1 **TITLE PAGE**

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3 **DNA methylation of PGC-1 α is associated with elevated mtDNA copy number and**
 4 **altered urinary metabolites in Autism Spectrum Disorder**

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12 **ABSTRACT**

13 **Background:** Autism Spectrum Disorder (ASD) is a complex disorder that is underpinned by
 14 numerous dysregulated biological pathways, including canonical mitochondrial pathways.
 15 Epigenetic mechanisms contribute to this dysregulation and DNA methylation is an important
 16 factor in the aetiology of ASD. We examined the relationship between DNA methylation of
 17 peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 α), an essential
 18 transcriptional regulator of mitochondrial homeostasis, and mitochondrial dysfunction in an
 19 ASD cohort of South African children.

20 **Results:** Using targeted Next Generation bisulfite sequencing, we found 12 highly variable
 21 CpG sites in PGC-1 α that were significantly differentially methylated ($p < 0.05$) between ASD

(n = 55) and controls (n = 44). In ASD, eight CpG sites were hypermethylated in the PGC-1 α promoter with a putative binding site for CAMP response binding element 1 (CREB1) spanning one of these CpG sites ($p = 1 \times 10^{-6}$). Mitochondrial DNA (mtDNA) copy number, a marker of mitochondrial function, was elevated ($p = 0.002$) in ASD compared to controls and correlated significantly with DNA methylation at the PGC-1 α promoter. There was a positive correlation between methylation at PGC-1 α at CpG#1 and mtDNA copy number (Spearman's $r = 0.2$, $n = 49$, $p = 0.04$) in ASD, but a negative correlation between methylation at PGC-1 α at CpG#4 promoter and mtDNA copy number in controls (Spearman's $r = -0.4$, $n = 42$, $p = 0.045$). While there was no relationship between mtDNA deletions and PGC-1 α methylation in ASD, mtDNA deletions correlated negatively with methylation at PGC-1 α at CpG#4 (Spearman's $r = -0.4$, $n = 42$, $p = 0.032$) in controls. Furthermore, levels of urinary organic acids associated with mitochondrial dysfunction correlated significantly ($p < 0.05$) with DNA methylation at PGC-1 α CpG#1 and mtDNA copy number in ASD ($n = 20$) and controls ($n = 13$) with many of these metabolites involved in altered redox homeostasis and neuroendocrinology.

Conclusions: These data show an association between PGC-1 α promoter methylation, elevated mtDNA copy number and metabolomic evidence of mitochondrial dysfunction in ASD. This highlights an unexplored link between DNA methylation and mitochondrial dysfunction in ASD.

41

42 **KEYWORDS**

43 Autism Spectrum Disorder, mitochondria, methylation, PGC-1 α , mtDNA copy number,
44 metabolomics, mitochondrial dysfunction

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46 **BACKGROUND**

47 Autism Spectrum Disorder (ASD) is defined by the presence of behavioural traits (1) despite
 48 being a highly heritable neurodevelopmental disorder (2). ASD is characterised by deficits in
 49 social communication and restrictive, repetitive behaviours (3). ASD is a complex disorder
 50 that affects the central nervous system as well as other organ systems, implying the
 51 dysregulation of pleiotropic biological and developmental pathways. ASD is underpinned by
 52 a heterogeneous genetic architecture that includes rare *de novo* genetic variations and low
 53 risk, common single nucleotide mutations (4,5). There is increasing evidence for the role of
 54 epigenetic alterations, and DNA methylation in particular, in modulating ASD phenotypes.
 55 This is evident from discordant identical ASD twin studies (6), studies using brain tissue
 56 from individuals with ASD (7,8), with recent reviews collating numerous reports on ASD
 57 epigenetics (9–12).

