THE EPIGENOMIC ANALYSIS OF HUMAN OBESITY

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ABSTRACT

The epigenome - the chemical modifications and packaging of the genome that can influence or indicate its activity - gives a molecular insight to cell-type specific activity. It can, therefore, reveal the pathophysiological mechanisms at work in disease. Detected changes can also represent physiological responses to adverse environmental exposures, thus enabling the epigenetic mark of DNA methylation to act as an epidemiological biomarker, even in surrogate tissue.

This makes epigenomic analysis an attractive prospect to further understand the pathobiology and epidemiological aspects of obesity. Furthermore, integrating epigenomic data with known obesity-associated common genetic variation can aid in deciphering their molecular mechanisms.

This review primarily examines epidemiological or population-based studies of epigenetic modifications in relation to adiposity-traits, as opposed to animal or cell models. It discusses recent work exploring the epigenome with respect to human obesity, which to date have predominately been array-based studies of DNA methylation in peripheral blood. It is of note that highly replicated BMI DNA methylation associations are not causal, but strongly driven by co-associations for more precisely measured intertwined outcomes and factors, such as hyperlipidaemia, hyperglycaemia and inflammation. Finally, the potential for the future exploration of the epigenome in obesity and related disorders will be considered.

INTRODUCTION

Epigenetic factors include the chemical modifications of DNA and the proteins that the DNA wraps around, which influence or may indicate the activity of genes. Deciphering these marks can be a powerful method to understand the specialised functioning of a cell and organs (1). Furthermore, they may inform how molecular mechanisms are impeded in disease or due to adverse environments.

Obesity rates have increased at such a magnitude that this clearly points towards non-genetic, or environmental factors, as the significant driver. Identified genetic susceptibilities are also modulated by these external influences (2, 3). This has led to considerable interest in the study of the epigenome with the potential to quantify gene activity changes due to this environment, as well as the possibility of gaining molecular understanding of the pathophysiological consequences of the obese state. Crucial biological understanding of obesity and its consequences are still lacking (4, 5) and would help focus vital preventative measures to counter obesity's substantial morbidity and mortality risk (6).

THE EPIGENOME

DNA is packaged at the strand level by wrapping around Histone proteins that comprise of 8 subunits, two of each of Histone 2A, 2B, 3 and 4. The histone proteins possess tails that can be post-translationally modified, including the addition of methyl or acetyl groups to Lysine (K) molecules on histone 3 (H3). These additions can be related to active or repressed regions depending upon which K is modified. Amino acid deviation in the protein structure of these histone molecules, termed Histone Variants, also modify activity and can as well be associated with disease processes (7).

Chemical modifications of DNA itself include the most common, stable and well-studied, DNA methylation, or the addition of a methyl group onto the 5' carbon of cytosine. This robust chemical mark is due to the strong covalent carbon-to-carbon bond that connects cytosine to the methyl group. In differentiated cells this occurs in the DNA sequence predominately

within the context of a CpG dinucleotide, that is where Guanine follows Cytosine in the 5' to 3' direction on one DNA strand. It acts canonically as a repressive mechanism within gene promoters. However, additional DNA modifications occur, and these are in fact progressive oxidative products of the active DNA demethylation process, driven by the TET enzymes, leading firstly to hydroxymethylcytosine (5hmC), formylcytosine (5fC) and then carboxycytosine (5caC). This final product is then recognised by the base-excision pathway to enable return to an unmodified cytosine (8). These less common modifications may also in themselves possess active roles, with 5hmC implicated functionally within enhancers (9).

High-throughput analysis of the epigenome is focused now on DNA methylation, particularly because of its stability in extracted DNA in comparison to other epigenetic marks, such as histone modifications. However, due to its biological plasticity it is an attractive biomarker with strong potential clinical utility (10).

CONSIDERATIONS IN THE ANALYSIS OF THE EPIGENOME

To powerfully examine the epigenome, especially in a human population setting (11), it is necessary to be clear how this mechanism contrasts from the genome. This understanding impacts significantly on design, analysis and interpretation. The differences can be broadly encapsulated in three principles: i) cell-type specificity ii) changeability and iii) sequence interactive (or positional) effects.

Firstly, as its function dictates, the epigenome is tissue or cell-type specific. Therefore, we wish, if at all possible, to be examining the cell-type(s) that are most relevant to the pathophysiology of the disease. We can only interpret any associated findings in tissue that is not the primary site of action as a surrogate measure for markers of exposure, which may also act on the organ of disease, or downstream physiological changes due to these exposures or disease outcomes. In the analysis of obesity this is not straightforward, as the intrinsic genetic predisposition is governed largely within the brain (12), with central control for energy balance directed by specific hypothalamic neurons, particularly within the arcuate nucleus (13).

Thus, these cells are inaccessible except in post-mortem samples. Adipose tissue is also an obvious focal interest for the dissection of obesity, with the physical and functional changes that occur to it and its role as an endocrine organ (5).

Secondly, the epigenome is changeable over time. This is most dramatic during development, where two rounds of epigenomic reprogramming occur, firstly in gamete formation and then secondly post-fertilisation. These are both characterised by global DNA demethylation (14). However, the epigenome is not static from this time-point. It is seen to suffer from 'epigenetic drift' with age, whereby hypomethylated regions gain and hypermethylated regions lose methylation over time stochastically throughout the genome (15). This process is proposed to be involved in age-related deterioration in function, and the concurrent increased risk of chronic diseases, such as obesity-related conditions. Though, on top of this are specific directional ageing changes that can be identified at defined functional loci across the genome (16, 17).

Thirdly, positional effects driven through sequence variation will be reflected in the measured epigenome. These can be direct, in *cis*, or *trans*, and can bedevil the analysis in human population samples, particularly across diverse ancestries (11) (Figure 1). Direct effects with respect to DNA methylation can be due to genetic polymorphism at the CpG dinucleotide itself. In fact, almost ~⅓ of SNPs occur at CpG locations, because of the hypermutability of methylated cytosines, and this contributes considerably to allelic variation in the DNA methylome (18, 19).

The observed epigenetic state may represent the activity or repression of local *cis*-regulatory elements (CREs) (usually 100-1000 bp in length) (20). Genetic variability within these regions can led to fixed obligatory epigenetic positions or facilitate epigenetic variation (21). Genetic effects on the methylome via transcription factors (TFs) has been known for decades, such as the role of SP1 within CpG islands (22). Motif changes in this and other Methylation Determining Regions (MDR), such as those for CTCF and RFX, give rise directly to methylation variation in CpG dense regions (23). TFs binding to transcription factor binding sites (TFBS) can also drive down DNA methylation (24) at distal regulatory regions and thus genetic variation in these binding

sites will also be influential. Some TFs are explicitly sensitive to DNA methylation (25) and some, in fact, require it in order to bind (26). Also SNPs affecting the expression of *trans* factors will impact on the epigenetic state within their distal TFBS or CRE regions (27). Genetic influence on the epigenome is in fact so strong that it can be observed in enhancer variation in only 19 individuals of diverse ancestry (28).

