

DETECTING OXIDATIVE STRESS IN CELL(S) USING EPIGENETIC MEANS**FIELD OF THE INVENTION**

The present invention relates to a method for detecting oxidative stress (OS) in a cell using epigenetic markers. In particular, the method is capable of identifying OS in cell by determining the methylation status of a CpG site in a test cell and comparing the resultant methylation status with a reference methylation status of a control cell without OS. Differential methylation of the CpG site in the test cell indicates that the test cell has OS.

BACKGROUND OF THE INVENTION

Living organisms are subjected continuously to a variety of stresses from the outside environment. In order to resist such stresses, they maintain their homeostasis by various regulatory systems. Oxidative stress refers to a serious imbalance between the levels of reactive oxygen species (ROS) in a cell and its antioxidant defense mechanism. In order to survive this stress, living organisms have a system called redox regulation to cope with the stress to maintain their homeostasis by regulating the redox state. This system functions to adapt to many external stress agents such as radiation, ultraviolet (UV) rays, environmental pollutants, high fever, low temperature, hypoxic condition, and infectious diseases as well as to oxidative stress from lifestyle-related diseases such as cancer, diabetes, arteriosclerosis, hypertension and obesity. However, if this regulation mechanism is broken for some reason or other, oxidative stress (OS) occurs. OS can lead to cellular damage, DNA fragmentation, apoptosis and cell death. Early detection of OS can prevent further damage in the living organism causing the organism to receive early treatment or start using protection.

There are some methods known in the art for early detection of OS. However, none of these methods known in the art have been officially used to detect OS in a cell by using a genomic sample of the body.

The human skin is constantly exposed to oxidative stress and free radicals, such as to high quantities of ROS, derived not only from ordinary metabolic reactions but also continuous exposure to air, radiation and UV rays, environmental pollutants, as well as physical and/or chemical agents (e.g., cosmetics). Under some conditions, the production of ROS may become so great that it may contribute to the pathogenesis of, for example, psoriasis or skin cancer. Oxidative damage caused by free radicals such as ROS is also a main cause of physical ageing in general, and of the skin in particular. Accordingly, there is a need in the art for detection of OS in cells, for example skin cells to prevent further damage to the cells.

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DESCRIPTION OF THE INVENTION

The present invention attempts to solve the problems above by providing a method of detecting Oxidative Stress (OS) in a test cell by comparing the methylation status of at least one CpG site in the test cell and the corresponding CpG site in a control cell with no OS, wherein the presence of hypomethylation or hypermethylation at the CpG site in the test cell is indicative of the test cell having OS.

Since environmental factors/ agents such as, UV light exposure, ageing, diet and the like, may trigger OS which can further induce an alteration in the promoter CpG methylation status of the gene by recruiting DNA methyltransferases (DNMTs) and TET enzymes to various promoters, biomarkers that result in differential methylation in a cell with OS is essential to overcome the problems mentioned above. In particular, CpG sites can be used as biomarkers for detecting OS in a cell. CpG sites in a cell with OS are differentially methylated (i.e. hypomethylated or hypermethylated) compared to the corresponding CpG sites in a cell without OS. Accordingly, these CpG sites may be effectively used to determine if a cell has OS. This is particularly advantageous as using epigenetics provides a means of predicting the onset of OS in a cell, thus allowing OS to be treated earlier before causing even more damage to the cell. Further, an epigenetic marker is a long-term biomarker, that is to say it is inheritable and can be used to detect OS in the next generation as well if need be.

According to one aspect of the present invention, there is provided a method of identifying oxidative stress (OS) in a test cell, comprising

- (a) determining the methylation status of at least one CpG site in a DNA sample obtained from the test cell,
- (b) comparing the methylation status of the CpG site from (a) with that of a control without OS,

wherein difference in the methylation status of the CpG site in the test cell compared to the CpG site in the control is indicative of the test cell having OS.

As used herein, the term "cell" refers to an intact live cell, naturally occurring or modified. The cell may be isolated from other cells, mixed with other cells in a culture, or within a tissue (partial or intact), or an organism. In particular, the cell may be a eukaryote cell. More in particular, the cell may be mammalian cell. The term "mammalian cell" refers to any cell derived from a mammalian subject. The cell may also be a cell derived from the culture and expansion of a cell obtained from a subject. The cell may also have been genetically modified to express a recombinant protein

and/or nucleic acid. The mammalian cell may be from humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; rodents such as mice, rats, rabbits, hamsters, and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. In particular, the subject is a mammal. More in particular, the mammal is selected from the group consisting of a mouse, a rat, a guinea pig, a dog, a mini-pig, a human being, a cow, a sheep, a pig, a goat, a horse, a donkey, and a mule. In particular, the mammalian cell may be a skin cell, a stem cell or a cell derived therefrom. More in particular, the mammalian cell may be a skin cell.

As used herein, a "CpG site" or "methylation site" is a nucleotide within a nucleic acid (DNA or RNA) that is susceptible to methylation either by natural occurring events *in vivo* or by an event instituted to chemically methylate the nucleotide *in vitro*. Some of these sites may be hypermethylated and some may be hypomethylated in a cell with OS compared to a cell with no OS.

As used herein, a "methylated nucleic acid molecule" refers to a nucleic acid molecule that contains one or more nucleotides that is/are methylated.

A "CpG island" as used herein describes a segment of DNA sequence that comprises a functionally or structurally deviated CpG density. For example, Yamada et al. have described a set of standards for determining a CpG island: it must be at least 400 nucleotides in length, has a greater than 50% GC content, and an OCF/ECF ratio greater than 0.6 (Yamada et al., 2004, Genome Research, 14, 247-266). Others have defined a CpG island less stringently as a sequence at least 200 nucleotides in length, having a greater than 50% GC content, and an OCF/ECF ratio greater than 0.6 (Takai et al., 2002, Proc. Natl. Acad. Sci. USA, 99, 3740-3745). In context of the present invention, the terms "methylation profile", "methylation pattern", "methylation state" or "methylation status," are used herein to describe the state, situation or condition of methylation of a genomic sequence, and such terms refer to the characteristics of a DNA segment at a particular genomic locus in relation to methylation. Such characteristics include, but are not limited to, whether any of the cytosine (C) residues within this DNA sequence are methylated, location of methylated C residue(s), percentage of methylated C at any particular stretch of residues, and allelic differences in methylation due to, e.g., difference in the origin of the alleles.

The term "methylation status" refers to the status of a specific methylation site (i.e. methylated vs. non-methylated) which means a residue or methylation site is methylated or not methylated. Then, based on the methylation status of one or more methylation sites, a methylation profile may be determined. Accordingly, the term "methylation profile" or also "methylation pattern" refers to the relative or absolute concentration of methylated C residues or unmethylated C residues at any particular stretch of residues in the genomic material of a biological sample. For example, if cytosine (C) residue(s) not typically methylated within a DNA sequence are methylated, it may be referred to as "hypermethylated"; whereas if cytosine (C) residue(s) typically methylated within a DNA sequence are not methylated, it may be referred to as "hypomethylated". Likewise, if the cytosine (C) residue(s) within a DNA sequence (e.g., the DNA from a sample nucleic acid from a

test subject) are methylated as compared to another sequence from a different region or from a different individual (e.g., relative to normal nucleic acid or to the standard nucleic acid of the reference sequence), that sequence is considered hypermethylated compared to the other sequence. Alternatively, if the cytosine (C) residue(s) within a DNA sequence are not methylated as compared to another sequence from a different region or from a different individual, that sequence is considered hypomethylated compared to the other sequence. These sequences are said to be "differentially methylated". Measurement of the levels of differential methylation may be done by a variety of ways known to those skilled in the art. One method is to measure the methylation level of individual interrogated CpG sites determined by the bisulfite sequencing method, as a non-limiting example.

As used herein, a "methylated nucleotide" or a "methylated nucleotide base" refers to the presence of a methyl moiety on a nucleotide base, where the methyl moiety is usually not present in a recognized typical nucleotide base. For example, cytosine in its usual form does not contain a methyl moiety on its pyrimidine ring, but 5-methylcytosine contains a methyl moiety at position 5 of its pyrimidine ring. Therefore, cytosine in its usual form may not be considered a methylated nucleotide and 5-methylcytosine may be considered a methylated nucleotide. In another example, thymine may contain a methyl moiety at position 5 of its pyrimidine ring, however, for purposes herein, thymine may not be considered a methylated nucleotide when present in DNA. Typical nucleotide bases for DNA are thymine, adenine, cytosine and guanine. Typical bases for RNA are uracil, adenine, cytosine and guanine. Correspondingly a "methylation site" is the location in the target gene nucleic acid region where methylation has the possibility of occurring. For example, a location containing CpG is a methylation site wherein the cytosine may or may not be methylated. In particular, the term "methylated nucleotide" refers to nucleotides that carry a methyl group attached to a position of a nucleotide that is accessible for methylation. These methylated nucleotides are usually found in nature and to date, methylated cytosine that occurs mostly in the context of the dinucleotide CpG, but also in the context of CpNpG- and CpNpN-sequences may be considered the most common. In principle, other naturally occurring nucleotides may also be methylated but they will not be taken into consideration with regard to any aspect of the present invention.

