyperthyroid patients with BAT activation. Among the upregulated proteins, 'highly similar to C-1-tetrahydrofolate synthase (B7Z809)' was significantly increased. $FDR < 0.05$ and $FC_{cutoff} = \log_2(1.2)$.

5 [0057] **Fig. 4.** depicts scatter plots showing correlations of exosomal protein expressions for cold vs capsinoid vs hyperthyroid states of BAT activation. Fig. 4A represents correlations of exosomal protein expressions for cold vs hyperthyroid states of BAT activation. Fig. 4B represents correlations of exosomal protein expressions for capsinoid vs hyperthyroid states of BAT activation. Fig. 4C represents correlations of exosomal protein expressions for cold vs
10 capsinoid states of BAT activation. The pattern of protein expression appears to be similar between cold-stimulated and capsinoid-stimulated BAT (i.e. Fig. 4B), as well as between capsinoid-stimulated and hyperthyroid-stimulated BAT (i.e. Fig. 4C). However, the protein expression profile for hyperthyroid-stimulated and cold-stimulated BAT (i.e. Fig. 4A) appears to be inversely correlated.

15 [0058] **Fig. 5.** depicts a Venn diagram showing common and uniquely expressed genes across the 3 different modes of BAT activation.

[0059] **Fig. 6.** depicts a Western blot analysis of MTHFD1L from isolated from plasma. (A) Western blot images showing comparison of the expression MTHFD1L from 2 weeks cold exposed group (4°C) and at thermoneutral condition. (B) Bar graphs showing Western blot
20 densitometry analysis showing significant increase of MTHFD1L in cold exposed group compared to the thermoneutral group ($* P < 0.05$; $** < 0.01$; $*** < 0.001$; $**** < 0.0001$).

[0060] **Fig. 7.** depicts a Bar graph showing mRNA expression of MTHFD1L from intrascapular brown adipose tissue obtained from animals exposed to 2 weeks of cold (4°C) and thermoneutral condition. There is a significant increase of MTHFD1L in cold exposed
25 group compared to the thermoneutral group ($P^* < 0.05$; $** < 0.01$; $*** < 0.001$; $**** < 0.0001$).

[0061] **Fig. 8.** depicts a schematic showing the process for the development of antibody test for detecting MTHFD1L protein, which is a biomarker shown to be useful in the present disclosure for detecting brown adipose tissue activation (BAT activation) and/or browning of white adipose tissue to brown adipose tissues (browning of WAT to BAT) in a subject.

30 [0062] **Fig. 9.** depicts an illustration showing a prototype of immunochemistry based Qualitative BAT Activity test kit. (A) shows a schematic diagram of the Qualitative BAT activity test kit (100) comprising a test strip A (110) and a test strip B (120). Each test strip

further comprises a sample receiving region (111 and 121) and an active reagents region (112 and 122). (B) shows an example of the positive and negative test result of the Qualitative BAT activity test kit. In this particular example, a positive test result will appear as a red band in the active reagents region B (122) and a blue band in the active reagents region A (112). A negative test result will appear as blue bands in both active reagents region A (112) and active reagents region B (122).

[0063] **Fig. 10.** depicts an illustration showing a prototype of enzymatic chemistry of MTHFD1L based Quantitative BAT Activity test.

EXAMPLES

[0064] Non-limiting examples of the disclosure will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the disclosure.

[0065] **Example 1 – Methods**

[0066] *Human subjects*

[0067] Healthy volunteers aged 21-40 years old with BMI ranging from 18.5 to 29.9 kg/m² were recruited into a study acronymed, TACTICAL-II that investigated BAT activation and energy expenditure elevation stimulated by cold and capsinoids (www.ClinicalTrials.gov identifier NCT 02964442) (2). Subjects were cold exposed for about 2 hours by wearing a cooling vest at a constant temperature of 14.5°C (Cool58, Polar Products, Ohio, US). On a separate study visit separated at least 48h apart, subjects were given capsinoids capsules (12 mg, 8 gel capsules) provided by Ajinomoto Inc. (Tokyo, Japan), the composition of which can has been described previously (3). Active BAT was detected using fusion positron emission tomography with magnetic resonance imaging scanner (PET-MRI). Participants with adipose tissue uptake exhibiting PET SUV mean ≥ 2 were defined to be BAT-positive as described in detail in the literature (1, 4, 5). Those below this cutoff were defined as BAT-negative. In total, twelve BAT-positive healthy subjects (6 males/ 6 females) were identified accordingly. The remaining 8 subjects (2 males/6 females) were BAT-negative. All subjects had measurements of PET SUV and magnetic resonance fat fraction (MR FF) via PET-MRI imaging after cold exposure for around 2 hours, infrared thermography maximal temperature (IR T_{scv} max), energy expenditure by whole body calorimetry (EE) and blood sampling for BAT secretome