58 Given the varied phenotypes and co-morbidities observed in individuals with ASD, numerous
 59 and diverse biological pathways have been implicated in ASD aetiology. These include gene
 60 regulatory-, signaling-, synaptic-, and mitochondrial-pathways (13-16). Mitochondrial
 61 dysfunction is emerging as a key contributor to ASD aetiology; deficiencies in oxidative
 62 phosphorylation (OXPHOS) can decrease the production of adenosine 5'-triphosphate (ATP),
 63 which is an essential requirement for brain function and neurodevelopment. The observation
 64 that congenital errors of mitochondrial metabolism contribute to > 5% of ASD cases (17) first
 65 implied a role for mitochondrial dysfunction in ASD. This has since been supported by
 66 clinical (18), biochemical (19,20), molecular (21) and more recently, epigenetic data (22).
 67 Citrigno *et al* (23) comprehensively reviewed the recent experimental data that support the
 68 Mitochondrial Dysfunction Hypothesis in ASD.

69 Mitochondrial homeostasis is dynamic and is regulated by interdependent pathways that
70 govern mitogenesis, mitophagy, mitochondrial fission and fusion (24). These dynamic
71 mechanisms enable cellular adaptation to changing energy demands, nutrient availability and
72 oxidative stress by regulating mitochondrial DNA (mtDNA) copy number (25,26). Abnormal
73 or fluctuating levels of mtDNA copy number is a marker of mitochondrial dysfunction
74 (27,28). An essential transcriptional regulator of mitochondrial homeostasis is peroxisome
75 proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 α) which regulates fatty
76 acid β -oxidation, OXPHOS, gluconeogenesis and antioxidant defense responses (29). PGC-
77 1 α catalyses mitogenesis by upregulating nuclear respiratory factors 1 and 2 (Nrf-1 and Nrf-
78 2), which promotes the transcription of mitochondrial transcription factors A (TFAM) and B2
79 (TFB2M) (24). Importantly, numerous studies report a correlation between DNA methylation
80 of the PGC-1 α promoter and PGC-1 α transcription, mtDNA copy number and metabolic
81 disease (30–33), suggesting that DNA methylation regulates PGC-1 α -driven mitogenesis.
82 Changes in mitochondrial morphology via fission and fusion, together with mitogenesis, also
83 contribute to maintaining mitochondrial metabolism and function by limiting reactive oxygen
84 species (ROS) damage (34). Mitochondrial fusion occurs in response to mild oxidative stress
85 and is mediated by mitofusins 1 and 2 (MFN1 and MFN2) and optic atrophy 1 (OPA1) (35–
86 37). These proteins work in conjunction with accessory proteins, such as stomatin-like
87 protein 2 (STOML2), which maintains the long isoforms of OPA1 needed to fuse the inner
88 mitochondrial membranes (38). Fission occurs during severe oxidative stress and separates
89 damaged mitochondrial components from the healthy mitochondrial network. Fission is
90 mediated by dynamin-related protein 1 (DRP1) which works with mitochondrial fission
91 protein 1 (FIS1) to divide the outer mitochondrial membrane (39,40). Recently, genes
92 involved in mitogenesis, fission and fusion have been implicated in neuropathology. PGC-1 α
93 is reported to play an important role in excitatory neurotransmitter signaling, neuroprotection,

neuroinflammation and neurogenesis and has been implicated in bipolar disorder, Parkinson's disease, Huntington's disease, schizophrenia and Alzheimer's disease (41). In addition, recent studies have implicated PGC-1 α (29), fusion and fission genes (42,43) in ASD aetiology (29). In view of the evidence implicating both differential DNA methylation and mitochondrial dysfunction in ASD, we examined the relationship between these two processes in a cohort of South African children with ASD. First, we first determined whether PGC-1 α was differentially methylated (DM) between ASD and matched controls from a South African population. Secondly, we addressed whether DNA methylation changed mitochondrial function, measured using mtDNA copy number and urinary metabolomics. Our aim was to test the hypothesis that DNA methylation of mitochondrial biogenesis genes changes mitochondrial function, thereby contributing to the aetiology of ASD.