Nearby positional effects such as polymorphic insertions of often heavily methylated retrotransposons will influence the local region (29). The density of CpGs also has a direct relationship with the DNA methylome (30), so CpG-SNPs (SNPs creating or abrogating a CpG dinucleotide (18, 31)) as well as affecting available methyl-C containing motifs, can impact on the rate of change of CpG density slopes. The influence of SNPs as mQTL (methylation QTL) (32), can capture impact upon *cis* or *trans* factors, the DNA methylation machinery, or haplotypic effects, such as regional density or proximity factors. Genetically-associated correlated CpGs can be observed to cluster, which have been termed 'GeMes' (33). Finally, the epigenome is a coordinated mechanism so the epigenetic layers should fit logically together, such that a DNA hypermethylated promoter should show lack of co-locating activating chromatin marks and vice versa (34).

CONFOUNDING ISSUES

All the above effects, tissue-specificity, changeability, and sequence interaction, can therefore confound or misattribute action or effect in epigenomic studies. To mitigate against these, rigorous study designs have been proposed and employed. However, due to practical, technical and cost considerations, it is often not possible perform the ideal study, but to compromise, acknowledge and work within these limitations (35, 36).

Isolating disease-relevant tissue cell-types for analysis is an obvious first starting point. However, many large epidemiological studies have DNA derived from peripheral blood available, with the positive that these are often sizeable numbers and possess deep phenotyping. Whilst these are the practicalities, an argument may be made that peripheral blood is of interest physiologically in obesity due to known inflammatory related changes (37) or

for detection of passive exposure biomarkers. Furthermore, the potential clinical utility of findings in blood is significant due to its comparative ease of access compared to fat-tissue biopsy. However, these DNA methylation differences will be unlikely to correlate with other target tissues. Postmortem samples or tissue biopsies are required to gain further insights into underlying mechanisms.

The additional factor regarding peripheral blood analysis is that it comprises of the various leukocyte cell-types and as such represents a meta-epigenome of these subsets contained (38). This mixture needs to be quantified and even in normal conditions these vary between individuals due to many factors including sex and genetic background. Thus, DNA methylation profiling in blood will strongly identify signals driven by sub-cell composition changes due to the disease state or immune responses associated with it. Whilst isolation of the most disease relevant cell-type is preferable (39), deconvolution algorithms have been devised using Differentially Methylated Positions (DMPs) that estimate leukocyte cell-type proportions (40). In fact, due to the cell-type specificity of epigenomes, once accurately delineated, this deconvolution is set to become one of the most powerful epigenomic tools. The mapping of these cell and tissue types is beginning through efforts such as the Epigenomics Roadmap (41), Blueprint (42) and future cell-type defining surveys (43). Kim *et al.* have recently shown that an expanded set of reference leukocytes, with pathologically important cell fractions comprising immature, memory plasma B cells, activated Natural killer cells and naïve T cells, can explain significantly more variability in peripheral blood methylomes (44). Additionally, these data improved case and control discrimination in immune-related disorders, including the metabolic syndrome.

Changeability in developed tissues encapsulates two often-intersecting mechanisms. Firstly, significant directional changes with time in proportions of a heterogeneous collection of cells, and secondly, the activation or representation of certain pathways. Relative change is seen clearly, as mentioned, in peripheral blood, with responses to acute infection or even chronic low-grade inflammation (45). Other significant proportional changes occur due to ageing, with a skew towards a greater myeloid cell

fraction in blood (46), but also a reduced ratio of neurons to glia in the brain (47). However, in many studies the phenotype of interest may alter the proportional composition of the cell types within the analysed target tissue (48). In fact, in some cases, correction for cell-type may impede the identification of cell-type specific modifications (49).

Finally, the influence of genetic factors on the epigenome is strong. Therefore considerable power comes from longitudinal studies (35), or monozygotic (MZ) or identical twin discordant analyses, to remove these significant influences (50, 51). In high-throughput DNA methylation array analysis the influence of genetic effects can be driven by SNPs at the investigated CpGs themselves, under the probes, or nearby (Figure 1). Drastic effects led to discrete genotypic grouping or "gap signals", with 11,007 seen in a recent Illumina Infinium Human Methylation450 bead chip (450k array) analysis (52). Previous 450k array analysis has found population genetic variation attributable in 66,877 probes (13.8%) (53). Chen *et al*. observed recently this strong genetic influence in a study of genetic drivers of epigenetic variation in human immune cells and it was noted to be a concern for EWAS interpretation (54). More complex haplotyptic effects may also exist, with strong consequences captured by "gap signals", though more subtle outcomes will converge. Although how these genetic influences cannot only confound studies, but also may facilitate functional variation, should also be borne in mind. CpG-SNPs are proposed to play a significant facilitative epigenetic role and these effects were recently seen be to enriched within GWAS regions in a number of studies, including Type 2 Diabetes (55-57)

CELL-TYPE SPECIFIC SIGNALS IN PERIPHERAL BLOOD

Dissecting the strong DNA methylation signals that can be identified with tobacco smoking in peripheral blood starts to reveal the biological intricacies of epigenetic changes in this heterogeneous tissue. Understanding these robust results is very useful in interpreting what may be possible in DNA methylation studies of obesity and other diseases. There are distinct results dependent upon which cellular subset is examined (58). The strongest

tobacco-associated signal in the gene *AHRR* appears so conspicuously as the epigenetic change occurs specifically within granulocytes and monocytes and is then amplified by the fact that smoking itself increases the proportion of granulocytes in peripheral blood. By contrast tobacco has little impact on *AHRR* T cell DNA methylation. In comparison, lymphoid-specific inflammatory changes, such as those seen in *GPR15*, where DNA methylation changes are only seen in T and B cells (58, 59). Biologically these changes are likely inflammatory responses driven by smoking injured tissues. On the myeloid side, monocyte to macrophage differentiation is influenced, as well as a proposed carbon monoxide environmental selection on bone marrow progenitor cells (58). Thus, the identification of these subtype specific signals enable novel hypotheses and mechanism to be proposed and explored.

OBESITY-INDUCED CHANGES IN FAT AND BLOOD

Fat tissue is not a homogenous organ. The adipocytes of subcutaneous fat are mostly white adipocytes, due to triglyceride storage, which are leptin and adipokine-secreting. Additionally, there are small but discernable levels of brown and beige fat (5). The role of brown fat in humans has gained significant interest due to its energy-dissipation properties and fat burning actions via uncoupling protein 1–containing mitochondria. Stimulation by the sympathetic nervous system after cold exposure leads to heat production. Beige, or brown-to-white thermogenic adipocytes, have a progenitor cell origin and conversion can be induced by cold temperatures, exercise and endocrine factors (5).

In obesity, adipose tissue becomes a large immunologically active endocrine organ (60). Infiltration by macrophages and other inflammatory immune cells occurs, partly due to obesity-related apoptosis of adipose cells (60). This leads to chronic adipose tissue inflammation (61) with the secreted proinflammatory cytokines contributing to the insulin resistant state that arises (62). Visceral fat deposits, including the omentum and mesenteric fat, are more detrimental than subcutaneous (63) and are associated with many of the systematic metabolic consequences of obesity (5).