analysis before and after 2 hours of BAT-activating stimulation. Hyperthyroid patients were recruited into a study acronymed, TRIBUTE (www.Clinical Trials.gov identifier NCT 03064542) (6), which investigated BAT activation due to thyroid hormone excess, and followed through from pre-treatment till euthyroidism was achieved as proven by standard thyroid function tests based on plasma free thyroxine, free triiodothyronine and TSH levels. The same protocol of PET-SUV, MR FF, IRT Tmax and EE were measured in the hyperthyroid and euthyroid states. Pooled plasma were collected from the three groups (cold, capsinoids, hyperthyroid). For the purpose of selecting the BAT-positive samples in the TACTICAL-II study: N=3-5 subjects' samples from the strongest PET-SUV response were pooled together to form 4 respective tubes, each containing at least 5mL pooled plasma for optimal exosome enrichment and mass spectrometry consisting of 1 tube pre-cold stimulation pooled plasma, 1 tube post-cold stimulation pooled plasma, 1 tube of pre-capsinoids pooled plasma and 1 tube of post-capsinoids pooled plasma. For TRIBUTE samples: N=3-5 patients with the strongest PET SUV were pooled to get 1 tube of 5 mL pooled plasma taken when they were pre-treated in the hyperthyroid state and 1 tube of 5 mL pooled plasma after the same patients were rendered euthyroid with carbimazole. Both studies were conducted according to the ethical guidelines of the Declaration of Helsinki, and all procedures were approved by the Domain-Specific Review Board of National Healthcare Group, Singapore (IRB codes C/2015/00715 and C/2015/00718). Written and witnessed informed consent was obtained from all subjects before their formal participation.

[0068] ***Human exosome isolation and tandem mass spectrometry***

[0069] Exosome analysis was performed via a well-established proteomic workflow. Separation of extracellular vesicles from human plasma was performed by differential centrifugation (7). Frozen individual plasma samples were thawed on ice and pooled in a group-wise manner as described above to obtain tubes containing around 5 ml of plasma specimens per group. The samples underwent sequential centrifugation to enrich the extracellular vesicles including microvesicles and exosomes using a modified protocol as previously described (8, 9). The purified exosomes were dissolved in TRIZOL buffer for protein isolation and purification. Exosomal proteome was profiled using Tandem Mass Tag (TMT) method coupled with Multi-Dimensional Protein Identification Technology (MuDPIT) (10). Each sample was run in 3 times. The labeled exosomal proteins with TMT tags were fractionated by HpHRP liquid chromatograph and analyzed by hybrid Quadrupole-Orbitrap LC-MS/MS using Q-Exactive

LC-MS/MS system. The raw data generated by Q-Exactive LC-MS/MS was analyzed by ProteomeDiscoverer with Sequest HT and Mascot software (Thermo-Fisher, San Jose, CA). The initial dataset was filtered through stringent elimination criteria of <1% false discovery rate (FDR) to obtain confident identification, CV < 10% and *p*-value <0.05 to get rid of the technical variations during quantization. Stringent statistical analysis such as volcano plot will be used to determine the significant cut-off value of fold-change. Proteins with significant fold-changes that passed the stringent filtering criteria were then be taken for further data mining and bioinformatics analysis such as gene ontology and pathway analysis. Potential candidate biomarkers were shortlisted from the significant expression changed proteins that are in critical nodes or switches of obesity pathology or WAT/BAT physiology. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (15) partner repository with the dataset identifier PXD023909.