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106 RESULTS

ASD cohort phenotype and demography: The individuals in our study with ASD spanned the full range of developmental phenotypes observed in ASD. This is reflected in both the number of different Autism Diagnostic Observation Schedule, Second Edition (ADOS-2) Modules used for assessments and the autism severity scores (Table S1, Additional File 1). Each ADOS-2 Module is tailored to match different developmental levels, ranging from pre-verbal individuals with ASD to those with fluent speech. Although our cohort comprised of different demographic groups, demography did not correlate with any ASD trait or any molecular marker (data not shown).

PGC-1 α promoter is hypermethylated in ASD: We investigated whether DNA methylation contributed to the regulation of mitochondrial biogenesis by measuring the methylation of PGC-1 α , a central transcriptional regulator of mitogenesis. We defined highly variable CpGs

as those sites where the methylation range exceeded 5% across all samples in order to identify functionally significant DM genes. This threshold is consistent with *in vitro* (44) and *in vivo* (45) methylation studies. There were 12 highly variable CpG sites in PGC-1 α that were significantly DM ($p < 0.05$) between ASD and controls ($n = 55$ ASD, $n = 43$ controls) (Table S2, Additional File 1). Of these, eight CpG sites were hypermethylated in ASD and clustered around the transcriptional start site (TSS), between the 5' untranslated region (UTR) and intron 1 (Fig. 1 a, b), while four sites, located at intron 2, intron 12, and the 3'UTR were hypomethylated in ASD (Fig. S1, Additional File 2).

The significant DM sites of PGC-1 α in our study is consistent with a role for DNA methylation in regulating mitochondrial biogenesis. To examine this hypothesis, we quantified DNA methylation of nuclear respiratory factor 2 alpha subunit (NRF2A), the transcriptional regulator of mitogenesis that acts directly downstream of PGC-1 α , as well as four genes involved in mitochondrial fission and fusion (STOML2, MFN2, OPA1, and FIS1) in a subset of our cohort ($n = 22$ ASD, $n = 22$ controls). STOML2 contained two DM CpG sites, located in intron 2 and exon 5 downstream of the TSS; these sites were hypermethylated in ASD (Fig. 2a; Table S2, Additional File 1). Significant DM sites between ASD and controls were also identified at one CpG site in NRF2A and FIS1, and at two sites in MFN2 and OPA1 (Fig. 2a; Table S2, Additional File 1). Therefore, we observed multiple DM mitogenesis, fission and fusion genes converging on the regulation of mitochondrial homeostasis in ASD (Fig. 2b), congruent with a role for DNA methylation in the dysregulation of mitochondrial function in our cohort.

Is PGC-1 α hypermethylation associated with altered mitochondrial function? We examined whether methylation affected mitochondrial biogenesis and function because DNA methylation was altered at several key regulators of mitogenesis in our ASD cohort. *In silico* transcription factor binding site analysis of DM CpG sites in the PGC-1 α promoter predicted

a putative binding site for the transcription factor CAMP response binding element 1 (CREB1) at the CpG#1 site ($p = 1 \times 10^{-6}$). This suggests that the DM site in ASD may have functional significance, which we examined by assessing mitochondrial function in our cohort. This was done by quantifying mtDNA copy number and deletions, as well as the levels of urinary metabolites typically associated with mitochondrial disease in ASD relative to controls.

Mitochondrial DNA copy number is increased in ASD: Increased mtDNA copy number is a compensatory response to mild oxidative stress (25,26) and is an established biomarker of mitochondrial function (46). Mitochondrial DNA copy number of NADH dehydrogenase 1 (ND1) relative to Beta-2-microglobulin (B2M) was significantly elevated in the ASD group compared to the control group ($p = 0.002$) (Fig. 3a). Mitochondrial deletions are typical of mitochondrial disease, therefore we examined whether mitochondrial deletions differed in ASD compared to controls by quantifying the copy number of the mitochondrial gene ND1 relative to NADH dehydrogenase 4 (ND4); the latter resides in the major mitochondrial deletion arc. While mtDNA deletions were not significantly elevated in ASD relative to controls ($p = 0.162$), we observed markedly elevated mtDNA deletions in some ASD individuals (Fig. 3b). Notably, mtDNA copy number correlated significantly (Spearman's $r = 0.9$, $n = 49$, $p = 8.814 \times 10^{-10}$) with mtDNA deletions (Fig. 3c), which suggests that elevated mtDNA copy number is associated with mitochondrial dysfunction in our cohort.