Obesity induces changes in innate immune cells, but also increases in $CD4^+$ and $CD8^+$ T cells and reduces tolerance-promoting regulatory B cells as well as inducing abnormal B cell function (64). There is a proposed role in obesity of lymphoblast-derived Natural Killer (NK) cells, which are shown to be significant regulators of macrophage polarization and insulin resistance (65), and their methylome is remodelled in conversion from naive to activated cells (66). Chronic low-grade inflammation is associated with obesity, and specific DNA methylation changes are observed, due to this state, assessed via C-reactive protein levels (CRP), within peripheral blood (45).

ANALYSIS OF THE EPIGENOME IN OBESITY

Studies of obesity epigenetics up to the beginning of this decade were predominately either targeted candidate genes or total measures of the epigenome, such as global DNA methylation (67). The later can be useful in gross abnormalities, such as cancer, when significant hypomethylation is strong enough to drive global variation. Though it is far less useful in more nuanced phenotypes and has not lead to consistent findings (68). With the former, as was the experience in complex trait genetics, the study of candidate genes has not been particularly fruitful, with weak and again inconsistent effects. In the exploration of imprinting genes or those involved in metabolism, few (if any) have been supported by subsequent genome-wide, more powered and confounding-aware studies (68). Many early studies therefore need reassessing in the light of current findings and knowledge.

All DNA methylation studies prior to at least 2012 are likely to have not taken the confounding effects of cell-type proportions into account. Furthermore, many of these studies are in small numbers, with added potential issues due to genetic heterogeneity. Whilst stronger effect size epigenetic associations can be found for phenotypic traits or complex diseases than genetic associations (69), lack of subsequent replication point to their weakness (68).

EPIGENOME-WIDE ASSOCIATION STUDIES FOR OBESITY (BMI) IN PERIPHERAL BLOOD

In 2014 Dick *et al*. published one of the first large Epigenome-wide Association Study (EWAS) for BMI using the 450k array (70). This was performed in whole blood derived DNA in European ancestry individuals. The initial discovery set was 479 individuals, followed by two rounds of replication, firstly in 339 and then secondly in 1,789 samples. The discovery set identified 5 CpGs across 3 genetic loci associated with BMI. Three CpGs replicated and these all resided in the first intron of *HIF3A*, the Hypoxia Inducible Factor 3 Alpha Subunit gene, involved in regulating hypoxia-inducible gene expression (cg22891070, cg27146050, and cg16672562). Potential lymphocyte cell-type effects on cg22891070 were tested for, after the initial analysis. Whilst a small association with leukocyte number was seen, adjustment for subtypes did not substantially reduce the BMI association.

Then to investigate *HIF3A* across different tissues, DNA from adipose tissue (n=635) and skin (n=395), was assessed, with significant methylation changes identified in adipose only. One driver of this may be the high level of inflammatory blood cell invasion into adipose tissue. Genetic effects were explored and 2 SNPs (rs8102595 & rs3826795) were both independently associated with cg22891070's DNA methylation state in all datasets, although were not themselves significantly associated with BMI.

As well as being replicated in a number of studies (71-73), including in adipose tissue (74), this result was also explored for causality in the ALSPAC cohort from Bristol, U.K. (75). Genetic and multiple time point DNA methylation data was available in □1,000 mother-offspring pairs. The DNA methylation changes were proposed to be secondary to differences in BMI, through examination of the temporal relationship of changes in sequential data, not causal, which was also supported by Mendelian Randomisation (MR) analysis. In further analysis by Main *et al.* a relatively high level of familiarity (h^2 51-64%) for *HIF3A* DNA methylation in blood was observed (76), in fact a similar level to obesity itself. Epidemiological evidence has also pointed at vitamin B2 and B12 levels influencing *HIF3A* epigenetic levels (73).

Another BMI EWAS analysis with the 450k array in peripheral blood DNA was performed in 2,097 African American adults in the Atherosclerosis Risk in Communities (ARIC) study (72). A similarly sized replication set of 2,377 European ancestry derived individuals from the Framingham Heart Study was employed, as well as isolated $CD4^+$ T cell DNA from 991 European ancestry individuals (Genetics of Lipid Lowering Drugs and Diet Network Study). This enabled 37 DMPs to be robustly identified with BMI and an additional DMP specifically associated with Waist Circumference only. 16 DMPs were also seen to be consistent in 648 adipose tissue samples. Novel results included *LGALS3BP*, *KDM2B*, *PBX1* and *BBS2*, and genes implicated in lipid metabolism, cytokine signalling and immune response.

In a recent study Wahl *et al.* analysed a collection of large GWAS cohorts with the 450k array in peripheral blood (77). The discovery cohort contained a mix of South East Asian (n=2,680) and European (n=2,707) ancestry individuals and identified 278 BMI-associated CpGs residing in 207 genetic loci whilst correcting for leukocyte cell-type proportions. Taking forward the strongest individual CpG associations within these regions to an additional 4,874 samples, 187 out of 207 replicated at a significance level of $p < 0.05$. The results did not vary significantly across differing ancestry groupings, except for seven DMPs where very strong population variation between Asian and European was seen (heterogeneity $p < 1 \times 10^{-7}$), hinting at population-specific or uncaptured genetic effects. All the changes were identified to be the potential consequence of obesity not causative, except for 1 CpG (cg26663590) in *NFATC2IP*. These results were strongly enriched for methylation scores within an intermediate level of 20-80%. This could point towards variation between certain cell subsets driving the signal, although analysis of 4 sub-fractions, monocytes, neutrophils, $CD4^+$ and $CD8^+$ T cells indicated that these results could in fact be seen across all these cell-types. Thus they differ from the biologically defined tobacco smoking results where cell-type specific divergent myeloid versus lymphoid signals could be found (58). A longitudinal sample set of 1,435 participants was evaluated over a period of seven years, showing a consistent relationship between change in methylation and change in BMI overtime in 178 of 187 CpGs.

Another large-scale BMI EWAS was recently reported by Mendelson *et al*., again using the 450k array in peripheral blood (78). The discovery set was in those of predominately European ancestry, including individuals from the Lothian Birth Cohort in Scotland and the US Framingham Heart Study. 135 CpGs were initially identified as BMI-DMPs. 83 DMPs replicated in at least one of the 3 additional replication cohorts, ARIC (n = 2,096), GOLDN (n = 992), and PIVUS (n = 967).