[0070] ***Animals***

[0071] All experimental procedures and animal research were approved by the institutional animal care and use committee. Animals were acquired from InVivos, Singapore and housed in designated holding room controlled for a 12 h-light, 12 h-dark cycle, temperature, humidity and quality of the air. Male Wistar rats (InVivos, Singapore) at seven weeks of age were randomized into two groups and assigned as thermoneutral (n=5) and cold exposed (n=5). Both the groups of animals were maintained on chow diet (CD) for further 8 weeks (indicated as 15 weeks of age). The diet composition of the CD (Altromin #1324 mod, Altromin, Germany) includes (6% fat and 18% protein) essential vitamins and proteins.

[0072] ***Cold exposure in rats***

[0073] The animals from thermoneutral group were maintained at 27-28°C. Prior to exposing the animals to 4°C, the animals were habituated to cold temperature to stabilize the metabolic alterations. The animals in the cold exposure group were subjected to cold exposure at 18°C for 3 days, 10°C for 3 days and 4°C for 1 day before the experiments. After cold exposure habituation, these animals were then exposed to 4°C for 2 weeks. The health condition of animals were monitored during entire period. All animals survived the cold exposure intervention. The protocol had been approved by the institutional animal ethics board (IACUC code number 181361) prior to the conduct of the study.

[0074] ***Blood and tissue collection in rats***

[0075] Blood was collected from the heart by cardiac puncture from the euthanized animal using 5 ml syringe with a 23G needle in sterile 2ml Eppendorf tubes containing 10 to 30 USP units of heparin/mL of blood. Blood samples were kept on ice and centrifuged at 12000 rpm for 10 minutes to collect the plasma. Plasma samples were aliquoted and stored at -80°C for further experiments. Brown adipose tissues were harvested from interscapular region and snap frozen with liquid nitrogen and stored at -80°C for further analysis.

[0076] ***Plasma exosome isolation in rats***

[0077] Isolation of exosomes from plasma was performed by using total exosome isolation kit (Thermo Fisher Scientific, catalogue # 4484450). The isolation was performed according to the protocol of the supplier. Plasma sample of 100 µl was used for the analysis and samples were centrifuged (Eppendorf centrifuge 5417R). Exosome pellets (kit isolates) were suspended in exosome resuspension buffer provided by supplier.

[0078] ***Exosome protein isolation in rats***

[0079] Isolation of protein from exosome was performed with the total exosome RNA & protein isolation kit (Fisher Scientific, catalogue # 4478545). Isolation of the protein was performed according to the protocol of the supplier. 5ul of each samples were loaded for Western blot experiment.

[0080] ***Western blot for exosomal proteins in rats***

[0081] Isolated exosome protein pellets were re-suspended in 30ul of exosome resuspension buffer provided by supplier. Equal amounts of protein (5 µl) were separated using 4-12% NuPage mini precast gels (Invitrogen Inc.) followed by dry transfer for Western blot experiments using the iBlot®2 Dry Blotting with Blot®2 NC regular Stacks. After transferring from dry blot, nitrocellulose membranes were stained with Ponceau S and images were acquired. Membranes were then blocked with 5% BSA in TBST for 1 hour at room temperature. Overnight incubation of membranes at 4°C with primary antibodies for Anti-MTHFD1L antibody+5% BSA (ab116615, Abcam, Cambridge, MA) and washed with TBST 5 min for 5 times, followed by 1 h exposure at room temperature with fluorescently-labeled secondary antibody 5% BSA buffer (IRDye® 800CW Donkey anti-Goat IgG); 1:10,000; cat. no. 925-32214 and washed with TBST 5 min for 5 times. Membrane scanned using an Odyssey Infrared Imaging system with IR fluorescence scan Odyssey 2.1 software (LI-COR Biotechnology).

[0082] The inventors have quantified the MTHFD1L protein expression levels by western blot technique. Western blot is utilized to detect proteins and their posttranslational modifications (PTM) in biological samples. Imaging devices that capture digital images of Western blots, visualized through chemiluminescence detection, have resulted in quantitative Western blotting. Each pixel in these digital images is assigned an intensity value that is related to the number of photons detected by the corresponding pixel in the sensor until it reaches saturation. Non-detector saturated images are used for quantitation, and protein/PTM abundance is most often measured using an optical density (O.D.) algorithm, which calculates O.D. values from the background-corrected band intensity and band area of the protein/PTM of interest. Western blot studies normalize levels of target protein(s)/PTM(s) to levels of loading control protein(s) or the total loading protein that do not change in abundance between comparisons. Normalization is routinely performed by dividing the O.D. value of the target protein/PTM by the O.D. value of loading control protein(s) that were detected in the same sample and ideally run in the same gel lane and detected in the same Western membrane. This ratio (target/loading control(s)) is used for comparisons of any difference between experimental samples.