PGC-1 α promoter methylation correlates significantly with mtDNA copy number and this relationship is altered in ASD: We examined whether PGC-1 α promoter methylation is associated with mtDNA copy number and/or deletions, and thus mitochondrial function in ASD. We observed a significant correlation between DNA methylation at the PGC-1 α promoter and mtDNA copy number, and this relationship differed between ASD and control groups. In the control group, PGC-1 α methylation at CpG#4 correlated negatively with both

mtDNA copy number (Spearman's $r = -0.4$, $n = 42$, $p = 0.045$) and mtDNA deletions (Spearman's $r = -0.4$, $n = 42$, $p = 0.032$) (Fig. S2, Additional File 3). However, in the ASD group there was a significant positive correlation between PGC-1 α methylation at CpG#1 and mtDNA copy number (Spearman's $r = 0.9$, $n = 49$, $p = 0.04$) (Fig. S2a, Additional File 3), with no correlation between PGC-1 α methylation and mtDNA deletions. This suggests that PGC-1 α methylation is associated with mitochondrial biogenesis and function and that this relationship is disrupted in our ASD group.

DNA methylation and mtDNA copy number are associated with metabolomic markers

of mitochondrial dysfunction: We investigated whether the differential methylation of PGC-1 α and elevated mtDNA copy number observed in ASD were associated with metabolomic evidence of mitochondrial dysfunction. We examined the correlation between PGC-1 α methylation, mtDNA copy number and levels of urinary organic acids which had previously been measured ($n = 20$ ASD, $n = 13$ controls) using gas chromatography - mass spectrometry (GC-MS) (22). DNA methylation at PGC-1 α CpG#1, which is DM in our samples, correlated significantly with three of the 55 urinary organic acids tested (Table S3b, Additional File 1). MtDNA copy number correlated with 22 urinary metabolites associated with mitochondrial dysfunction (Table S3a, Additional File 1). Notably, both PGC-1 α methylation and mtDNA copy number are associated with metabolites derived from BCAA metabolism, including 3-hydroxy-3-methylglutaric acid (3-H-3-MGA), which correlated significantly with both PGC-1 α CpG#1 ($p = 0.009$) and with mtDNA copy number ($p = 0.004$) (Fig. 4a, b). In addition, mtDNA copy number is associated most significantly ($r < -0.3$ or $r > 0.3$; $p < 0.01$) with metabolites derived from four metabolic pathways: fatty acid oxidation, phenylalanine-dopamine synthesis pathway, glycine-glutamine metabolism and branched-chain amino acids (BCAAs) (Table S3a, Additional File 1). These pathways converged on mitochondrial OXPHOS, one-carbon metabolism and neurotransmitter synthesis (Fig. 5). Our

data are consistent with an established metabolomic model for altered mitochondrial metabolism and neuroendocrinology (47) and supports an association between DNA methylation, elevated mtDNA copy number and mitochondrial dysfunction in ASD.

DISCUSSION

ASD is a heritable, complex phenotype with numerous molecular pathways contributing to its aetiology (2). Despite the high heritability of ASD, there is no single or simple genetic mutation that accounts for ASD, and the disorder is characterised by phenotypic and clinical heterogeneity. This implies that epigenetic mechanisms may be important in ASD, and DNA methylation is known to contribute to ASD aetiology (11). DNA methylation dysregulates many biological pathways in ASD, including, but not limited to, immune function (48), chromatin remodeling (49), synaptic signaling and neuronal regulation (50). Increasingly, mitochondrial biological pathways have also been implicated in ASD (18,20,21). The link between mitochondrial dysfunction and ASD is unsurprising given that efficient ATP production is essential for brain development and function. However, the relationship between DNA methylation and mitochondrial function is not fully understood. Our data examined this relationship by testing the hypothesis that mitochondrial biogenesis and fusion genes are DM between ASD and controls, and that differential methylation affects mitochondrial function in ASD.