Gene ontology enrichment was identified for lipid metabolism in those DNA methylation variation associated genes that also had concordant expression changes. 77 of the CpGs that were non-redundant (*i.e*. correlation $|r| < 0.7$) captured ~18% of the inter-individual variation in BMI. The previous *HIF3A* result replicated, with the strongest association seen in the youngest subset (cg22891070, $p = 0.003$). Just 11 CpGs revealed a recognised three-way association whereby the DNA methylation was associated with BMI and expression, as well as BMI–associated expression changes. These included *ABCG1*, *CACNA2D3*, *CPT1A (79)*, *DHCR24*, *SARS*, *SLC1A5* and *SREBF1*. No enrichment for blood or adipose-specific regulatory features was seen using eFORGE (80) to detect tissue-specificity of the DMPs through co-localisation with tissue-specific DNase-I Hypersensitivity Sites. DMPs were more enriched in enhancer regions (H3Kme1) than promoter, although this may indicate the less dynamic nature of these promoter CpGs (81). The top cis-mQTL for the replicated BMI-DMPs, selected by lowest *p*-value ±500 kb from the CpG, was not strongly attenuated by BMI association in 81 of the 83 CpGs. One gene, *SREBF1* (Sterol regulatory element-binding transcription factor 1), a lipid metabolism transcription factor was proposed as possibly causal. It is known to induce conversion of acetyl-CoA to triglycerides thus promoting glycolysis, lipogenesis and adipogenesis. Also, a role in adiposity, insulin resistance, coronary artery disease, obesity-related dyslipidaemia is supported in model organism and human studies (78).

A comparison of the results from these three recent large EWAS studies (72, 77, 78) identified ten CpGs that were robust and replicated within each as well as being common to all (See Table 1). These ten CpGs are located in ten unique genes, predominately within intragenic intronic loci and in almost half within CpG island shores. They also are generally supported in other BMI-

related studies (70, 82-85). Gene ontology enrichment analysis via GREAT for these ten CpGs (compared with the 450k array probes as background) identified nominal significance of biological processes, such as regulation of cholesterol and lipids, human phenotypes including hyperlipidemia, and diseases of the hepatobilary system (Supplementary Table S1).

In fact, many of these same CpGs are associated with lipid-related traits from specific EWAS's performed for these measures, assumedly due to their relationship to the development of adiposity or downstream-induced epigenetic changes from BMI-associated altered blood lipid profiles. Four of the ten DMPs are significantly related to triglyceride (TG) levels in a recent study by Dekkers *et al*. (49), as well as in earlier and contemporary lipid studies (79, 86-89). Furthermore, four CpGs are related to glucose-related phenotypes in a study of Kriebel *et al*. (90). This clearly shows the power of EWAS to identify more precise biochemical phenotypes (38) and the benefit of closely examining the distinct biological changes associated with broad epidemiological measures, such as BMI. A number of these exact CpGs also are supported by additional studies in or related to BMI, such as an analyses in Arabs (84), Metabolic Syndrome (83), and hypertriglyceridemic waist in Mexican Americans (91). Of interest is that one of the CpGs, cg06192883, in *MYO5C*, was recently identified in an EWAS for the inflammatory marker CRP (45). Another, cg09349128, in *CRELD2*, is associated with Inflammatory Bowel Disease (92), which is known itself to be connected to lipid abnormalities (93). Two additional genes, *LGALS3BP* (Lectin galactoside-binding soluble 3 binding protein) and *SBNO2* (strawberry notch homolog 2), were consistently identified across all 3 studies but represented by differing CpGs.

EWAS ANALYSIS IN ADIPOSE TISSUE

Agha *et al.* analysed adipose tissue with the 450k array and associated measures of adiposity obtained by dual-energy X-ray absorptiometry-assessed android fat mass, android:gynoid fat ratio and trunk:limb fat ratio, as well as BMI (71). This was in 106 individuals (64% women, 68% white) after adjusting for smoking, race, and sex as well as reference-free adipose cell-mixture effects. A gene focused analysis identified association with these

adiposity traits including *ANGPT2*, *AOC3*, *AQP7*, *CETP*, *LIPE*, and *SOD3*. *HIF3A* was also explored and found to be positively associated with BMI at the same 3 CpGs as identified by Dick *et al*. (70)

Ronn *et al*. investigated age, BMI and HbA1c levels, all risk factor for disease, in both adipose tissue and blood DNA methylation (74). The DNA methylation ageing signals seen previously in *ELOVL2*, *FHL2*, *KLF14*, *GLRA1* in blood were identified in adipose. This study supported the *HIF3A* finding in adipose within females only and found HbA1c associations with 711 sites in adipose tissue. There was minimal overlap in signal between HbA1c and BMI or age.

A study of post gastric bypass DNA methylation variation in adipose tissue identified changes within genes associated with obesity by pre- and post-surgery weight-loss analysis (94). Both omental and subcutaneous adipose tissue were compared and these give further insight to the dramatic physiological changes that occur with the rapid post-operative weight loss.

OBESITY AND AGEING ASSOCIATED CHANGES

Horvath *et al*. observed that obesity accelerates the detected epigenetic ageing of the liver when assessed by the Horvath 'Epigenetic Clock', but interestingly not in the other tissue types tested, including blood, adipose tissue, or muscle (95). Obesity-related nonalcoholic fatty liver disease did not drive these hepatic changes. Although, it was postulated to be associated with obesity-driven liver comorbidities, including insulin resistance and hepatocellular carcinoma. The lack of changes in other tissues was hypothesized as due to liver-specific oxidative stress and also perhaps the suboptimal measure of obesity via BMI.

A study by Simpkin *et al*. identified that an accelerated epigenetic age measure at birth is also associated with a more rapid increase in BMI in childhood (96). Furthermore, obesity is proposed to contribute to the 'exposome' that influences epigenetic ageing, with increased BMI from young adulthood to middle age contributing to a greater age acceleration (97). In an analysis integrating blood derived ageing–related differentially

methylated regions (DMRs) within GWAS associated regions, 3 were identified within loci for fasting glucose-related traits that interact with BMI (17).

DEVELOPMENTAL AND *IN UTERO* INFLUENCES

Critical windows may exist for environmental or intrauterine factors to impact on the epigenome, as it being defined, during early development (98). These early changes are proposed to have the ability to act as 'metastable epialleles' that then propagate through the all germ layers to effect all cell types (99). Although evidence for a role in DNA methylation in this process in human is not strong, the possibility for some repetitive elements such as SVAs (SINE-VNTR-Alu) to escape the global demethylation, subsequent reprogramming, and then be vulnerable to environmental influence cannot be excluded at this stage (14).

Epidemiological data has proposed that *in utero* exposure to under- and overnutrition affects obesity risk in later life, potentially through epigenetic modifications (100). Several studies have explored this through the unique model of contrasting children born before or after maternal gastric bypass surgery. Guénard *et al*. compared DNA methylation from siblings born from 25 mothers adjusting for age and sex and puberty (101). Children born prior to the procedure possessed a significantly higher z-score BMI, as well as poorer insulin resistance values. Temporal DNA methylation variation was identified, although in blood and not adjusted for cell-type effects, but gene enrichment for glucose homeostasis and immune function was seen. However, more recent and powerful studies have not borne out the findings of these earlier studies. In a direct obesity analysis, Willmer *et al*. in a study from Scandinavia of 164 children born before and 176 born after surgery at four years of age did not show any improving effect of bariatric surgery on weight development in children (77). Sharp *et al*. examined 1,018 neonatal cord blood derived DNA methylomes with the 450k array for the relationship between maternal and later childhood adiposity. Both high and low maternal weight extremes were seen to led to significant neonatal epigenetic changes, although weight gain during pregnancy was not influential (102). A large Danish Birth cohort of 30,655 trio families examined children at seven

years to also directly explore the influence of parental BMI through pregnancy. This supported a role for the intrauterine environment in fetal and child growth trajectories, but that later parent-child associations in weight were likely to be contributed to by shared known genetic or environmental factors with both parents (103).