In context of the present invention, the terms "methylation profile", "methylation pattern", "methylation state" or "methylation status," are used herein to describe the state, situation or condition of methylation of a genomic sequence, and such terms refer to the characteristics of a DNA segment at a particular genomic locus in relation to methylation. Such characteristics include, but are not limited to, whether any of the cytosine (C) residues within this DNA sequence are methylated, location of methylated C residue(s), percentage of methylated C at any particular stretch of residues, and allelic differences in methylation due to, e.g., difference in the origin of the alleles.

The term "hypermethylation" refers to the average methylation state corresponding to an increased presence of 5-mCyt at one or a plurality of CpG dinucleotides within a DNA sequence of a test DNA sample, relative to the amount of 5-mCyt found at corresponding CpG dinucleotides within a normal control DNA sample. In particular, control refers to a cell with no indication of OS.

The term "hypomethylation" refers to the average methylation state corresponding to a decreased presence of 5-mCyt at one or a plurality of CpG dinucleotides within a DNA sequence of a test DNA sample, relative to the amount of 5-mCyt found at corresponding CpG dinucleotides within a normal control DNA sample. In particular, control refers to a cell with no indication of OS.

- 5 As used herein, the term "genomic material" refers to nucleic acid molecules or fragments of the genome of the subject or group of subjects. In particular, such nucleic acid molecules or fragments are DNA or RNA or hybrids thereof, and most preferably are molecules of the DNA genome of a subject or group of subjects.

- As used herein, the "DNA sample" refers to the DNA extracted from the cell according to any
10 aspect of the present invention using known methods in the art.

- In particular, when there is differential methylation detected in a test cell, that is to say that the cell displays hypermethylation or hypomethylation at, at least one CpG site in comparison to the control (i.e., a cell without indication of OS), then the test cell has OS. More in particular, when the CpG site displays hypomethylation in the test cell in comparison to the corresponding CpG site in the
15 control cell, the test cell has OS. In another example, when the CpG site displays hypermethylation in the test cell in comparison to the corresponding CpG site in the control cell, the test cell has OS.

- In particular, in the method according to any aspect of the present invention, in step (a) the methylation status of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47,
20 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 100 CpG sites are determined. A skilled person would be capable of determining the number of CpG sites that need to be used in step (a) according to any aspect of the present invention. Even more in particular, the methylation status of at least two CpG sites are determined in step (a) of the method
25 according to any aspect of the present invention.

More in particular, in step (a) the CpG site is selected from the list provided in Table 2:

Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position
cg10435849, chr21, 47518747	cg02339482, chr3, 191068318	cg23441673, chr11, 134831429	cg24637319, chr11, 88071195
cg11349857, chr19, 2388952	cg01955286, chr3, 125475060	cg11785465, chr1, 51717051	cg09332835, chr11, 784136
cg00848693, chr16, 69400894	cg23268879, chr7, 5359951	cg01429408, chr1, 156860314	cg22425466, chr9, 140115987
cg21149107, chr14, 71228406	cg01944087, chr15, 69110737	cg02075410, chr4, 151504138	cg18117149, chr6, 41746516
cg02585025, chr16, 74347151	cg06544141, chr1, 27320384	cg17773376, chr3, 107941759	cg15268826, chr4, 132897691
cg18693345, chr5, 2754148	cg20076186, chr3, 128372563	cg12365107, chr3, 52720035	cg23636099, chr2, 114359251
cg25126321, chr11, 66462313	cg20906710, chr12, 50065474	cg23023126, chr19, 308808	cg09788788, chr4, 87468529
cg22155376, chr11, 17591837	cg25319564, chr13, 114214573	cg08721076, chr2, 172967670	cg26163843, chr14, 34944819
cg17251097, chr10, 81838583	cg21598631, chr2, 242154679	cg09325695, chr14, 37825967	cg13864229, chr14, 37051830
cg16098051, chr17, 39663178	cg20393002, chr3, 120909792	cg11434693, chr1, 226349343	cg17942925, chr5, 180611441
cg23153556, chr11, 64900163	cg27626141, chr8, 103876469	cg04110544, chr5, 42424942	cg18659937, chr11, 66669653
cg11271430, chr4, 187984718	cg14075645, chr21, 47552343	cg07758428, chr11, 68193549	cg03611733, chr15, 45585637
cg22545649, chr4, 96470459	cg08351474, chr20, 4666916	cg10811474, chr19, 8428787	cg13514165, chr9, 130544369
cg05736120, chr6, 170125165	cg15047751, chr3, 52395045	cg23807890, chr2, 228736357	cg04360557, chr1, 33282743
cg14417917, chr9, 140065568	cg23247704, chr1, 116518985	cg16112558, chr17, 18157478	cg11578055, chr4, 10447440
cg23526353, chr4, 100484905	cg05791356, chr3, 11578196	cg14751914, chr18, 46477404	cg01283246, chr5, 135266135
cg02884346, chr4, 6271501	cg01728507, chr4, 3563357	cg10853608, chr16, 713101	cg21606780, chr1, 197881327
cg12528713, chr4, 81187605	cg12833948, chr16, 1384648	cg11986082, chr2, 66652819	cg09009011, chr9, 117030773
cg05943476, chr2, 70780904	cg08714407, chr2, 24307071	cg12271199, chr1, 59248280	cg11926460, chr2, 234378272
cg03379894, chr5, 141131868	cg18908499, chr1, 247712237	cg25458727, chr3, 10261761	cg11718315, chr15, 65579480
cg17135325, chr3, 160939158	cg15709169, chr12, 125549783	cg14418275, chr11, 66243281	cg22054008, chr9, 131873921
cg16362899, chr10, 121554998	cg12383788, chr1, 22471748	cg19936912, chr4, 152330012	cg07896133, chr16, 2060022
cg25546329, chr19, 40175243	cg05831191, chr16, 67201012	cg02318866, chr17, 74723064	cg25109721, chr17, 48125064
cg00532451, chr1, 228528913	cg06818207, chr6, 6003238	cg18129198, chr1, 63249693	cg20671801, chr12, 51041328

Table 2. list of 96 differentially methylated CpG sites in OS

The method according to any aspect of the present invention, further comprises the step of:

- (i) performing bisulfite modification to the DNA sample before step (a).
- 5 'Bisulfite treatment' of genomic DNA used interchangeably with the term 'bisulfite modification', refers to the treatment of the genomic DNA with a deaminating agent such as a bisulfite that may be used to treat all DNA, methylated or not. In particular, the term "bisulfite" as used herein encompasses any suitable type of bisulfite, such as sodium bisulfite, or other chemical agents that are capable of chemically converting a cytosine (C) to an uracil (U) without chemically modifying a
- 10 methylated cytosine and therefore can be used to differentially modify a DNA sequence based on the methylation status of the DNA, e.g., U.S. Pat. Pub. US 2010/0112595. As used herein, a reagent that "differentially modifies" methylated or non-methylated DNA encompasses any reagent that modifies methylated and/or unmethylated DNA in a process through which distinguishable products result from methylated and non-methylated DNA, thereby allowing the identification of the
- 15 DNA methylation status. Such processes may include, but are not limited to, chemical reactions (such as a C to U conversion by bisulfite) and enzymatic treatment (such as cleavage by a methylation-dependent endonuclease). Thus, an enzyme that preferentially cleaves or digests methylated DNA is one capable of cleaving or digesting a DNA molecule at a much higher efficiency when the DNA is methylated, whereas an enzyme that preferentially cleaves or digests
- 20 unmethylated DNA exhibits a significantly higher efficiency when the DNA is not methylated.

Accordingly, before step (a) according to any aspect of the present invention is carried out, the genomic DNA contained/ obtained or extracted from the cell, is first bisulfite treated.

An alternative method available in the art may be used instead of bisulfite treatment. A skilled person will understand which other methods to use. In one example, TET-assisted pyridine borane

sequencing (TAPS) may be used for detection of 5mC and 5hmC (Yibin Liu, et al., *Nature Biotechnology*, 37: 424–429 (2019)).

The cell used according to any aspect of the present invention is obtained from a biological sample selected from the group consisting of blood, brain, sperm and any other tissue or sample that
5 provides genomic DNA to be used in the method according to any aspect of the present invention. In particular, the biological sample may comprise any biological material obtained from the subject that contains DNA, and may be liquid, solid or both, may be tissue or bone, or a body fluid such as blood, lymph, etc. In particular, the biological sample useful for the present invention may comprise biological cells or fragments thereof.

10 The term “test” used in conjunction with the term cell herein refers to a cell that is subjected to the method according to any aspect of the present invention and is the basis for an analysis application of the present invention. A ‘test cell’ is therefore a cell or a group of cells being tested according to any aspect of the present invention or a profile being obtained or generated in this context. Conversely, the term “reference” or ‘control’ shall denote, mostly predetermined, entities which are
15 used for a comparison with the test entity. In particular, a ‘test cell’ refers to a cell being tested for OS where the methylation status has to be determined and a ‘control’ refers to a cell without OS where the methylation status is already known and used as a reference.