[0083] ***Real time PCR for rat samples***

[0084] Total RNA was isolated from the interscapular brown adipose tissue using RNeasy Lipid Tissue Mini Kit (Qiagen 74804) and cDNA conversion using a revertAid H minus first strand cDNA synthesis kit (Thermo Scientific k1632) with oligo d(T) 18 primer according to the manufacturer's instructions. Real-time qPCR, cDNA samples were analyzed in duplicate using the SYBR Green PCR Master Mix reagent kit (Applied Biosystems 4367659) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative mRNA levels were calculated and normalized to 36B4 Forward (TTCCCACTGGCTGAAAAGGT; SEQ ID NO: 1) and (GCCGCAGCCGCAAATGC; SEQ ID NO: 2), used as an endogenous control gene. The primer sequences used for the Methylenetetrahydrofolate Dehydrogenase (NADP+ Dependent) 1-Like were as follows: MTHFD1L (TGCCGAGGGACTTCATTCTG; SEQ ID NO: 3) and (ACCTGGCATTGTGCTCATCA; SEQ ID NO: 4). Fold change in mRNA expression was calculated by **delta-delta** Ct method. Statistical significance was assessed by two-tailed Welch's *t* tests to analyze the differences between two groups and statistical significance was defined as $P < 0.05$.

[0085] ***Data analysis***

[0086] For rat samples, Western blot data was analysed using Odyssey software as per manufactures guidelines. Panceau intensity was analysed using ImageJ software. The average values were expressed as mean $\pm$ SD. Statistical significance was assessed by two-tailed Welch's *t* tests to analyze the differences between the two groups and statistical significance defined as $P < 0.05$. For the human data, these are presented as mean $\pm$ standard deviation (SD) unless otherwise stated. A P value < 0.05 was considered statistically significant. Statistical analysis was performed in Stata MP V14.0 (Stata Corp, Texas, USA) for the confirmatory analysis and other statistical analysis was performed in SPSS software version 23 (IBM SPSS Inc.).

10 [0087] ***Statistical Analysis***

[0088] Only proteins that were identified in at least two technical replicates from controls and at least two technical replicates from cases (defined as the group where the inventors anticipate higher BAT activity) were used for statistical analysis. The mass-by-charge (M/Z) abundance data was scaled within for each sample. The fold-change between cases and controls was calculated as the ratio of the group means. The difference in the group means was tested using a two sample *t*-test. The corresponding p-value was adjusted for multiple hypothesis testing using the false discovery rate method (FDR) (11). Proteins with FDR $< 5\%$ and at least 1.2 fold change in the capsinoid stimulation study was considered significant. Similarly, FDR $< 5\%$ and 1.3 fold change was used for the cold exposure stimulation study and FDR $< 5\%$ and 1.5 fold change for hyperthyroid treatment study. Volcano plots were generated using the Enhanced Volcano plot package (12). The p-value corresponding to FDR of 5% and the study specific fold change cutoffs are represented as horizontal and vertical lines. All statistical analysis was carried out using R package version 3.6.0. (13).

[0089] **Example 2 – Results**

25 [0090] ***Profiling the activated BAT proteome to identify a unique signature or protein that characterizes BAT activation***

[0091] In the quest to unravel the BAT secretome, the inventors analyzed the detailed proteomic dataset generated from mass spectrometry to discover the existence of novel exosomal-based biomarkers. Using a standardized bioinformatics approach, the inventors represented the data of the BAT secreted proteome in the cold-stimulated, capsinoid-stimulated and hyperthyroid patients in the form of 3 volcano plots (Fig. 1-3). In regard to the protein expression levels in pooled plasma of human subjects under acute cold stimulation with

resultant BAT activation, 9 exosomal proteins showed unique expression with acute cold stimulation. Among the upregulated and downregulated exosomal proteins, 3 were upregulated and 6 were downregulated significantly. For the subjects under acute capsinoids stimulation with resultant BAT activation, 8 exosomal proteins showed unique expression under capsinoids stimulation. Among the upregulated and downregulated exosomal proteins, 1 were upregulated and 7 were downregulated significantly. As for hyperthyroid patients with resultant BAT activation, 97 exosomal proteins showed expression unique to the hyperthyroid patients. Among the upregulated and downregulated exosomal proteins, 81 were upregulated and 16 were downregulated significantly.