The genetic component of these maternal adiposity epidemiological observations has been recently dissected through large-scale GWAS and was seen to be a significant, but not complete, contributor (104). Furthermore, Richmond *et al*. performed a direct MR analysis, using a weighted genetic score from variants for BMI, for maternal obesity influences in children from ages 7-18 years. In over 2,000 samples in both discovery and replication sets, they found little support for a strong causal intrauterine effect of increasing maternal BMI with increased childhood adiposity (105). The complexity of this MR analysis in this scenario was recently discussed by Lawlor *et al*. (106).

LIMITATIONS AND FUTURE DIRECTIONS

Current large-scale high-throughput analysis is limited to DNA methylation array-based techniques, commonly the 450k array and the more recent 850k array. These provide only a partial story with regards to the DNA methylome as ~28 million CpGs reside within the genome, as well as the additional potential for non-CG changes. An obvious area of under-exploration by this approach is repetitive elements, which are proposed to conceal significant functionality, such as the strong overlap of chromatin enhancer evidence within LTR repeat class member LTR12C (107). Examples also include a potential epigenetic role for an Alu repetitive element in *POMC* in childhood obesity (108).

Furthermore, co-ordinated variation across a functional region, such as an enhancer, by the delineation of a significant DMR can increase statistical and biological confidence of the result (109). However, sparse array data are not the most powerful for robust DMR calling (110). Enrichment for disease ontology based results is stronger using sequencing-derived DMRs rather than isolated DMPs (58). Thus, larger and more in depth sequencing-focused analyses could identify further novel findings.

External confounding factors in EWAS analysis include tobacco, alcohol, diet, medications, and other chemicals (68), though these signatures are now beginning to be precisely defined. Strong and replicated effects from prenatal exposures with smoking that persist into childhood exist, although these are not yet robust for diet. However, evidence exists in a number of murine and other animal models which have explored prenatal dietary exposures (111). DNA methylation associations with blood serum metabolites have been identified in humans through EWAS (112).

Major areas for future exploration are the influence of rare cell types, and an issue not yet resolved in biology, at what point does a change in the epigenome of a cell then indicate a new cell type? Epigenetic variability is occurring even in assumed homogenous cell-types, such as neutrophils (113). It is of important note that when robustly controlled experiments reduce genetic and cell-type variability by the use of isolated cell-types in MZ twin discordant models, minimal significant directional disease-associated DMPs are identified (39). Although, increased variability at distinct positions can be found, it is not attributable therefore could be technical artefact, or unaccounted environmental effects. Underlining these cell-type specific effects, the Mendelson *et al*. BMI EWAS only saw 22 of the initial 135 DMPs replicated in isolated $CD4^+$ T cells (78). Validation and most crucially independent replication are required for robust findings to be reported, with subsequent functional investigation in appropriate biological models.

The integration of genetic and epigenetic data is another area where powerful insights can be made. Analysis within GWAS loci to determine allelic or haplotype epigenetic differences was first shown with DNA methylation in the obesity-related *FTO* locus (31). This amalgamation of epigenetic and haplotypic data has now become a valuable tool in the dissection of GWAS traits (114). Incorporating chromatin segmentation data across multiple tissue types, including fat, enabled the localisation of enhancer variation in adipocytes influencing *IRX3* and *IRX5* expression, within the large linkage disequilibrium (LD) block region of *FTO*. These regulatory genes were shown to be involved with the browning of fat and thermogenesis (115).

Few studies to date have analysed histone variants or post-translational modifications with respect to human obesity, and have been limited to

extremely small numbers and/or only global measures (116). Murine studies have proposed potential biological insights that may merit human exploration, such as histone variant MacroH2A1, which may have an anti-adipogenic role within differentiating adipose tissue (117). However, large-scale human studies are on the horizon, as can be seen by the example of a recent successful post-mortem study for Autism Spectrum Disorder in the brain of the active chromatin mark H3K27ac, or Histone Acetylome-wide Association Study (HAWAS) (118). Also, the use of Histone Deacetylase Inhibitors in inflammation-related diseases, as well the potential of targeted epigenetic drugs via CRISPR, point to future epigenomic therapeutic possibilities (119).

CONCLUSION

Epigenomic analysis is a significant tool in the hunt to improve risk prediction, as well as prognosis, beyond the rudimentary measure of BMI for obesity-related diseases. By observing at a molecular level the biologically perturbed mechanisms associated with this disorder, we may understand more precisely, for instance, the significant pathogenic influence of visceral fat. On the epidemiological side, whilst population-based strategies have borne the greatest improvements in human health in the past, there is now evidence that this may not be the case for obesity (120). More focused policies may be required due to its widening distribution over time.

Large-scale DNA methylation array analyses in blood have revealed the precise sequelae of the obese state, such as epigenetic changes driven by dyslipidemia, hyperglycaemia and chronic inflammation. This points towards the use of more precisely defined biochemical phenotypes in EWAS analysis. Nevertheless, further study may implicate additional novel disease-associated outcomes, exposures or even causes. These could include or exclude unproven but intriguingly proposed factors, such as air pollution (121), metal exposure (122), or other "obesogens" in the environment and food chain (123), with a role in obesity, or other diseases. The powerful potential of robustly identified DNA methylation biomarkers, even if not causal, can be seen for tobacco-associated *AHRR* cg05575921 capturing

future smoking-related morbidity and mortality (124). With regards to obesity, potential to predict future cardiovascular disease exists in the lipid-related DNA methylation changes that influence *ABCG1* expression (125).

The analysis of the epigenome is constantly improving and being refined, such as recent discussion by van Iterson *et al*. on controlling genomic bias in DNA methylation EWAS (69). This trajectory will enable us to more accurately define the epigenome and interpret these findings. Increased high-throughput access to distal regulatory regions via the 850k array, larger sample-sized sequencing-based DNA methylome studies, and the analysis of additional modifications, including population chromatin data, will drive forward this progress. Also, the potential of 3rd generation sequencing to directly assess DNA modifications will be a significant step for epigenomics (126). We will be able to more accurately define currently abstractly lumped together 'environmental' change, by more precisely excluding or integrating genetic effects, as well as defining cell-type specific and cell proportion changes, including increasingly rare cell-types. With this will come improved biological interpretation and understanding of critical pathological changes within defined cell-type(s), and genetic pathways. This knowledge will hopefully help reduce the chronic burden of obesity worldwide.

FIGURE LEGEND

Figure 1 – Factors influencing DNA methylation array analysis A: Direct CpG-SNPs; B: SNPs under probe sequences. C: *Cis* and *Trans* SNP effects with increased (orange) or decreased (grey) transcription factor (TF) expression influencing the methylation state of binding site; D: *Cis* effects can include regional effects from Hypermethylated Repeats or Methylation Determining Region motifs within CpG dense regions; E: Density change within Intermediate CpG density regions such as CpG Island shores will also influence methylation state.