The OS according to any aspect of the present invention may be caused or may be a result of UV light exposure, ageing, H₂O₂ exposure and a combination thereof (i.e. UV light and H₂O₂ exposure,
20 UV light exposure and ageing, H₂O₂ exposure and ageing or UV light and H₂O₂ exposure and ageing.

According to a further aspect of the present invention, there is provided a method of detecting the incidence of oxidative stress (OS) in a cell, the method comprising detecting an epigenetic change in at least one CpG site in the cell, wherein detection of the epigenetic change is indicative of the
25 incidence of OS and wherein the epigenetic change is methylation.

In particular, the methylation is hypomethylation. DNA hypomethylation profiling may be very useful for stratifying cell cultures systems ranging from 1D to 3D, stem cells to differentiated skin tissue models under stress.

The term ‘epigenetic change’ as used herein refers to a chemical (e.g., methylation) change or
30 protein (e.g., histones) change that takes place to a gene body or a promoter thereof. Through epigenetic changes, environmental factors like diet, stress and prenatal nutrition can make an imprint on genes passed from one generation to the next.

BRIEF DESCRIPTION OF FIGURES

Figure 1A is a scatter plot showing that a large number of probes have a different methylation
35 status in cell with where artificial OS (high UV for 24hrs) was induced according to Example 1. As can be seen, there is an equally large number of probes that are hypomethylated as there are probes hypermethylated.

Figure 1B is a box-plot confirming the results in Figure 1A that a large number of probes have a different methylation status in cell with where artificial OS (high UV for 24hrs) was induced according to Example 1.

Figure 2A is a scatter plot showing that a large number of probes have a different methylation status in cell with where artificial OS (low UV for 72hrs) was induced according to Example 1. As can be seen, there is an equally large number of probes that are hypomethylated as there are probes hypermethylated.

Figure 2B is a box-plot confirming the results in Figure 2A that a large number of probes have a different methylation status in cell with where artificial OS (low UV for 72hrs) was induced according to Example 1.

Figure 3A is a scatter plot showing that a large number of probes have a different methylation status in cell with where artificial OS (low H₂O₂ for 24hrs) was induced according to Example 2. As can be seen, there is a large number of probes that are hypomethylated in cells with OS compared to cells without OS.

Figure 3B is a box-plot confirming the results in Figure 3A that a large number of probes have a different methylation status in cell with where artificial OS (low H₂O₂ for 24hrs) was induced according to Example 2.

Figure 4A is a scatter plot showing that a large number of probes have a different methylation status in cell with where artificial OS (high H₂O₂ for 24hrs) was induced according to Example 2. As can be seen, there is a large number of probes that are hypomethylated in cells with OS compared to cells without OS.

Figure 4B is a box-plot confirming the results in Figure 4A that a large number of probes have a different methylation status in cell with where artificial OS (high H₂O₂ for 24hrs) was induced according to Example 2.

Figure 5A is a scatter plot showing that a large number of probes have a different methylation status in cell with where artificial OS according to Example 3 with Medox® in cells was induced. As can be seen, there is a large number of probes that are hypomethylated in cells with OS compared to cells without OS.

Figure 5B is a box-plot confirming the results in Figure 5A that a large number of probes have a different methylation status in cell with where artificial OS according to Example 3 with Medox® in cells was induced.

EXAMPLES

The foregoing describes preferred embodiments, which, as will be understood by those skilled in the art, may be subject to variations or modifications in design, construction or operation without departing from the scope of the claims. These variations, for instance, are intended to be covered by the scope of the claims.

Example 1*Oxidative Stress on Human Tissue with UV*

Artificial oxidative stress was induced in the cell culture system and skin tissue model to analyze the methylation status of promoters.

- 5 T-Skin models were obtained from Episkin SA, France which is composed of reconstructed human skin. Each skin model consists of a dermal equivalent overlaid by a stratified, well-differentiated epidermis derived from normal human keratinocytes. Upon receiving the skin models, it was recovered by incubating in T-Skin culture medium overnight at 37°C in a 5% CO₂ incubator.

- To induce oxidative stress on a human tissue system, skin models (5x replicates) were exposed to
10 UV radiation (UVA 24 J/cm² + UVB 50mJ/cm²) (high UV) daily for 24 hrs.

In another group, skin models (5x replicates) were exposed to UV radiation (UVA 12 J/cm² + UVB 25mJ/cm² daily) (low UV) daily for 72 hrs.

- Exposure to UV radiation leads to the generation of ROS which finally results in the development of oxidative stress within the cells. A control set of skin models (5x replicates) were maintained for
15 72hrs without any exposure to UV radiation. Followed by the treatment, skin models were collected, and genomic DNA was purified from the tissue samples using the DNeasy® Blood & Tissue Kit (Qiagen). The genomic DNA was quantified using the PicroGreen® or NanoDrop™ 2000.

- The genomic DNA (500ng) from tissue samples were subjected to bisulfite conversion using the EZ
20 DNA Methylation-Gold™ Kit (Zymo Research). The methylation levels were quantified using Infinium MethylationEPIC v2.0 Kit (Illumina) which can analyze over 850,000 methylation sites quantitatively across the genome at single-nucleotide resolution.

Quality control and data processing

- Methylation EPIC array data processing was performed in R version 4.1.2 (2021-11-01) using the
25 minfi version 1.40.0. The raw intensity data (IDAT) were imported into the R (4.1.2), processed using the minfi (1.4.0) Bioconductor package,¹⁸. Quality check on samples was performed to keep probes that have a detection P-value < 0.01 in one or more samples or have a mean detection P-value < 0.05 in all samples. Then samples were normalized using functional normalization (implemented by preprocessFunnorm function in minfi) for type-bias correction and background
30 correction.

- Prior to differential methylation analysis, the probes with non-specific binding, cross reactive probes, probes affected by common SNPs, and probes annotated to the X,Y chromosomes were also filtered out. Beta-value and M-value of normalized and filtered samples were calculated using
35 getBeta and getM function respectively, the samples were then subjected to further downstream analysis.

Differential methylation analysis

Differential methylation analysis was performed using packages limma version 3.50.1 and DMRcate version 2.8.5. Contrast matrix was set up by comparing each corresponding treatment and control group and empirical Bayesian algorithm was used to fit the M-values based on the design and contrast model. Probes with adjusted P-value lower than 0.05 were considered as differentially methylation positions (DMPs). Annotation was performed using IlluminaHumanMethylationEPICanno.ilmn12.hg19 and annotatr package (1.20.0).

As seen in Figures 1A and B and 2A and 2B there are many probes that were differentially methylated in the cell with OS compared to a control cell with no OS. The results also show that there were equally as many hypomethylated probes as hypermethylated probes.

10 Example 2

Oxidative Stress on Human Tissue with H₂O₂

Another way to induce oxidative stress on a human tissue system is through Hydrogen peroxide treatment which leads to the generation of ROS within the cells. The skin models (5x replicates) were treated with two different concentrations of Hydrogen peroxide [100µM (low) and 200µM (high)] for 2hrs and were maintained for 24hrs. A control set of skin models (5x replicates) were maintained for 24hrs without any treatment with Hydrogen peroxide. Followed by the treatment, skin models were collected, and genomic DNA was purified from the tissue samples using the DNeasy® Blood & Tissue Kit (Qiagen). The genomic DNA was quantified using the PicroGreen® or NanoDrop™ 2000.

20 Same method of quality control and data processing as that disclosed in Example 1 was carried out on the samples here. Further, the same differential methylation analysis as disclosed in Example 1 was carried out on the data obtained from Example 2.

As seen in Figures 3A and 3B and 4A and 4B, there are many probes that were differentially methylated in the cell with OS compared to a control cell with no OS. There is a large number of probes that are hypomethylated in cells with OS compared to cells without OS.

Example 3

Oxidative Stress on cell culture system with Medox®

To investigate oxidative stress in the cell culture system, Mesenchymal Stem Cells (MSCs) were treated with Medox (Evonik, Batch:H-080719) which contains a lot of natural anthocyanins, associated with antioxidative and anti-inflammatory properties. Bone marrow derived MSCs were cultured for 1 week in Mesencult ACF Plus Medium with two doses of Medox (4x replicates): 25 µg/ml (low) and 100 µg/ml (high). The media with Medox was replaced every second day for 1 week. As a control (4x replicates), MSCs were cultured for 1 week in Mesencult ACF Plus Medium without any Medox® treatment. Medox® treatment is expected to produce the opposite reaction to OS.

This was followed by collection of cell pellet and genomic DNA was purified from the cell pellet using the DNeasy® Blood & Tissue Kit (Qiagen). The genomic DNA was quantified using the PicroGreen® or NanoDrop™ 2000.

- 5 The genomic DNA (500ng) from the cell pellet was subjected to bisulfite conversion using the EZ DNA Methylation-Gold™ Kit (Zymo Research). The methylation levels were quantified using Infinium MethylationEPIC v2.0 Kit (Illumina) which can analyze over 850,000 methylation sites quantitatively across the genome at single-nucleotide resolution.

- 10 DNA methylation profiling has been proven to be a powerful analytical tool to accurately identify the origin of tissue and the effect of environmental factors. It has several advantages as a biomarker classifier as it is a stable marker, and it can facilitate quantitative analysis at single-nucleotide resolution.