10 [0092] Using recommended statistical cutoffs, a common exosomal protein, B7Z809 (UniProtKB taxonomy symbol for 'highly similar to C-1-tetrahydrofolate synthase, Homo sapiens'), was found to be present in the plasma following cold-stimulation and capsinoids ingestion in healthy lean human subjects as confirmed BAT-positive by PET-MRI. The inventors also found this same protein to be over-expressed among hyperthyroid patients whose
15 BAT was activated by thyroid hormones. Notably, the thyroid hormone, triiodothyronine (T3), is known to be a critical factor required for brown adipocyte differentiation as well as activation (14). Hence, it is likely to have activated BAT among those with thyroid hormone excess, a state which could partly explain the rapid weight loss despite good appetite in these cases due to fat catabolism by activated BAT. As B7Z809 is the UniProtKB taxonomy symbol for the
20 protein, "highly similar to C-1-(methylene)-tetrahydrofolate synthase, Homo sapiens", the inventors deduced that the enzyme, methylene tetrahydrofolate dehydrogenase 1-like (MTHFD1L), is the protein that is over-expressed by activated BAT. This is because MTHFD1L is a mitochondrial enzyme, and BAT which is richly endowed with mitochondria can conceivably release it during activation under the appropriate stimuli. Among the
25 upregulated proteins, B7Z809 was significantly increased (FDR < 0.05 and FC cutoff = log₂ (1.2)) with all the 3 modes of BAT activation.

[0093] ***Proteome correlation differs by method of BAT activation***

[0094] Despite having found B7Z809 as a unique protein over-expressed in cold-stimulated, capsinoid-stimulated and thyroid hormone-stimulated BAT, it would be insightful to determine
30 if other proteins secreted by activated BAT are also similar or different based on the method of BAT activation. The inventors therefore correlated the proteome signatures of these differently activated BAT against each other. Enigmatically, the secreted proteome of BAT

activated by the hyperthyroid state varied inversely with that by cold stimulation (Fig.4A). However, the secreted proteome of capsinoids-activated BAT correlated to the secreted proteome of cold-activated BAT and also BAT activated by hyperthyroidism (Fig. 4B, 4C). It is possible that the mechanisms and gene programs that are activated in cold-stimulated and hyperthyroid-stimulated BAT may differ in the upstream pathways (as reflected by the different correlation pattern), but these pathways somehow converged downstream such that the final end result was the same, which is BAT activation that led to increased thermogenesis.

[0095] ***B7Z809 is the common exosomal protein of BAT activation***

[0096] Using a Venn diagram representation, the inventors overlapped all the secreted proteome of BAT activated by all 3 modes of stimulation and this revealed 2 proteins at the intersection of the 3 groups, namely B7Z809 (highly similar to C-1-tetrahydrofolate synthase, Homo sapiens) and P23284 (PPIB). As *PPIB* (peptidylpropyl isomerase B) encodes for cyclophilin B protein which has a stable expression in peripheral whole blood and used to be an internal standard for whole blood mRNA quantification assays, this implied that the only protein that is over-expressed when BAT is activated is B7Z809 (Fig. 5). As B7Z809 is an enzyme with C-1-tetrahydrofolate synthase activity, this matches mitochondrial monofunctional C-1-tetrahydrofolate dehydrogenase-1-like (MTHFD1L) perfectly.

[0097] ***MTHFD1L protein is also over-expressed in activated BAT of rodents***

[0098] To further explore if MTHFD1L is a protein secreted by activated BAT into the circulation in other vertebrates, the inventors examined the exosomes in plasma of rats exposed to cold. Using Western blots, the inventors confirmed that MTHFD1L isolated from rat plasma was significantly higher in rats exposed to 2 weeks of cold at 4°C compared to the thermoneutral condition (Fig. 6A and 6B).