The transcriptional regulator of biogenesis, PGC-1 α , was significantly DM between ASD and controls in our cohort, with the promoter region being hypermethylated in ASD. The promoter region included a CpG site (CpG#1) containing a putative transcription binding site for CREB1, which is a potent activator of PGC-1 α transcription (51). Although we were not able to examine PGC-1 α transcription in our cohort, this CREB1 site is reported to be DM in

217 metabolic disease (31) which suggests that DM sites at the PGC-1 α promoter region and TSS
218 in our ASD cohort could affect gene transcription and subsequently, mitochondrial biogenesis
219 and function.

220 Mitochondria are adaptive to changing cellular metabolic demands, thus they are dynamic
221 organelles that are remodeled by biogenesis, fission and fusion (52). Therefore, we measured
222 the methylation of additional genes involved in mitochondrial biogenesis, fission and fusion
223 and found that NRF2A, which facilitates mtDNA replication downstream of PGC-1 α in ASD,
224 was DM (24). We also found that genes involved in mitochondrial fission (STOML2, MFN2,
225 OPA1) and fusion (FIS1) were DM in ASD. Collectively, these DM genes converge on the
226 pathways regulating mitochondrial homeostasis in response to metabolic and oxidative stress
227 (Fig 2). Of note, STOML2 was hypermethylated at two CpG sites downstream of the TSS in
228 ASD. STOML2 plays an important role in mitochondrial fusion by stabilizing the OPA1
229 protein, which facilitates fusion of the inner mitochondrial membranes (36–38). STOML2-
230 deficient cells fail to undergo mitochondrial fusion during stress, leading to mitochondrial
231 fragmentation (38). STOML2 is also well-established as an anti-apoptotic gene in cancer
232 cells, highlighting the importance of fusion to re-establish mitochondrial homeostasis and
233 prevent mitophagy (autophagy of mitochondria) under stress. Our data is consistent with
234 previous work showing that both fission and fusion genes are differentially expressed in ASD
235 (20,42,43). This supports the link between ASD and mitochondrial fusion and fission which
236 highlights the differential methylation of mitochondrial genes on an integrated pathway level.

237 Our data show genes that regulate mitochondrial biogenesis, fission and fusion are DM in our
238 cohort. While we were unable to measure gene expression because of the low integrity of
239 RNA extracted from the tissue source (buccal swabs) used in our study, previous studies have
240 shown that differential methylation alters the expression of these genes (20,42,43,53). We

investigated whether mitochondrial DNA copy number, a marker of mitochondrial function (46), was altered in our cohort. Changes in mtDNA copy number have been reported in ASD, with both increases (54–56) and decreases (57,58) observed in ASD. These discrepancies can be attributed to several factors that differed across studies, including the age of the participants studied, the presence of co-morbidities and the degree of mitochondrial dysfunction in participants. We observed a significant increase in mtDNA copy number in our ASD group compared to controls, which represents an established compensatory mechanism in response to mitochondrial dysfunction. We also observed a significant positive correlation of mtDNA copy number with mtDNA deletions in ASD, suggesting that elevated mtDNA copy number is indicative of mitochondrial dysfunction in our cohort. Increased mtDNA copy number is observed as a response to oxidative stress in animal models and *in vitro* studies (59–61), as well as in human clinical studies using buccal samples (62). This compensatory mechanism has also been reported in both mitochondrial diseases (63–65), neuropsychiatric and neurodevelopmental disorders (66–69).