TABLE

chr	start	Stop	CpG	Gene	Methyl BMI	CGI	Location	Additional BMI	Other Phenotype
chr11	68607621	68607622	cg00574958	*CPT1A*	-	Shore	Intronic	BMI & WC*(82);	TG(49, 86, 87); VLDL-C(87); Lipoprotein Subfractions(79); HTG waist(91); MetS in Eur(83)
chr15	52554170	52554171	cg06192883	*MYO5C*	+	-	Intronic	BMI in Arab(84)	CRP(45)
chr21	43656586	43656587	cg06500161	*ABCG1*	+	Shore	Intronic		TG, HDL-C(49, 88, 89); Glucose-related(90); HTG waist(91)
chr16	11422408	11422409	cg06946797	*RMI2*	-	-	Intronic	BMI in Arab(84)	Glucose-related(90)
chr17	40927698	40927699	cg08857797	*VPS25*	+	-	Intronic	BMI in Arab(84)	TG(49)
chr22	50327985	50327986	cg09349128	*CRELD2*	-	Shore	Intergenic		Glucose-related(90); IBD(92)
chr17	2612405	2612406	cg09664445	*CLUH*	+	Shore	Intronic	BMI in Arab(84); & in Eur(70)	
chr17	17730093	17730094	cg11024682	*SREBF1*	+	Shelf	Intronic	BMI in Eur(85)	TG(49, 89); Glucose-related(90)
chr6	31681881	31681882	cg13123009	*LY6G6F*	+	-	Intronic	BMI in Arab(84)	
chr5	158634084	158634085	cg26403843	*RNF145*	+	Shelf	Intronic		

Table 1: Consistent BMI-DMPs from the studies of Demerath *et al*. (72), Wahl *et al*. (77), and Mendelson *et al.* (78).
WC: Waist circumference, TG: Triglycerides; LDL-C: Low Density Lipoprotein Cholesterol; HDL-C: Low Density Lipoprotein Cholesterol; VLDL-C: Very Low Density Lipoprotein Cholesterol; HTG: Hypertriglyceridemic.
*This study was in fact performed earlier with many of the same cohorts at Wahl *et al.*

APPENDIX

cis-regulatory elements = binding sites of transcription factors that enhance or repress transcription.
CpG-SNP = SNP that creates or abrogates a CpG dinucleotide
CTCF = CCCTC-binding factor transcription factor
DMP – Differentially Methylated Position
DMR – Differentially Methylated Region
EWAS – Epigenome-Wide Association study
GWAS – Genome-wide Association study
LD = Linkage Disequilibrium
LTR = Long Terminal Repeats
MDR = Methylation Determining Regions
mQTL = methylation Quantitative Trait Loci
MR = Mendelian Randomisation
RFX = regulatory factor X family transcription factor
SNP = Single Nucleotide Polymorphism
SVA = SINE-VNTR-Alu repeat element
trans-regulatory elements = DNA sequences that encode transcription factors