[0099] ***MTHFD1L mRNA is increased in BAT of rats exposed to cold***

[00100] To demonstrate that MTHFD1L protein is a biomarker of BAT activation, the inventors needed to show that MTHFD1L mRNA is increased in BAT itself. Analysis of the cold-exposed rats revealed that MTHFD1L mRNA from intrascapular brown adipose tissue obtained from animals exposed to 2 weeks of cold (4°C) was 3-folds higher in cold-exposed state compared to the thermoneutral state (Fig. 7). This helps to support that the over-expression of MTHFD1L was due to activated BAT rather than secreted from other tissues.

[00101] **Example 3 – Discussion**

[00102] Over the last decade, BAT has garnered great interest as a therapeutic target to combat obesity and type 2 diabetes as numerous studies have established an association between BAT activity and metabolic health. In addition to its metabolic function, BAT is also a source of signaling molecules that can modulate BAT function or even other distant tissues like the liver. Based on recent research from other groups having described exosomes being secreted by active BAT, the inventors examined their own mass spectrometry dataset of the human plasma samples to determine if any unique proteins of BAT activation could be present. In this study, the inventors demonstrate the existence of a common exosomal protein, MTHFD1L (Fig. 7) that was over-expressed in all 3 different modes of BAT activation, thereby implying that this circulating exosomal protein may serve as a unique plasma biomarker for BAT activity in rats and humans. The pattern of secreted BAT proteome appears to be similar for capsinoids and cold stimulation methods, as well as between capsinoids and hyperthyroidism. This implies that there are probably commonalities in the genes that are actively transcribed during BAT activation by these different modes of stimulation. However, the exosomal proteome of BAT stimulated by cold and by the hyperthyroid state seems to correlate inversely with each other. To describe the potential cause for such inverse correlation, the inventors note that the key purpose of BAT activation by cold is a survival thermogenic response meant to defend the body from hypothermia. On the other hand, in hyperthyroid patients whose BAT is activated by thyroid hormones, these patients are not exposed to the cold. Quite the contrary, hyperthyroid patients often experience heat intolerance with a preference for cold weather, and also exhibit hyperhidrosis as a thermoregulatory response instead. Hence, the diametrically opposite situation with respect to thermoregulation could potentially lead to BAT proteomes in cold-stimulated and hyperthyroid-stimulated BAT to correlate inversely. It is possible that the mechanisms and gene programs that are activated in these 2 methods of BAT stimulation differs in the upstream pathways, but that these pathways somehow converged downstream such that the final end result was BAT activation that led to increased thermogenesis.

[00103] Exosomes are found in various body fluids including blood and play a role in exchanging information between cells and tissues. Classically, communication between tissues and organs occur by means of circulating hormones and cytokines as encountered in endocrinology and immunology, and via neural transmission as well described in neurology. Exosomes represent a novel paradigm for such inter-organ cross-talk. Several studies have

shown that exosomes can regulate the function of the recipient/target cell and miRNAs are considered as major exosomal signals and effectors. For example, exosomes play a pivotal role in adipose *Sirt1* deficiency-mediated obesity and insulin resistance. In this disclosure, the inventors find a range of secreted exosomal proteins in all 3 modes of BAT activation.

5 Importantly, the inventors are the first to show that exosomal MTHFD1L is a common protein over-expressed in all the 3 different modes of BAT activation in humans. The large repertoire of the activated BAT secretome include exosomal cargoes of peptides, proteins and even nucleic acids such as ncRNA (eg. miRNA) from BAT that are likely to exert certain effects on other target tissues of the body with various physiological consequences. For instance, the

10 inventors had demonstrated that lncRNA is intimately linked to BAT physiology.