Same method of quality control and data processing as that disclosed in Example 1 was carried out on the samples here. Further, the same differential methylation analysis as disclosed in Example 1 was carried out on the data obtained from Example 3.

- 15 With the low treatment to Medox® on MSCs, 35,532 differentially methylated probes ($p < 0.05$) were identified, out of which 15,368 probes were hypermethylated and 20,164 probes were hypomethylated.

- 20 As seen in Figures 5A and B there are many probes that were differentially methylated in the cell with OS compared to a control cell with no OS. There is a large number of probes that are hypomethylated in cells with OS compared to cells without OS.

Example 4

Oxidative Stress on Human Tissue with UV rays

Artificial oxidative stress was induced in the cell culture system and skin tissue model to analyze the methylation status of promoters.

- 25 T-Skin models were obtained from Episkin SA, France which is composed of reconstructed human skin. Each skin model consists of a dermal equivalent overlaid by a stratified, well-differentiated epidermis derived from normal human keratinocytes. Upon receiving the skin models, it was recovered by incubating in T-Skin culture medium overnight at 37°C in a 5% CO₂ incubator.

- 30 To induce oxidative stress on a human tissue system, skin models (5x replicates) were exposed to UV radiation (UVA 24 J/cm² + UVB 50mJ/cm²) (high UV) and cultured for 24 hrs.

In another group, skin models (5x replicates) were exposed to UV radiation (UVA 12 J/cm² + UVB 25mJ/cm² daily) (low UV) and cultured for 24 hrs.

- 35 Exposure to UV radiation leads to the generation of ROS which finally results in the development of oxidative stress within the cells. A control set of skin models (5x replicates) were maintained for 24hrs without any exposure to UV radiation. Followed by the treatment, skin models were

collected, and genomic DNA was purified from the tissue samples using the DNeasy® Blood & Tissue Kit (Qiagen). The genomic DNA was quantified using the PicroGreen® or NanoDrop™ 2000.

- 5 The genomic DNA (500ng) from tissue samples were subjected to bisulfite conversion using the EZ DNA Methylation-Gold™ Kit (Zymo Research). The methylation levels were quantified using Infinium MethylationEPIC v2.0 Kit (Illumina) which can analyze over 850,000 methylation sites quantitatively across the genome at single-nucleotide resolution.

Oxidative Stress on Human Tissue with H₂O₂

- 10 Another way to induce oxidative stress on a human tissue system is through Hydrogen peroxide treatment which leads to the generation of ROS within the cells. The skin models (5x replicates) were treated with two different concentrations of Hydrogen peroxide [100µM (low) and 200µM (high)] for 2hrs and were maintained for 24hrs. A control set of skin models (5x replicates) were maintained for 24hrs without any treatment with Hydrogen peroxide. Followed by the treatment, skin models were collected, and genomic DNA was purified from the tissue samples using the
- 15 DNeasy® Blood & Tissue Kit (Qiagen). The genomic DNA was quantified using the PicroGreen® or NanoDrop™ 2000.

- The genomic DNA (500ng) from tissue samples were subjected to bisulfite conversion using the EZ DNA Methylation-Gold™ Kit (Zymo Research). The methylation levels were quantified using Infinium MethylationEPIC v2.0 Kit (Illumina) which can analyze over 850,000 methylation sites
- 20 quantitatively across the genome at single-nucleotide resolution.

Oxidative Stress on Human Tissue with Particulate Matter 2.5

- Another way to induce oxidative stress on a human tissue system is through Particulate Matter 2.5 (PM_{2.5}) treatment which leads to the generation of ROS within the cells by its chemical components and metals. The skin models (5x replicates) were treated with two different
- 25 concentrations of PM_{2.5} [15 µg/cm² (low) and 30 µg/cm² (high)] and were maintained for 24hrs. A control set of skin models (5x replicates) were maintained for 24hrs without any treatment with PM_{2.5}. Followed by the treatment, skin models were collected, and genomic DNA was purified from the tissue samples using the DNeasy® Blood & Tissue Kit (Qiagen). The genomic DNA was quantified using the PicroGreen® or NanoDrop™ 2000.

- 30 The genomic DNA (500ng) from tissue samples were subjected to bisulfite conversion using the EZ DNA Methylation-Gold™ Kit (Zymo Research). The methylation levels were quantified using Infinium MethylationEPIC v2.0 Kit (Illumina) which can analyze over 850,000 methylation sites quantitatively across the genome at single-nucleotide resolution.

Oxidative Stress on Human Tissue with Glyoxal

- 35 Another way to induce oxidative stress on a human tissue system is through Glyoxal treatment which provokes oxidative stress by increasing the level of ROS within the cells by producing advanced glycation end-products. The skin models (5x replicates) were treated with two different

concentrations of glyoxal [0.5 mM (low) and 1 mM (high)] and were maintained for 24hrs. A control set of skin models (5x replicates) were maintained for 24hrs without any treatment with glyoxal. Followed by the treatment, skin models were collected, and genomic DNA was purified from the tissue samples using the DNeasy® Blood & Tissue Kit (Qiagen). The genomic DNA was quantified
5 using the PicroGreen® or NanoDrop™ 2000.

The genomic DNA (500ng) from tissue samples were subjected to bisulfite conversion using the EZ DNA Methylation-Gold™ Kit (Zymo Research). The methylation levels were quantified using Infinium MethylationEPIC v2.0 Kit (Illumina) which can analyze over 850,000 methylation sites quantitatively across the genome at single-nucleotide resolution.

10 *Oxidative Stress on Human Tissue with ageing*

Another way to induce oxidative stress on a human tissue system is through ageing which leads to the generation of Reactive oxygen and nitrogen species (RONS) within the cells.

The skin models were maintained in the deep well plate with media 14 days (6x replicates) with media being renewed after 7days to induce ageing in the skin tissue. Skin models were collected
15 after 14 days, and genomic DNA was purified from the tissue samples using the DNeasy® Blood & Tissue Kit (Qiagen). The genomic DNA was quantified using the PicroGreen® or NanoDrop™ 2000.

The genomic DNA (500ng) from tissue samples were subjected to bisulfite conversion using the EZ DNA Methylation-Gold™ Kit (Zymo Research). The methylation levels were quantified using
20 Infinium MethylationEPIC v2.0 Kit (Illumina) which can analyze over 850,000 methylation sites quantitatively across the genome at single-nucleotide resolution.

Quality control and data processing

A total of 103 samples from 16 different treatments and their respective controls were analyzed. The treatments can be grouped into two batches. Batch 1 consisted of all 24 hour treatments, at
25 high and low concentrations. All treatments in batch 1 shared the same control group. Table 1 shows the sample information for batch 1.

Methylation EPIC array data processing was performed in R version 4.2.2 (2021-11-10 r83330) using the minfi version 1.42.0. The raw intensity data (IDAT) were imported into the R (4.2.2), processed using the minfi (1.42.0) Bioconductor package. Quality check on samples were
30 performed to keep probes that had a detection P-value <0.01 in one or more samples or had a mean detection P-value <0.05 in all samples. The samples were then normalized using functional normalization (implemented by preprocessFunnorm function in minfi) for type-bias correction and background correction.

Prior to differential methylation analysis, the probes with non-specific binding, cross reactive
35 probes, probes affected by common SNPs, and probes annotated to the X,Y chromosomes were also filtered out. Beta-value and M-value of normalized and filtered samples were calculated using

getBeta and getM function respectively, the samples were then subjected to further downstream analysis.

Table 1: Sample information for batch 1

Group	Treatment	Replicates
Control	Untreated, 24h	5
Pollution Treatment	Treated with 30 $\mu\text{g}/\text{cm}^2$ particulate matter 2.5 for 24h	5
	Treated with 15 $\mu\text{g}/\text{cm}^2$ particulate matter 2.5 for 24h	5
H ₂ O ₂ Treatment	Treated with systemic exposure to 200 μM H ₂ O ₂ for 2h and cultured for 24h	5
	Treated with systemic exposure to 100 μM H ₂ O ₂ for 2h and cultured for 24h	5
Glyoxal Treatment	Treated with systemic exposure to 1 mM glyoxal 1 mM for 24h	5
	Treated with systemic exposure to 0.5 mM glyoxal 1 mM for 24h	5
UV-radiation Treatment	Exposed to UVA 24 J/cm ² + UVB 50mJ/cm ² and cultured for 24h	5
	Exposed to UVA 12 J/cm ² + UVB 25mJ/cm ² and cultured for 24h	5
Aged	Untreated, cultured for 2 weeks in media	6

5 Differential methylation analysis

Pair-wise differential methylation analysis (total of 16 pairs) was performed using the limma package version 3.52.4 . The batch 1 samples were analyzed together. Contrast matrix was set up by comparing each corresponding treatment and control group and empirical Bayesian algorithm was used to fit the M-values based on the design and contrast model. Probes with adjusted P-value lower than 0.05 were considered as differentially methylation positions (DMPs). After which, the DMPs within the batch 1 comparisons, batch 1 High concentration comparisons, and batch 1 Low concentration comparisons were compared to identify common DMPs that are present in all comparisons within each group and have the same methylation status throughout.