CONFLICT OF INTEREST STATEMENT

The author declares no conflict of interest

SUPPLEMENTARY TABLE S1

Gene Enrichment Analysis for 10 Consistent BMI-associated CpGs

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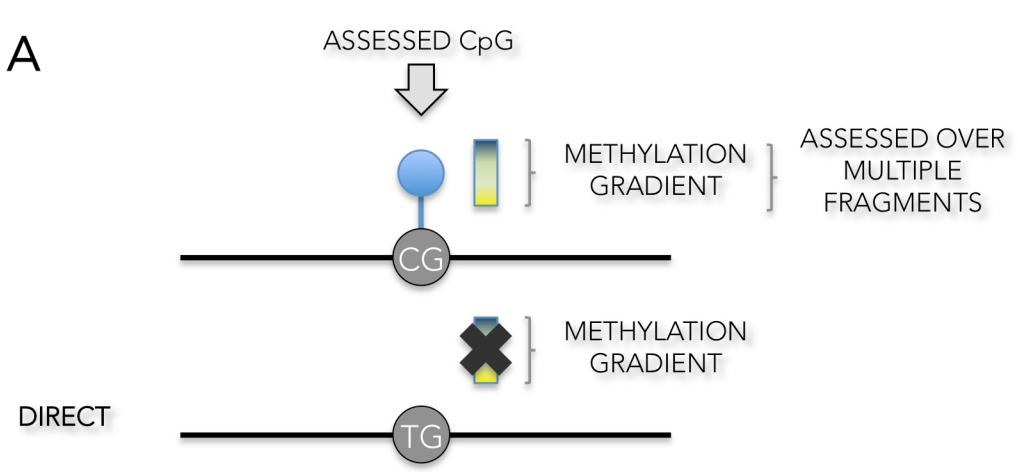
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CG
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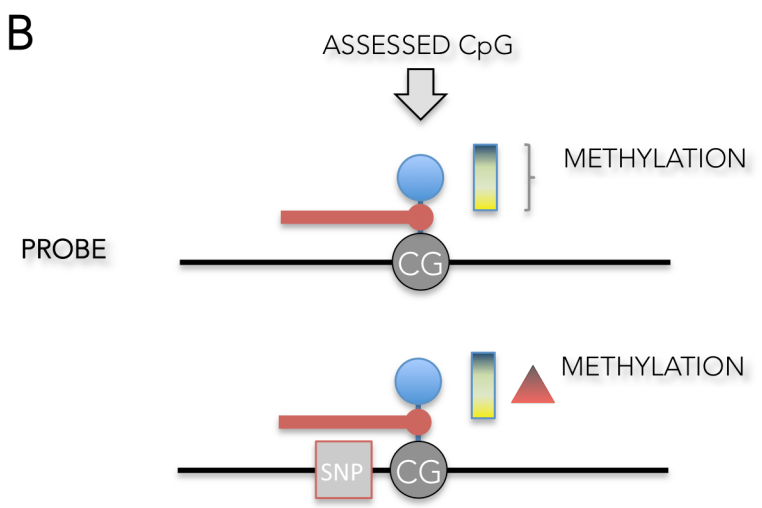
DIRECT
TG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SNP
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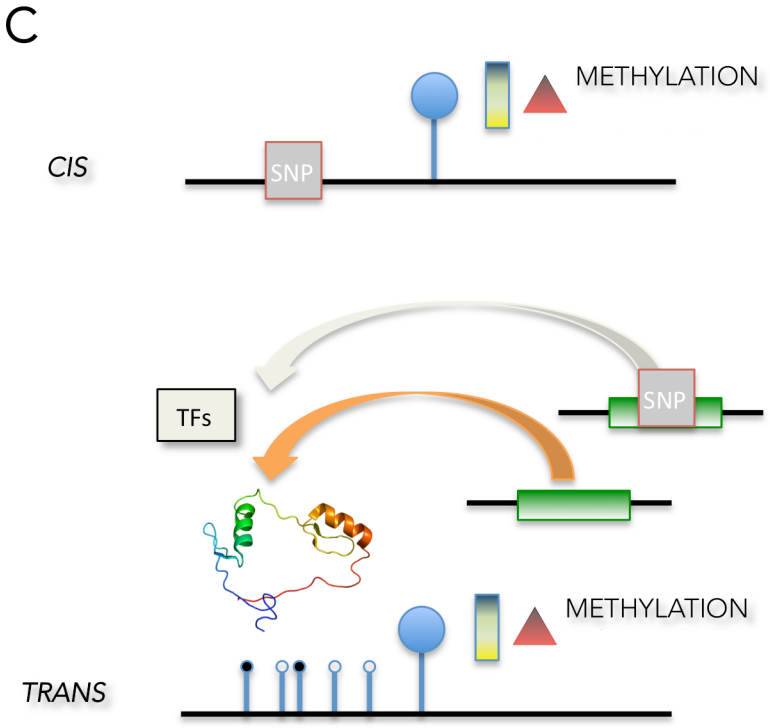
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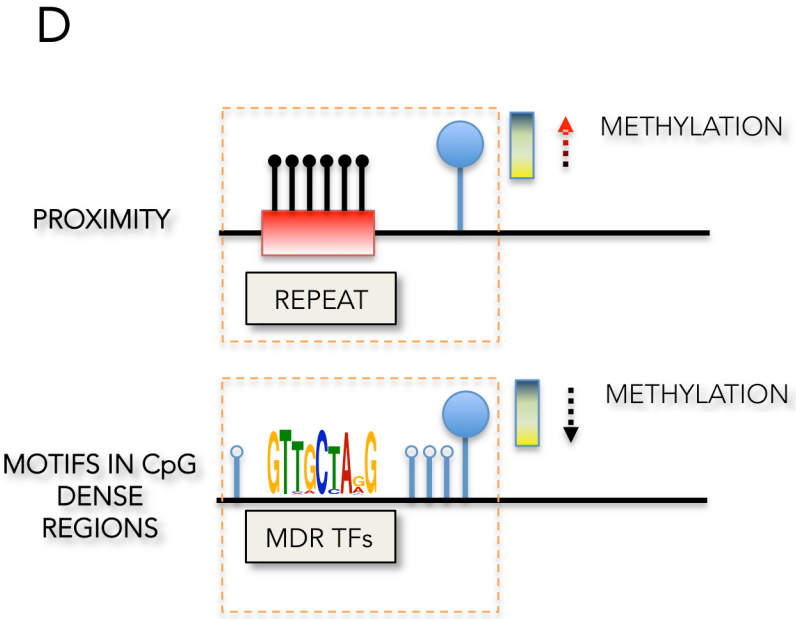
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METHYLATION
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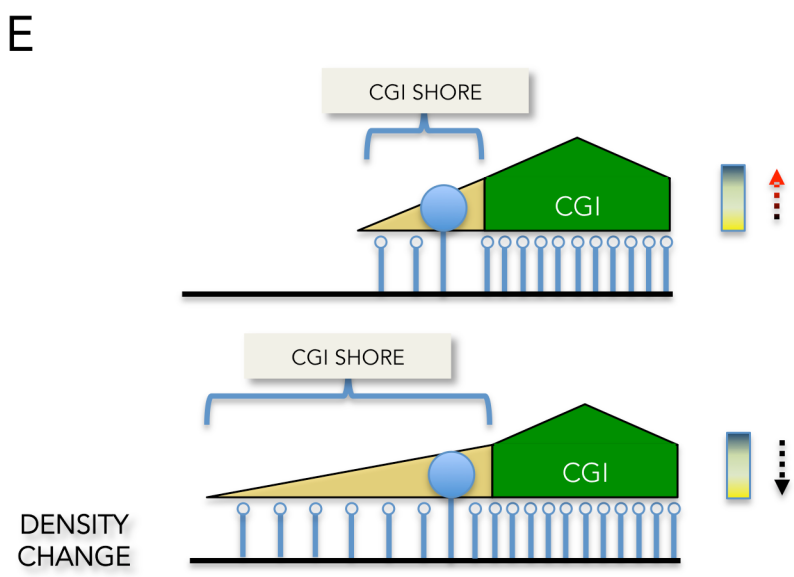
MDR TFs


E
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CGI SHORE
CGI
DENSITY CHANGE

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extension, curated regulatory domains included
# Ontology	Term Name	Hyper Rank	Hyper Raw P-Value
Hyper Bonferroni P-Value	Hyper FDR Q-Val	Hyper Fold Enrichment
Hyper Expected	Hyper Foreground Region Hits	Hyper Total Regions
Hyper Region Set Coverage	Hyper Term Region Coverage	Hyper Foreground
Gene Hits	Hyper Background Gene Hits	Total Genes Annotated
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GO Molecular Function	glycoprotein transporter activity	2
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96	0.1	0.01041667	1	2	2
GO Molecular Function	sterol response element binding	3
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GO Molecular Function	carnitine O-palmitoyltransferase activity	4
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189	0.1	0.005291005	1	4	4
GO Molecular Function	dynein intermediate chain binding	5
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5	5
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process	2	0.0000385532	0.402495408	0.201247704
215.3047	0.009289163	2	451	0.2	0.00443459	2
6	6
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process	6	0.0003000956	1	0.5221663440000001	76.88234
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4

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0.003007135 1 146 0.1 0.006849315 1 5
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2 4343 0.2 0.0004605112 2 69 69
GO Biological Process distal tubule morphogenesis 37
0.003598635 1 1 277.4354 0.003604442 1
175 0.1 0.005714286 1 3 3
GO Biological Process negative regulation of transport 38
0.00372896 1 1 8.997072 0.3334418 3 16189
0.3 0.000185311 3 302 302
GO Biological Process regulation of transport 39
0.003999272 1 1 4.098324 1.220011 5 59233
0.5 0.00008441241 5 1216 1218
GO Biological Process intracellular distribution of mitochondria
40 0.004091252 1 1 243.9759 0.004098766 1
199 0.1 0.005025126 1 4 4
GO Biological Process negative regulation of digestive system process
41 0.004563138 1 1 218.6991 0.004572493 1
222 0.1 0.004504505 1 9 9
GO Biological Process anion transport 42 0.004633303 1
1 8.32925 0.3601765 3 17487 0.3 0.000171556 3
394 394
GO Biological Process regulation of cholesterol esterification
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GO Biological Process renal sodium ion absorption 44
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417 419
GO Biological Process male gamete generation 46
0.004979527 1 1 8.118025 0.369548 3 17942
0.3 0.0001672054 3 418 420
GO Biological Process cholesterol metabolic process 47
0.00498986 1 1 18.45001 0.108401 2 5263
0.2 0.0003800114 2 107 107
GO Cellular Component apical lamina of hyaline layer 1
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GO Cellular Component astral microtubule 2 0.001296854 1