A connection between PGC-1 α methylation and mtDNA copy number is established, and a negative correlation is reported between PGC-1 α promoter methylation and both PGC-1 α transcription and mtDNA copy number in neurological (53) and metabolic disorders (31,33,70). Consistent with this, we report a negative correlation between PGC-1 α promoter hypermethylation and both mtDNA copy number and deletions in our control group. Thus, hypomethylation of PGC-1 α promoter is associated with elevated mtDNA copy number and deletions, suggesting that this is an adaptive mechanism to upregulate PGC-1 α dependent mitochondrial biogenesis under conditions of mild mitochondrial dysfunction. However, this relationship between PGC-1 α promoter methylation and mtDNA copy number was perturbed in ASD. We observed a significant positive correlation between PGC-1 α promoter methylation at CpG#1 and mtDNA copy number. Hypermethylation at the PGC-1 α promoter

could inhibit the PGC-1 α -dependent activation of antioxidant response elements (71) which is congruent with the evidence for elevated oxidative stress in ASD (72). Oxidative stress can induce mitogenesis via Nrf-2 (73) which upregulates NRF2A independently of PGC-1 α in a redox-sensitive manner (62). We found no relationship between PGC-1 α methylation and mtDNA deletions in ASD, suggesting an absence of the adaptive hypomethylation to compensate for metabolic and putative oxidative stress.

To further explore the link between differential methylation, mtDNA copy number and mitochondrial function, we used metabolomic analysis, which directly reflects the biochemical activity, including mitochondrial activity, of a biological sample (74). We examined whether differential methylation and elevated mtDNA copy number correlated with metabolomic evidence of mitochondrial dysfunction in our cohort, using urinary organic acids that had been associated with mitochondrial disease in South Africans (75). We found a metabolomic profile associated with mtDNA copy number that was consistent with a link between DNA methylation, mitochondrial dysfunction and neuropathology in our ASD cohort. Although the metabolomic analysis was performed on a smaller sample size than the methylation and mtDNA copy number experiments, it was a functional and exploratory way to corroborate the mitochondrial dysfunction reflected by elevated mtDNA copy number in our cohort. Consequently, we focused on the metabolic pathways, rather than specific metabolites, that correlated most significantly with mtDNA copy number ($p < 0.01$) in our cohort. Metabolites derived from tyrosine, tryptophan and glycine significantly ($p < 0.01$) correlated with mtDNA copy number. These are precursors of dopamine, serotonin, melatonin and glutamate synthesis, which are all important neurotransmitters implicated in ASD aetiology (76). The notable enrichment of metabolites derived from the dopamine pathway, which is closely tied to cellular oxidation state (77), is consistent with altered redox homeostasis in ASD. Moreover, glycine serves as the precursor to one-carbon metabolism

(cysteine, methionine, and glutathione pathways). These are essential regulators of oxidative state which have been implicated in the metabolomic profile associated with ASD severity in two independent cohorts (78,79) and have been identified as a link between DNA methylation and mitochondrial dysfunction (80,81). In addition, both PGC-1 α methylation and mtDNA copy number correlate significantly with metabolites derived from BCAA catabolism. BCAAs provide nitrogen to the glutamate-glutamine cycle and have been implicated as important regulators of glutamatergic neurotransmission (82), which contribute to ASD aetiology (83). Two metabolites (3-H-3-MGA and 3-methylglutaconic acid (3-MGA)) were significantly elevated in our ASD cohort (22) and are characterized as urinary biomarkers of mitochondrial respiratory chain deficiencies (75,84). Altered BCAA metabolism has also been linked to oxidative stress resulting from perturbed NAD⁺/NADH redox ratios (85). Of note, mtDNA copy number correlated significantly with the direct precursor of *de novo* NAD⁺ synthesis, QA (p=0.008), which is a neurotoxin (86) that is also implicated in ASD etiology (87). NAD⁺ has also been identified as a metabolic link between changes in mtDNA copy number, methionine metabolism and DNA methylation (88). Therefore, both PGC-1 α methylation and mtDNA copy number are associated with metabolomic evidence of mitochondrial dysfunction, oxidative stress and neuropathology. Together, our metabolic data are consistent with a dysregulation of the link between methionine metabolism, mitochondrial dysfunction and neurotransmitter synthesis in ASD.