Batch 1 comparisons had a total of 96 common DMPs, Batch 1 High comparisons had a total of 616 common DMPs and Batch 1 Low comparisons had a total of 238 common DMPs. Tables 2,3 and 4 show the list of common DMPs within each group for Batch 1 comparisons, Batch 1 High comparisons, and Batch 1 Low comparisons respectively.

Table 2 List of common DMPs within group for Batch 1 comparisons (i.e. all treatments)

Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position
cg10435849, chr21, 47518747	cg02339482, chr3, 191068318	cg23441673, chr11, 134831429	cg24637319, chr11, 88071195
cg11349857, chr19, 2388952	cg01955286, chr3, 125475060	cg11785465, chr1, 51717051	cg09332835, chr11, 784136
cg00848693, chr16, 69400894	cg23268879, chr7, 5359951	cg01429408, chr1, 156860314	cg22425466, chr9, 140115987
cg21149107, chr14, 71228406	cg01944087, chr15, 69110737	cg02075410, chr4, 151504138	cg18117149, chr6, 41746516
cg02585025, chr16, 74347151	cg06544141, chr1, 27320384	cg17773376, chr3, 107941759	cg15268826, chr4, 132897691
cg18693345, chr5, 2754148	cg20076186, chr3, 128372563	cg12365107, chr3, 52720035	cg23636099, chr2, 114359251
cg25126321, chr11, 66462313	cg20906710, chr12, 50065474	cg23023126, chr19, 308808	cg09788788, chr4, 87468529
cg22155376, chr11, 17591837	cg25319564, chr13, 114214573	cg08721076, chr2, 172967670	cg26163843, chr14, 34944819
cg17251097, chr10, 81838583	cg21598631, chr2, 242154679	cg09325695, chr14, 37825967	cg13864229, chr14, 37051830
cg16098051, chr17, 39663178	cg20393002, chr3, 120909792	cg11434693, chr1, 226349343	cg17942925, chr5, 180611441
cg23153556, chr11, 64900163	cg27626141, chr8, 103876469	cg04110544, chr5, 42424942	cg18659937, chr11, 66669653
cg11271430, chr4, 187984718	cg14075645, chr21, 47552343	cg07758428, chr11, 68193549	cg03611733, chr15, 45585637
cg22545649, chr4, 96470459	cg08351474, chr20, 4666916	cg10811474, chr19, 8428787	cg13514165, chr9, 130544369
cg05736120, chr6, 170125165	cg15047751, chr3, 52395045	cg23807890, chr2, 228736357	cg04360557, chr1, 33282743
cg14417917, chr9, 140065568	cg23247704, chr1, 116518985	cg16112558, chr17, 18157478	cg11578055, chr4, 10447440
cg23526353, chr4, 100484905	cg05791356, chr3, 11578196	cg14751914, chr18, 46477404	cg01283246, chr5, 135266135
cg02884346, chr4, 6271501	cg01728507, chr4, 3563357	cg10853608, chr16, 713101	cg21606780, chr1, 197881327
cg12528713, chr4, 81187605	cg12833948, chr16, 1384648	cg11986082, chr2, 66652819	cg09009011, chr9, 117030773
cg05943476, chr2, 70780904	cg08714407, chr2, 24307071	cg12271199, chr1, 59248280	cg11926460, chr2, 234378272
cg03379894, chr5, 141131868	cg18908499, chr1, 247712237	cg25458727, chr3, 10261761	cg11718315, chr15, 65579480
cg17135325, chr3, 160939158	cg15709169, chr12, 125549783	cg14418275, chr11, 66243281	cg22054008, chr9, 131873921
cg16362899, chr10, 121554998	cg12383788, chr1, 22471748	cg19936912, chr4, 152330012	cg07896133, chr16, 2060022
cg25546329, chr19, 40175243	cg05831191, chr16, 67201012	cg02318866, chr17, 74723064	cg25109721, chr17, 48125064
cg00532451, chr1, 228528913	cg06818207, chr6, 6003238	cg18129198, chr1, 63249693	cg20671801, chr12, 51041328

Table 3 List of common DMPs within group for Batch 1 high comparisons

Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position
cg10435849, chr21, 47518747	cg12350116, chr12, 110035956	cg23153556, chr11, 64900163	cg01955286, chr3, 125475060	cg21674250, chr1, 44877746	cg21333026, chr2, 230715536
cg04542433, chr16, 66460861	cg16098051, chr17, 39663178	cg19254558, chr3, 183602222	cg23268879, chr7, 5359951	cg24819967, chr10, 135190860	cg21221085, chr17, 16939856
cg08524698, chr22, 21213167	cg18155210, chr20, 3223112	cg06905970, chr19, 41222748	cg211334654, chr2, 178128211	cg22501927, chr1, 21583342	cg02668075, chr2, 109558595
cg02759622, chr19, 4831885	cg27401784, chr1, 46974443	cg11271430, chr4, 187984718	cg24078065, chr21, 43372534	cg214355360, chr12, 124586893	cg27598661, chr4, 184404666
cg11349857, chr19, 2388952	cg05718255, chr22, 35790141	cg22545649, chr4, 96470459	cg12330281, chr1, 200708527	cg2798069, chr4, 56412099	cg22415302, chr13, 51796293
cg03297966, chr4, 77818419	cg19641582, chr12, 71003638	cg04454086, chr5, 140345966	cg19412946, chr14, 81637075	cg10959866, chr9, 90245483	cg15709169, chr12, 125549783
cg00848693, chr16, 69400894	cg07146974, chr7, 51275251	cg09253914, chr2, 37311580	cg03982350, chr16, 87184047	cg07973336, chr7, 23348405	cg04529955, chr4, 147442738
cg21149107, chr14, 71228406	cg25546329, chr19, 40175243	cg02948541, chr16, 16427335	cg02264567, chr1, 156863732	cg27649346, chr18, 46303409	cg12383788, chr1, 22471748
cg08521332, chr16, 33965573	cg24119722, chr22, 42347272	cg04964471, chr12, 54769641	cg00681792, chr19, 55677269	cg22407574, chr21, 34960583	cg05831191, chr16, 67201012
cg02585025, chr16, 74347151	cg09194750, chr11, 34937341	cg05736120, chr6, 170125165	cg17341676, chr20, 43991995	cg05137064, chr16, 29814872	cg18634296, chr10, 25305078
cg26071963, chr14, 88945568	cg00532451, chr1, 228528913	cg14010550, chr19, 1009642	cg01973174, chr1, 34411168	cg23247704, chr1, 116518985	cg06580782, chr3, 39093495
cg18761773, chr12, 56498536	cg02339482, chr3, 191068318	cg16454084, chr6, 58147041	cg01944087, chr15, 69110737	cg11980897, chr1, 32237457	cg10909625, chr5, 76370281
cg13544851, chr7, 154002372	cg08351474, chr20, 4666916	cg11032208, chr5, 95997736	cg06544141, chr1, 27320384	cg08739693, chr1, 110165726	cg16452987, chr9, 132959967
cg11807318, chr7, 2566252	cg15047751, chr3, 52395045	cg12277998, chr20, 33999883	cg20076186, chr3, 128372563	cg05368794, chr1, 231374794	cg09047214, chr15, 101688732
cg19705215, chr14, 61747501	cg27297626, chr2, 23785055	cg23631636, chr2, 217363620	cg20906710, chr12, 50065474	cg01065828, chr8, 86089846	cg25554740, chr10, 135047333
cg17177084, chr14, 100604840	cg18895813, chr21, 28339705	cg14417917, chr9, 140065568	cg22253436, chr8, 140659783	cg04093159, chr5, 166211825	cg00023345, chr17, 61518492
cg18693345, chr5, 2754148	cg03609398, chr8, 145913076	cg23526353, chr4, 100484905	cg01528287, chr6, 108010793	cg13865352, chr18, 22006621	cg00460690, chr11, 16909174
cg25317268, chr10, 134258313	cg22879676, chr17, 81009871	cg13715127, chr17, 72856825	cg13999880, chr12, 25802509	cg05791356, chr3, 11578196	cg06818207, chr6, 6003238
cg12405421, chr1, 35222918	cg09469984, chr1, 9796095	cg11313080, chr15, 38794891	cg25319564, chr13, 114214573	cg14867604, chr14, 100438440	cg23441673, chr11, 134831429
cg25126321, chr11, 66462313	cg08714407, chr2, 24307071	cg02884346, chr4, 6271501	cg21598631, chr2, 242154679	cg15246232, chr2, 88991544	cg20311251, chr10, 105218160
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cg08521046, chr2, 86362005	cg18908499, chr1, 247712237	cg05943476, chr2, 70780904	cg24451141, chr18, 13756072	cg24810646, chr10, 131363388	cg00656264, chr16, 87715203
cg01406836, chr13, 42145979	cg17287998, chr5, 59711350	cg14462321, chr9, 137232372	cg20800088, chr15, 47438146	cg08639839, chr1, 149069849	cg03740761, chr20, 47237270
cg22155376, chr11, 17591837	cg02363737, chr6, 116210122	cg03379894, chr5, 141131868	cg19026959, chr19, 13049204	cg12833948, chr16, 1384648	cg14884931, chr22, 42323359
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cg17637107, chr8, 20054583	cg11785465, chr1, 51717051	cg13573073, chr12, 8940075	cg19055951, chr2, 20832097	cg18557054, chr1, 201347529	cg08331513, chr11, 2906981
cg20251110, chr12, 124813301	cg27570984, chr16, 55125644	cg25307468, chr19, 3604804	cg27626141, chr8, 103876469	cg15578811, chr17, 79981292	cg02741216, chr10, 98945465
cg10842775, chr6, 21761442	cg01429408, chr1, 156860314	cg17135325, chr3, 160939158	cg07786657, chr1, 167487633	cg06299882, chr20, 61474428	cg09005159, chr7, 73790765
cg08806153, chr2, 176971961	cg15772361, chr18, 61328223	cg07573061, chr13, 3240690	cg00121148, chr3, 113603874	cg05629982, chr2, 735726792	cg09554774, chr12, 49215832
cg04283403, chr6, 150326126	cg15822010, chr7, 206484	cg16362899, chr10, 121554998	cg14075645, chr21, 47552343	cg11188443, chr20, 54824646	cg04828041, chr4, 40143600
cg17251097, chr10, 81838583	cg20122548, chr15, 45694411	cg01485266, chr19, 2476132	cg08092446, chr19, 58987653	cg03681544, chr8, 144287457	cg09869051, chr1, 109289141
cg16879432, chr19, 33786219	cg17022540, chr1, 2144214	cg04862885, chr11, 1248653	cg07508081, chr1, 161194409	cg04386130, chr3, 156209042	cg22934677, chr14, 54859664
cg15641162, chr4, 185942800	cg05782361, chr2, 54773839	cg04747007, chr19, 7585845	cg04717777, chr1, 16090836	cg01744617, chr3, 58151629	cg10375321, chr16, 132464

Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position
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cg13937996, chr2, 153266209	cg06322988, chr3, 138725189	cg20139425, chr17, 43212881	cg23807890, chr2, 228736357	cg04485850, chr20, 60372565	cg03922095, chr7, 1525238
cg22427191, chr22, 36494224	cg16362030, chr22, 38136754	cg18272518, chr15, 71659231	cg16112558, chr17, 18157478	cg01640506, chr7, 150417635	ch.19.36684304F, chr19, 31992464
cg24930197, chr3, 41240705	cg18118621, chr11, 64011997	cg23023126, chr19, 308808	cg13311494, chr17, 17460905	cg14418275, chr11, 66243281	cg05390516, chr13, 115042160
cg08885316, chr7, 50487054	cg22159364, chr7, 44097635	cg24567198, chr2, 133417634	cg14751914, chr18, 46477404	cg19936912, chr4, 152330012	cg16191143, chr1, 25297315
cg15240033, chr6, 3623985	cg10811474, chr19, 8428787	cg08721076, chr2, 172967670	cg15788149, chr17, 116312273	cg15664967, chr2, 58468431	cg22425466, chr9, 140115987
cg06966413, chr9, 100395677	cg24843474, chr1, 241520598	cg15383980, chr1, 113741483	cg11741902, chr3, 57113245	cg11692435, chr8, 1105844	cg25989749, chr11, 62550245
cg09796800, chr4, 128651518	cg10537820, chr5, 213707	cg25885449, chr18, 77638415	cg18747378, chr6, 31239247	cg02925268, chr12, 132301999	cg18117149, chr6, 41746516
cg11083745, chr5, 149494527	cg20938846, chr19, 50000840	cg05202597, chr1, 21890260	cg01231552, chr9, 133577566	cg09918917, chr17, 33456630	cg13625974, chr22, 20771271
cg04543516, chr20, 21589093	cg17045801, chr7, 43153223	cg05863464, chr8, 142390089	cg10853608, chr16, 713101	cg03617037, chr6, 89980404	cg15268826, chr4, 132897691
cg19683251, chr17, 60762219	cg21249176, chr1, 21998094	cg07134887, chr2, 234664020	cg11986082, chr2, 66652819	cg02318866, chr17, 74723064	cg23951305, chr6, 170581890
cg087743107, chr9, 91933643	cg25054736, chr15, 52577927	cg00750074, chr16, 89608354	cg16280683, chr5, 61708653	cg12311032, chr3, 149687905	cg26284844, chr13, 24829503
cg22056112, chr13, 52707286	cg23710710, chr1, 40723602	cg15461699, chr13, 81278703	cg24862510, chr1, 164290545	cg15241633, chr7, 86415945	cg18346693, chr20, 62286145
cg16156418, chr3, 50404342	cg26840672, chr6, 73867020	cg13387232, chr4, 1387823	cg12271199, chr1, 59248280	cg26488985, chr16, 89584852	cg24689021, chr11, 64043199
cg19859579, chr15, 40074661	cg22396663, chr7, 2107835	cg09325695, chr14, 37825967	cg13099445, chr16, 58529666	cg09583267, chr12, 132349953	cg10911103, chr7, 2082116
cg03698539, chr16, 68012273	cg21499281, chr20, 17600475	cg23230362, chr11, 119060239	cg10314139, chr10, 71560231	cg02746308, chr3, 32827258	cg07588857, chr1, 227163884
cg09055236, chr7, 2673197	cg10188284, chr14, 64505421	cg13750850, chr22, 19879377	cg02547521, chr7, 44147661	cg16088254, chr2, 238662099	cg04373657, chr19, 2800910
cg22304730, chr20, 56287294	cg18795727, chr14, 73065288	cg06150299, chr16, 30993485	cg21454030, chr4, 17812573	cg14525059, chr3, 128485954	cg00573191, chr3, 132246926
cg08826875, chr13, 113366920	cg04146151, chr16, 2155961	cg21103170, chr14, 101521464	cg25431093, chr15, 62982653	cg13323902, chr5, 140090859	cg23636099, chr2, 114359251
cg09291566, chr21, 44891501	cg25116223, chr17, 45940213	cg02045416, chr2, 10355362	cg16964533, chr22, 24647456	cg16252905, chr1, 1150936	cg08111895, chr11, 31531177
cg26156154, chr12, 6587462	cg15148156, chr17, 33415442	cg11434693, chr1, 226349343	cg11697038, chr1, 27931284	cg15660669, chr21, 17102764	cg02347105, chr3, 21557425
cg05603630, chr10, 52751341	cg01519223, chr6, 35351379	cg04944766, chr13, 35516489	cg01451391, chr17, 53343561	cg18129198, chr1, 63249693	cg00796030, chr11, 44608074
cg20605947, chr14, 73727793	cg25648436, chr19, 58554527	cg26904914, chr5, 39425374	cg17504999, chr4, 134072723	cg10161889, chr10, 30331911	cg26798702, chr1, 1373044
cg09590286, chr11, 45275416	cg21730366, chr19, 52097802	cg16670155, chr19, 18557220	cg25458727, chr3, 10261761	cg19502932, chr11, 6640663	cg11504739, chr11, 62186060
cg02075410, chr4, 151504138	cg25995375, chr22, 38347490	cg09419983, chr3, 148847273	cg04901136, chr12, 46384453	cg24637319, chr11, 88071195	cg17436224, chr14, 23299104
cg26061430, chr9, 128193128	cg04899896, chr4, 79860379	cg20900852, chr1, 11723557	cg14839905, chr11, 68183723	cg11800251, chr1, 200116926	cg01443832, chr7, 29725145
ch.1.250064R, chr1, 6704940	cg00212264, chr3, 117988595	cg07124045, chr10, 103595509	cg20775755, chr6, 31660252	cg23452480, chr17, 43921637	cg13225272, chr1, 83729633
cg20121920, chr12, 124833935	cg10217913, chr9, 125026985	cg20582655, chr1, 156390237	cg03255128, chr16, 31227411	cg06364063, chr20, 36795219	cg01711850, chr13, 80726306
cg17193163, chr11, 66056724	cg02789926, chr17, 48464057	cg25084108, chr14, 33668205	cg09012071, chr19, 2171984	cg09332835, chr11, 784136	cg26729372, chr10, 131637200
cg15609471, chr19, 45606565	cg10439889, chr22, 39105684	cg04110544, chr5, 42424942	cg07262320, chr5, 621696	cg24159561, chr3, 32188219	cg22835523, chr15, 85668287
cg17773376, chr3, 107941759	cg26159271, chr2, 47586936	cg25794571, chr13, 29233160	cg27460056, chr7, 1038959	cg06835772, chr16, 85296220	cg19496566, chr19, 48249018
cg19577984, chr14, 80669228	cg14493975, chr11, 63879234	cg25632986, chr16, 67229444	cg27051886, chr22, 42308024	cg24987626, chr9, 137021144	cg02196694, chr8, 60031540
cg12365107, chr3, 52720035	cg20435334, chr1, 1312097	cg07758428, chr11, 68193549	cg07193511, chr11, 64017619	cg18655025, chr14, 91008005	cg06984849, chr16, 2017213

Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position
cg05062179, chr17, 76987630	cg22513229, chr19, 7447757	cg01496536, chr9, 140672566	cg12790440, chr16, 67464868	cg14661811, chr6, 31619002
cg27374486, chr21, 36281988	cg06689990, chr16, 1547486	cg18719814, chr4, 47916640	cg00487727, chr19, 36391260	cg12084866, chr17, 39485627
cg25029315, chr17, 33288471	cg19713585, chr16, 2298791	cg09989011, chr10, 104677906	cg10244978, chr16, 1353946	cg13368085, chr19, 1470810
cg15495741, chr11, 78590580	cg11687406, chr20, 10199434	cg13516768, chr20, 61599125	cg13735608, chr1, 110200767	cg09637723, chr3, 49967744
cg11631579, chr5, 118309902	cg09706428, chr21, 44892086	cg18532480, chr8, 66556501	cg11969813, chr17, 79816559	cg07269567, chr6, 30886161
cg07773052, chr10, 49514483	cg02458396, chr4, 710003	cg10108042, chr16, 1808309	cg08952467, chr13, 50384748	cg18178844, chr6, 32014100
cg21606780, chr1, 197881327	cg15759988, chr8, 144717698	cg23912435, chr1, 150601613	cg15191887, chr5, 664666	cg22535104, chr7, 75300290
cg14250783, chr7, 157722718	cg17885211, chr1, 156479602	cg09394728, chr3, 128615971	cg02247881, chr19, 18599725	cg26251208, chr15, 77933917
cg08963419, chr19, 10402829	cg12492007, chr5, 180023958	cg27264462, chr1, 2106365	cg26582975, chr3, 158520111	ch.11.1112034R, chr11, 58387925
cg03997071, chr22, 25752084	cg17512109, chr11, 124938944	cg02932840, chr10, 28591940	cg00370047, chr11, 1481436	cg08982252, chr17, 73845418
cg16015474, chr6, 33264213	cg17223541, chr4, 3228890	cg09009011, chr9, 117030773	cg23496597, chr20, 57463725	cg20515846, chr10, 134605837
cg153320905, chr12, 114162813	cg18519957, chr1, 203486002	cg05971212, chr16, 30995097	cg07896133, chr16, 2060022	cg211438681, chr9, 138404917
cg20034440, chr10, 124717069	cg11578055, chr4, 10447440	cg16576106, chr16, 4736778	cg03059876, chr20, 61905353	cg15645344, chr4, 165109574
cg06023279, chr18, 12896925	cg11196113, chr4, 13629736	cg15967278, chr8, 29886272	cg00862312, chr17, 79847431	cg06459490, chr12, 124122371
cg23391171, chr13, 41634374	cg01283246, chr5, 135266135	cg02714988, chr4, 85556351	cg07415687, chr6, 7910841	cg02668843, chr17, 40575479
cg22054008, chr9, 131873921	cg23840854, chr1, 161414152	cg13882691, chr16, 75681773	cg25109721, chr17, 48125064	cg21665850, chr2, 731073
cg23832749, chr10, 43608967	cg17208360, chr15, 25483088	cg10034572, chr2, 160921789	cg10062258, chr1, 65885702	cg24814328, chr1, 32104077
cg23381664, chr6, 33281712	cg21179492, chr9, 138393651	cg24126199, chr20, 48771253	cg08379517, chr19, 18509505	cg20671801, chr12, 51041328
cg24352985, chr2, 220116772	cg20094085, chr10, 60936248	cg11926460, chr2, 234378272	cg04588356, chr2, 3473852	cg18463820, chr14, 58619219
cg00904548, chr3, 49720819	cg25201056, chr5, 14997275	cg01444801, chr10, 135216882	cg22330533, chr22, 50173996	cg21362926, chr1, 894026
cg14628323, chr17, 79906610	cg10608004, chr19, 49991389	cg10902252, chr2, 27527527	cg05533001, chr7, 2019608	cg16901014, chr8, 146051423
cg09813248, chr12, 121164194	cg24025896, chr5, 87976294	cg11718315, chr15, 65579480	cg21657580, chr19, 45748941	cg14871650, chr4, 170541668
cg26159074, chr5, 1554307	cg01848616, chr3, 14191828	cg00207893, chr17, 74015638	cg17704839, chr19, 9939038	
cg02469461, chr2, 231589895	cg17249224, chr5, 1257061	cg26062048, chr5, 153418412	cg13016048, chr9, 140054056	
cg03011594, chr16, 56370697	cg19510820, chr11, 107047888	cg04203210, chr16, 2079199	cg194555421, chr7, 30174596	
cg04084170, chr2, 220143147	cg16530128, chr11, 2925951	cg14405625, chr9, 136855902	cg07387931, chr3, 140785160	
cg15966876, chr18, 13824072	cg12146100, chr16, 30712867	cg05288192, chr17, 80893564	cg20968354, chr14, 104688848	
cg11924470, chr1, 151684408	cg25400013, chr11, 108093439	cg18529846, chr14, 23057979	cg06934003, chr12, 106871374	
cg18927317, chr7, 75987610	cg20281040, chr1, 16533063	cg26401541, chr6, 91078974	cg05464720, chr4, 1349298	
cg13796518, chr10, 51827657	cg05557618, chr9, 136326006	cg20847581, chr19, 42746215	cg15277778, chr12, 48169438	
cg27392559, chr17, 27410759	cg05134567, chr19, 45449165	cg17076573, chr2, 241640172	cg19343212, chr10, 73012347	
cg07381470, chr1, 1483130	cg13412213, chr10, 119598364	cg17414431, chr19, 49622715	cg24066474, chr11, 134104369	
cg03914014, chr19, 10420513	cg08130630, chr1, 2312475	cg26635518, chr12, 110232895	cg02996897, chr12, 32832074	

Table 4 List of common DMPs within group for Batch 1 low comparisons

Probe ID, chromosome, positi	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position
cg10435849, chr21, 47518747	cg14417917, chr9, 140065568	cg23807890, chr2, 228736357	cg23268879, chr7, 5359951	cg14696850, chr12, 7585134	cg05276408, chr10, 1994537
cg22155376, chr11, 17591837	cg13073261, chr15, 25201732	cg23659696, chr6, 39083005	cg05791356, chr3, 11578196	cg12335414, chr10, 134359618	cg16112558, chr17, 18157478
cg18693345, chr5, 2754148	cg25106538, chr17, 5116354	cg19388017, chr19, 15285100	cg252321670, chr13, 68682018	cg12079885, chr12, 125328475	ch.6.609614F, chr6, 24693000
cg00848693, chr16, 69400894	cg20671801, chr12, 51041328	cg02742186, chr13, 21100191	cg00720322, chr17, 2154955	cg10416894, chr13, 20437958	cg19086321, chr15, 57842332
cg05424199, chr16, 30759608	cg08714407, chr2, 24307071	cg12365107, chr3, 52720035	cg02318866, chr17, 74723064	cg12499716, chr19, 30096869	
cg07964219, chr21, 46847898	cg07896133, chr16, 2060022	cg24637319, chr11, 88071195	cg00260888, chr20, 30865478	cg25376334, chr3, 71429536	cg22425466, chr9, 140115987
cg06380459, chr17, 78234294	cg01728507, chr4, 3563357	cg00794209, chr8, 255189	cg17251097, chr10, 81838583	cg11101514, chr10, 27180907	cg12078605, chr2, 80531500
cg01558827, chr12, 89746108	cg11986082, chr2, 66652819	cg20393002, chr3, 120909792	cg18882457, chr6, 136247622	cg19936912, chr4, 152330012	cg23526353, chr4, 100484905
cg21392845, chr17, 7227224	cg01690573, chr6, 151771312	cg11926460, chr2, 234378272	cg05474055, chr11, 64611972	cg23441673, chr11, 134831429	cg26079579, chr1, 33896226
cg08188779, chr15, 45004577	cg24432158, chr3, 51892692	cg11761615, chr7, 5348924	cg18321533, chr14, 21162163	cg25319564, chr13, 114214573	cg04731570, chr5, 139487691
cg21149107, chr14, 71228406	cg11349857, chr19, 2388952	cg11683563, chr19, 9435051	cg19496491, chr1, 12695499	cg01283246, chr5, 135266135	cg19524023, chr6, 170592108
cg02884346, chr4, 6271501	cg14418275, chr11, 66243281	cg12271199, chr1, 59248280	cg08721076, chr2, 172967670	cg12833948, chr16, 1384648	cg08700690, chr15, 60884630
cg23237353, chr4, 6712888	cg18129198, chr1, 63249693	cg01089702, chr8, 42552401	cg19289969, chr7, 1892989	cg15006118, chr11, 78387290	cg09325695, chr14, 37825967
cg07744695, chr19, 44229909	cg27336518, chr16, 722515	cg27553048, chr1, 111772613	cg12940822, chr5, 127873397	cg17289308, chr11, 2588628	cg00633933, chr22, 50280082
cg07758428, chr11, 68193549	cg18117149, chr6, 41746516	cg07344096, chr1, 7724122	cg01263854, chr20, 54824316	cg15524632, chr19, 19117770	cg04551967, chr17, 1837764
cg16362899, chr10, 121554998	cg05831191, chr16, 67201012	cg27380530, chr5, 173099221	cg16624187, chr3, 139108582	cg05736120, chr6, 170125165	cg10811474, chr19, 8428787
cg25546329, chr19, 40175243	cg27300841, chr16, 30905180	cg25803700, chr11, 117233980	cg13250001, chr3, 119810597	cg22630823, chr18, 56459606	cg26163843, chr14, 34944819
cg11820257, chr12, 124836411	cg15268826, chr4, 132897691	cg21606780, chr1, 197881327	cg01944087, chr15, 69110737	cg13823617, chr1, 6421969	cg21573582, chr11, 77300763
cg17942925, chr5, 180611441	cg23247704, chr1, 116518985	cg12010548, chr14, 105838058	cg04110544, chr5, 42424942	cg09179999, chr15, 77811681	cg25458727, chr3, 10261761
cg05512065, chr6, 92104129	cg18659937, chr11, 66669653	cg20979652, chr8, 40791285	cg25432518, chr7, 1894300	cg07818050, chr5, 134685323	cg06485995, chr7, 27547647
cg06544141, chr1, 27320384	cg11785465, chr1, 51717051	cg08575696, chr12, 121087382	cg05588111, chr10, 112678594	cg24938752, chr3, 128294769	cg23023126, chr19, 308808
cg02585025, chr16, 74347151	cg25972327, chr8, 94233755	cg26460816, chr5, 65892187	cg10853608, chr16, 713101	cg13514165, chr9, 130544369	cg27106230, chr13, 99229068
cg17930710, chr20, 30678020	cg18855351, chr13, 30920638	cg02339482, chr3, 191068318	cg16098051, chr17, 39663178	cg27464104, chr16, 67926805	cg05870631, chr17, 20450466
cg04929760, chr9, 97562312	cg15399131, chr5, 170293065	cg04819250, chr13, 111089384	cg08351474, chr20, 4666916	cg15635599, chr9, 97020433	cg08987887, chr6, 76311629
cg17125331, chr16, 13861325	cg23685282, chr3, 46205005	cg02075410, chr4, 151504138	cg00763120, chr22, 23637135	cg15047751, chr3, 52395045	cg16335841, chr2, 233246214
cg15635052, chr3, 32433105	cg06754987, chr1, 44444250	cg00661228, chr7, 20166735	cg06753949, chr19, 15334309	cg04640920, chr15, 37180889	cg24549058, chr14, 70006946
cg16090620, chr22, 47197221	cg04360557, chr1, 33282743	cg03352322, chr19, 52693048	cg03757430, chr11, 93269784	cg23847795, chr12, 3736446	cg20892288, chr9, 128003619
cg12163823, chr2, 242673721	cg15973898, chr10, 104121704	cg22678932, chr22, 2206201	cg21067709, chr13, 21714627	cg18864497, chr6, 7313384	cg12194824, chr19, 5691122
cg11271430, chr4, 187984718	cg14751914, chr18, 46477404	cg01955286, chr3, 125475060	cg18154328, chr11, 60609349	cg13724220, chr2, 3383413	cg11578055, chr4, 10447440
cg26140833, chr13, 25861650	cg19131736, chr7, 1902888	cg13864229, chr14, 37051830	cg05321808, chr4, 187647457	cg14322961, chr9, 91789383	cg09788788, chr4, 87468529
cg18908499, chr1, 247712237	cg23636099, chr2, 114359251	cg23325796, chr12, 115442768	cg22545649, chr4, 96470459	cg06533586, chr3, 99630775	cg26201787, chr12, 77157648
cg25927164, chr17, 17685407	cg24784794, chr3, 79068831	cg19539972, chr4, 7069911	cg09423221, chr3, 78078946	cg03611733, chr15, 45585637	cg21594328, chr2, 206830726
cg16083044, chr6, 168788815	cg09009011, chr9, 117030773	cg22054008, chr9, 131873921	cg17869339, chr1, 148897999	cg12197666, chr13, 40511553	cg01429408, chr1, 156860314