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0.01587302          1         1         1
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63        0.1       0.01587302          1         1         1
Mouse Phenotype     abnormal hippocampus stratum oriens morphology  2
0.001769931         1         1         564.5488  0.001771326         1
86        0.1       0.01162791          1         2         2
Mouse Phenotype     decreased pro-B cell number  3         0.001999572         1
1         29.46068  0.0678871 2         3296      0.2       0.0006067961        2
58        58
Mouse Phenotype     increased interleukin-23 secretion     4
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164       0.1       0.006097561         1         5         5
Mouse Phenotype     decreased pre-B cell number  5         0.004297771         1
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Mouse Phenotype     increased early pro-B cell number      6
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218       0.1       0.004587156         1         4         4
Mouse Phenotype     abnormal lipid level         7         0.004678304         1
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765       767
Mouse Phenotype     decreased circulating magnesium level  8
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230       0.1       0.004347826         1         7         7
Mouse Phenotype     abnormal pro-B cell morphology         9
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0.2       0.0003835091        2         99        99
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Human Phenotype     Hyperlipidemia      1         0.0003578343        1         1
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Human Phenotype     Recurrent encephalopathy     2         0.001811059         1
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1         1         1
Human Phenotype     Transient hyperlipidemia     2         0.001811059         1
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1         1         1
Human Phenotype     Pseudohypoaldosteronism      4         0.002283917         1
1         437.3982  0.002286246         1         111       0.1
0.009009009         1         4         4
Human Phenotype     Abnormality of lipid metabolism        5
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Human Phenotype     Head-banging        6         0.002674387         1         1
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1         1
Human Phenotype     Hyperchloremia      7         0.003003097         1         1
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4         4
Human Phenotype     Sporadic  8         0.00302621          1         1
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Human Phenotype     Hyperacusis         9         0.004440057         1         1
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2         2
Human Phenotype     Hypoketotic hypoglycemia     10        0.004993814         1
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Disease Ontology lissencephaly 4 0.004501599 1 1
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BioCyc Pathway mitochondrial L-carnitine shuttle pathway 1
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MSigDB Pathway Genes involved in RORA Activates Circadian Expression 1
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MSigDB Pathway Genes involved in Circadian Clock 2
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3715 0.2 0.000538358 2 52 52
MSigDB Pathway Genes involved in Transcriptional Regulation of White
Adipocyte Differentiation 3 0.00312285 1 1
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72 72
MSigDB Pathway Genes involved in PPARA Activates Gene Expression 4
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3649 0.2 0.0005480954 2 37 37
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1016 1018
MGI Expression: Detected TS22_thymus primordium 8
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MGI Expression: Detected TS20_glossopharyngeal IX nerve 9
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1131 1133
MSigDB Perturbation Genes down-regulated between two breast carcinoma
subtypes: metaplastic (MCB) and ductal (DCB). 1 0.00007821423
0.26311266971999997 0.26311266971999997 34.06305 0.08807197 3
4276 0.3 0.0007015903 3 106 106
MSigDB Perturbation Genes which best discriminated between two groups of
breast cancer according to the status of ESR1 and AR [GeneID=2099;367]: basal
(ESR1- AR-) and luminal (ESR1+ AR+). 2 0.0002138256
0.7193093184 0.3596546592 12.09622 0.3306818 4 16055
0.4 0.0002491436 4 322 322
MSigDB Perturbation Genes up-regulated in bulk samples from early primary
breast tumors expressing ESR1 [GeneID=2099] vs the ESR1 negative samples. 3
0.0003067409 1 0.3439587958666666 76.03947 0.02630213 2
1277 0.2 0.001566171 2 26 26
MSigDB Perturbation Genes up-regulated in the luminal B subtype of breast
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MSigDB Perturbation Up-regulated genes from the optimal set of 550 markers
discriminating breast cancer samples by ESR1 [GeneID=2099] expression: ER(+)
vs ER(-) tumors. 5 0.0005055934 1 0.34016323951999994
18.0376 0.1663193 3 8075 0.3 0.000371517 3
156 156
MSigDB Perturbation Genes up-regulated in the liver tissue from 10 week old
male mice with KLF10 [GeneID=7071]. 6 0.0006357855 1
0.35646373699999995 52.65857 0.03798052 2 1844 0.2
0.001084599 2 50 50
MSigDB Perturbation Genes important for spermatid differentiation, based on
mouse models with male reproductive defects. 7 0.0007224791 1
0.3472028132 49.36573 0.04051393 2 1967 0.2
0.001016777 2 37 37
MSigDB Perturbation Genes up-regulated in group C of tumors arising from
overexpression of BCL2L1 and MYC [GeneID=598;4609] in plasma cells. 8
0.00132112 1 0.55553096 36.36794 0.05499349 2
2670 0.2 0.0007490637 2 46 46
MSigDB Perturbation Genes up-regulated in hepatocellular carcinoma (HCC)
induced by ciprofibrate [PubChem=2763]. 9 0.00136041 1
0.5084910266666667 35.83114 0.05581736 2 2710 0.2
0.0007380074 2 59 59
MSigDB Perturbation Cluster 5: genes changed in primary keratinocytes by UVB
irradiation. 10 0.001690353 1 0.5686347491999999
32.08936 0.06232596 2 3026 0.2 0.0006609385 2
46 46
MSigDB Perturbation Genes correlated with the early tumor onset in the Emu-myc
transgenic mouse lymphoma model. 11 0.003537915 1 1
22.01369 0.09085254 2 4411 0.2 0.0004534119 2
108 108
MSigDB Perturbation Genes up-regulated in immature bone marrow progenitor
cells upon knock out of CBFA2T3 [GeneID=863]. 12 0.004276857 1
1 8.569875 0.3500634 3 16996 0.3 0.0001765121 3
376 376
MSigDB Predicted Promoter Motifs Motif TGACGTYA matches JUN: jun

oncogene
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MSigDB Predicted Promoter Motifs Motif TGGGGTYACTNNCGGTCA matches NR1H3:
nuclear receptor subfamily 1, group H, member 3 2 0.002617446 1
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0.0005289606 2 76 76
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4984 0.2 0.0004012841 2 115 115
MSigDB Predicted Promoter Motifs Motif CTGCAGY (no known TF) 4
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TreeFam platelet-activating factor acetylhydrolase, isoform Ib, alpha
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TreeFam RNF139, RNF145, SYVN1 5 0.001543702 1 1
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1 1
Ensembl Genes SREBF1 18 0.003270102 1 1
305.3535 0.003274893 1 159 0.1 0.006289308 1
1 1
MSigDB Immunologic Signatures Genes down-regulated in comparison of dendritic cells (DC) stimulated with poly(I:C) (TLR3 agonist) at 12 h versus DC cells stimulated with Pam3Csk4 (TLR1/2 agonist) at 12 h. 1
0.0008032368 1 1 15.38053 0.1950518 3 9470
0.3 0.0003167899 3 197 197
MSigDB Immunologic Signatures Genes up-regulated in comparison of dendritic cells (DC) stimulated with CpG DNA (TLR9 agonist) at 0.5 h versus those stimulated with CpG DNA (TLR9 agonist) at 4 h. 2
0.0008679936 1 0.828933888 14.97415 0.2003452 3
9727 0.3 0.0003084199 3 197 197