CONCLUSION

Our study is one of the few molecular studies from Sub-Saharan Africa to examine ASD in an understudied African population. We present the first report of an association between the methylation of genes involved in mitochondrial biogenesis and remodeling, and

mitochondrial function in a South African ASD cohort. While our results are correlative and cannot establish causality, they contribute to, and are supported by, the growing body of evidence that point to aberrant DNA methylation and mitochondrial dysfunction in ASD aetiology. Our results highlight the value of epigenetic research in under-studied populations to highlight novel associations. The central role of DNA methylation in modulating mitochondrial function highlights the potential to explore existing mitochondrial medications as putative therapeutic interventions for ASD symptomatology.

METHODS

Participants and Sample collection: The ASD vs controls design and study participants were previously described (22). This study examined South African boys with ASD (n=59) and age-matched typically developing controls (n=40) from three demographic groups: African -, European- and Mixed- ancestry. All participants completed an ADOS-2 assessment which was used for phenotyping in the ASD group and ensured the absence of ASD traits in the control group. The study protocol had University of Cape Town Ethics, as well as the Western Cape Government approval to recruit participants at schools. Buccal cells were collected from participants for DNA extraction; this is a minimally invasive collection method suited for DNA methylation studies (89,90). Urine was collected for organic acids extraction for metabolomic analysis using GC-MS.

Targeted Next-Generation Bisulfite Sequencing: DNA methylation was quantified using tNGBS for PGC-1 α and STOML2 in ASD (n=55) and controls (n=44) as well as for FIS1, MFN2, OPA1 and NRF2A in ASD (n=22) and controls (n=22). The tNGBS was completed by EpigenDx, Inc. (MA, USA) who designed a total of 32 tNGBS assays to analyse 171 CpG sites across six genes. They designed seven assays that analysed 26 CpG sites for PGC-1 α ,

eight assays that analysed 45 CpG sites for STOML2, four assays covering 30 CpG sites for FIS1, five assays covering 26 CpG sites for MFN2, five assays covering 25 CpG sites for OPA1 and three assays that analysed 19 CpG sites for NRF2A. Methylation levels were calculated by dividing the number of methylated reads by the number of total reads. Unpaired two-tailed t-tests with unequal variance were used to determine the significant DM CpG sites between ASD and control ($p < 0.05$).

Mitochondrial DNA copy number and deletion: mtDNA copy number and mitochondrial deletions were measured using multiplex real-time quantitative polymerase chain reaction (RT-qPCR). Mitochondrial genes, ND1 and ND4, were amplified by RT-qPCR and normalized to the nuclear gene, B2M, in the same PCR reaction. The probes were coupled to nonfluorescent quenchers (BHQ®, LGC BioSearch) and both the primers and probes used were previously reported by Grady *et al.* (64). Each DNA sample (20ng/μl) was amplified in triplicate, with standard curves set for each gene using equimolar pooled DNA from ASD and controls in a 10-fold dilution series. A two-step thermal profile was used with denaturation at 95°C for 10 min, followed by 40 cycles of 10s at 95°C, 30s at 60°C on the Rotor-Gene Q 6-plex (QIAGEN). Mitochondrial copy number was calculated using the equation $2 \times 2^{\Delta Ct}$, where $\Delta Ct = Ct(\text{nuclear DNA gene}) - Ct(\text{mtDNA gene})$. Significance was determined using a two-tailed unpaired t-test.

Metabolomic correlation analysis: We examined the correlation between PGC-1α methylation, mtDNA copy number and levels of urinary organic acids associated with mitochondrial dysfunction. Urinary organic acids were previously extracted and quantified by GC-MS for 35 participants (21 ASD and 13 controls) (22). The GC-MS data had been analyzed and deconvoluted using a standard metabolomics-based data processing workflow (75) and was log 2 transformed before statistical analysis. The Shapiro-Wilks test was used to

363 test for normality, after which the Spearman Rank Correlation analysis was performed in
364 SPSS (v26).