[00104] MTHFD1L is methylenetetrahydrofolate dehydrogenase 1-like protein, a mitochondrial monofunctional enzyme encoded by the *MTHFD1L* gene localized on chromosome 6q25.1 with N(10)-formyltetrahydrofolate synthetase activity. This catalyzes the synthesis of tetrahydrofolate (THF) in the mitochondria, a crucial step necessary for the de

15 novo synthesis of purines and thymidylate and hence, mitochondrial mtDNA. N(10)-formyltetrahydrofolate is also required for the formation of the mitochondrial initiator methionine tRNAs to drive the translation of mitochondrially encoded proteins. Presumably, when BAT becomes activated, it leads to a cascade of events that orchestrate mitochondrial fusion-fission dynamics to adapt to the heightened catabolic thermogenic state. MTHFD1L is

20 translocated into the mitochondria soon after nuclear transit of MTHFD1L mRNA into the cytoplasm for translation at the rough endoplasmic reticulum and is very likely critical for BAT activity. The newly synthesized MTHFD1L preprotein in the cytosol is kept soluble by molecular chaperones such as heat shock protein-90 or heat shock cognate-70 (Hsp90 or Hsc70) which then guide it through the mitochondria where it interacts with the mitochondrial

25 outer membrane translocase (Tom) complex, the main protein gateway of the mitochondria, with Tom40-formed channel being the key portal for MTHFD1L entry. The translocase of the inner membrane (Tim) then import the preprotein to the mitochondrial inner membrane where MTHFD1L is shown to be tightly associated with the matrix side of the mitochondrial inner membrane. The inventors' data supported that this mitochondrial enzyme, MTHFD1L, is the

30 common protein released into the bloodstream upon activation of BAT. The actual molecular mechanisms of the release of MTHFD1L into the circulation is however presently unresolved. While being speculative, MTHFD1L might find its way out from the mitochondria to the

cytoplasm during the dynamic processes of mitochondrial fission-fusion with changes in BAT activity and leaks out of active brown adipocytes to the surrounding dense capillary network richly supplying brown fat tissues. Alternatively, because MTHFD1L is encoded by the nuclear chromosomes and not by mitochondrial DNA, it is also possible that the heightened rate of

5 *MTHFD1L* gene transcription and subsequent mRNA translation in the ribosomes of the rough endoplasmic reticulum within the cytoplasm could result in some MTHFD1L protein being channeled into endosomes fated for the exosomal secretory pathway instead of being fully shuttled into the mitochondria of active brown adipocytes.

[00105] The demonstration of MTHFD1L over-expression in rats exposed to cold showed

10 that this protein is not merely a specific finding confined to humans, but potentially a conserved BAT secretome response in other vertebrates as well. It is therefore useful to investigate if the over-expression of MTHFD1L is also applicable to other BAT-possessing non-human mammals surviving and/or hibernating in cold wintry climates.

[00106] The detection of BAT has relied mainly on PET-CT imaging of ¹⁸F-FDG uptake into

15 metabolically active BAT for over a decade. This imaging technique was originally developed—and is still mainly used for detection of metastasis in oncology. However, PET-CT suffers from a major drawback of exposing people to large doses of ionizing radiation contributed by both the ¹⁸F-FDG tracer and the CT scan, and thus cannot be used for screening purposes or in the longitudinal study of a large number of healthy subjects in a population.

20 Thus, novel diagnostic biomarkers of BAT activity that are safe, highly repeatable and of low cost and will be important to help accelerate BAT research in order to better understand the physiological role of human BAT in health and disease, as well as for clinical trials to stratify subjects and to quantify the effects of drug candidates on BAT. In this regard, the exosomal protein, MTHFD1L, in human and rat blood samples is highly promising.

[00107] The inventors' disclosure has shown that secretion of MTHFD1L by activated BAT

25 is not restricted to humans but represents a general response in BAT biology. Furthermore, the mass spectrometry mass-to-charge (M/Z) ratio of B7Z809 as determined by the system was consistent with MTHFD1L as per bioinformatics classification. Moreover, by the inventors' use of an appropriate model organism for BAT activation study (Wistar rats), the inventors

30 unequivocally showed that MTHFD1L was the mitochondrial protein proven to be secreted into plasma via exosomes when BAT is activated. This strongly supports that MTHFD1L is the protein over-expressed by activated BAT in human beings.

[00108] Taken together, MTHFD1L represents a potential promising and useful brown fat biomarker for basic science and clinical applications, which can be engineered into a test-kit for BAT activity detection with relative ease in large cohorts of patients. This can catalyze BAT research and assist with clinical correlation of BAT activity in metabolic disorders such as thyroid dysfunction, obesity and diabetes where BAT may play an important role.

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