CLAIMS

1. A method of identifying oxidative stress (OS) in a test cell, comprising
 - (a) determining the methylation status of at least one CpG site in a DNA sample obtained from the test cell,
 - 5 (b) comparing the methylation status of the CpG site from (a) with that of a control without OS,

wherein difference in the methylation status of the CpG site in the test cell compared to the CpG site in the control is indicative of the test cell having OS.
2. The method according to claim 1, wherein the difference in methylation status is
 - 10 hypomethylation or hypermethylation of the CpG site in the test cell and the hypomethylation or hypermethylation of the CpG site is indicative of OS in the test cell.
3. The method according to either claim 1 or 2, wherein the CpG site is selected from the list of CpG sites in the table below:

Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position	Probe ID, chromosome, position
cg10435849, chr21, 47518747	cg02339482, chr3, 191068318	cg23441673, chr11, 134831429	cg24637319, chr11, 88071195
cg11349857, chr19, 2388952	cg01955286, chr3, 125475060	cg11785465, chr1, 51717051	cg09332835, chr11, 784136
cg00848693, chr16, 69400894	cg23268879, chr7, 5359951	cg01429408, chr1, 156860314	cg22425466, chr9, 140115987
cg21149107, chr14, 71228406	cg01944087, chr15, 69110737	cg02075410, chr4, 151504138	cg18117149, chr6, 41746516
cg02585025, chr16, 74347151	cg06544141, chr1, 27320384	cg17773376, chr3, 107941759	cg15268826, chr4, 132897691
cg18693345, chr5, 2754148	cg20076186, chr3, 128372563	cg12365107, chr3, 52720035	cg23636099, chr2, 114359251
cg25126321, chr11, 66462313	cg20906710, chr12, 50065474	cg23023126, chr19, 308808	cg09788788, chr4, 87468529
cg22155376, chr11, 17591837	cg25319564, chr13, 114214573	cg08721076, chr2, 172967670	cg26163843, chr14, 34944819
cg17251097, chr10, 81838583	cg21598631, chr2, 242154679	cg09325695, chr14, 37825967	cg13864229, chr14, 37051830
cg16098051, chr17, 39663178	cg20393002, chr3, 120909792	cg11434693, chr1, 226349343	cg17942925, chr5, 180611441
cg23153556, chr11, 64900163	cg27626141, chr8, 103876469	cg04110544, chr5, 42424942	cg18659937, chr11, 66669653
cg11271430, chr4, 187984718	cg14075645, chr21, 47552343	cg07758428, chr11, 68193549	cg03611733, chr15, 45585637
cg22545649, chr4, 96470459	cg08351474, chr20, 4666916	cg10811474, chr19, 8428787	cg13514165, chr9, 130544369
cg05736120, chr6, 170125165	cg15047751, chr3, 52395045	cg23807890, chr2, 228736357	cg04360557, chr1, 33282743
cg14417917, chr9, 140065568	cg23247704, chr1, 116518985	cg16112558, chr17, 18157478	cg11578055, chr4, 10447440
cg23526353, chr4, 100484905	cg05791356, chr3, 11578196	cg14751914, chr18, 46477404	cg01283246, chr5, 135266135
cg02884346, chr4, 6271501	cg01728507, chr4, 3563357	cg10853608, chr16, 713101	cg21606780, chr1, 197881327
cg12528713, chr4, 81187605	cg12833948, chr16, 1384648	cg11986082, chr2, 66652819	cg09009011, chr9, 117030773
cg05943476, chr2, 70780904	cg08714407, chr2, 24307071	cg12271199, chr1, 59248280	cg11926460, chr2, 234378272
cg03379894, chr5, 141131868	cg18908499, chr1, 247712237	cg25458727, chr3, 10261761	cg11718315, chr15, 65579480
cg17135325, chr3, 160939158	cg15709169, chr12, 125549783	cg14418275, chr11, 66243281	cg22054008, chr9, 131873921
cg16362899, chr10, 121554998	cg12383788, chr1, 22471748	cg19936912, chr4, 152330012	cg07896133, chr16, 2060022
cg25546329, chr19, 40175243	cg05831191, chr16, 67201012	cg02318866, chr17, 74723064	cg25109721, chr17, 48125064
cg00532451, chr1, 228528913	cg06818207, chr6, 6003238	cg18129198, chr1, 63249693	cg20671801, chr12, 51041328

- 15 4. The method according to any one of the preceding claims, wherein in step (a) the methylation status of at least 2 CpG sites are determined.
5. The method according to any one of the preceding claims, wherein in step (a) the
 - 20 methylation status of at least 3 CpG sites are determined.
6. The method according to any one of the preceding claims, wherein in step (a) the methylation status of at least 5 CpG sites are determined.

7. The method according to claim 3, wherein in step (a) the methylation status of all the CpG sites listed is determined.
8. The method according to any one of the preceding claims, further comprising the step of:
5 (i) performing bisulfite modification to the DNA sample before step (a).
9. The method according to any one of the preceding claims, wherein the cell is obtained from a biological sample selected from the group consisting of blood, brain, sperm and any other tissue or sample that provides genomic DNA.
10
10. The method according to any one of the preceding claims wherein the cell is a eukaryote.
11. The method according to any one of the preceding claims, wherein the cell is from a mammal.
15
12. The method according to claim 11, wherein the mammal is a mouse, a rat, a guinea pig, a dog, a mini-pig, a human being, a cow, a sheep, a pig, a goat, a horse, a donkey, and a mule.
- 20 13. The method according to any one of the preceding claims, wherein the cell is a skin cell, a stem cell or a cell derived therefrom.