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367 **ABBREVIATIONS**

368 ADOS-2: Autism Diagnostic Observation Schedule, Second Edition

369 ASD: Autism Spectrum Disorder

370 ATP: Adenosine 5'-triphosphate

371 BCAAs: Branched-chain amino acids

372 B2M: Beta-2-microglobulin

373 CA: Citramalic acid

374 CREB1: CAMP response binding element 1

375 DM: Differentially methylated

376 DRP1: Dynamin-related protein 1

377 FIS1: Mitochondrial fission protein 1

378 GABPA: GA-binding protein α -chain

379 GC-MS: Gas chromatography – mass spectrometry

380 MFN1: Mitofusin 1

- 381 MFN2: Mitofusin 2
- 382 mtDNA: Mitochondrial DNA
- 383 ND1: NADH dehydrogenase 1
- 384 ND4: NADH dehydrogenase 4
- 385 Nrf-1: Nuclear respiratory factor 1
- 386 Nrf-2: Nuclear respiratory factor 2
- 387 NRF2A: Nuclear respiratory factor 2 alpha subunit
- 388 OPA1: Optic atrophy 1
- 389 OXPHOS: Oxidative phosphorylation
- 390 PGC-1 α : Peroxisome proliferator-activated receptor gamma coactivator –1 alpha
- 391 ROS: Reactive oxygen species
- 392 RT-qPCR: Real-time quantitative polymerase chain reaction
- 393 STOML2: Stomatin-like protein 2
- 394 TFAM: Mitochondrial transcription factor A
- 395 TFB2M: Mitochondrial transcription factor B2
- 396 tNGBS: targeted Next Generation Bisulfite Sequencing
- 397 TSS: Transcriptional start site
- 398 UTR: Untranslated region

399 3-H-3-MGA: 3-hydroxy-3-methylglutaric acid

400 3-MGA: 3-Methylglutaconic acid

401

402

403 **DECLARATIONS**

404 **Ethics approval and consent to participate**

405 This study was approved by the University of Cape Town (FSREC076-2014) and Western
406 Cape Government (20141002-37506). We obtained informed, written consent from the
407 parents of all the participants prior to their participation in the study.

408 **Consent for publication**

409 Not applicable.

410 **Availability of data and other materials**

411 The molecular datasets used and analysed during the current study are available from the
412 corresponding author on reasonable request.

413 **Competing interests**

414 The authors declare that the research was conducted in the absence of any commercial or
415 financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

CO conceptualised the overall study design, was responsible for the phenotype data, supervised the laboratory work and data analysis and was the major contributor in writing the manuscript. These authors contributed equally to the manuscript: SB, EM and CM. SB assisted with the design, data acquisition and analysis of the mitochondrial DNA copy number data and contributed to writing the manuscript. EB assisted with the design and analysis of the methylation data for the mitochondrial fission and fusion genes and contributed to writing the manuscript. CM assisted with the design and analysis of the methylation data for the mitochondrial biogenesis genes, analysed the urinary metabolic data, and contributed to writing the manuscript. All authors read and approved the final manuscript.

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REFERENCES

1. Lord C, Brugha TS, Charman T, Cusack J, Dumas G, Frazier T, *et al.* Autism Spectrum Disorder. Nat Rev Dis Primers. 2020;6:5.

- 439 2. Sandin S, Lichtenstein P, Kuja-Halkola R, Hultman C, Larsson H, Reichenberg A. The
440 Heritability of Autism Spectrum Disorder. *Jama*. 2017;318(12):1182–4.
- 441 3. American Psychiatric Association. Diagnostic and Statistical Manual of Mental
442 Disorders. 5th ed. Arlington, VA. 2013.
- 443 4. Gaugler T, Klei L, Sanders SJ, Bodea CA, Goldberg AP, Lee AB, *et al*. Most genetic
444 risk for autism resides with common variation. *Nat Genet*. 2014;46(8):881–5.
- 445 5. Feliciano P, Zhou X, Astrovskaya I, Turner TN, Wang T, Brueggeman L, *et al*. Exome
446 sequencing of 457 autism families recruited online provides evidence for autism risk
447 genes. *npj Genom Med*. 2019;4:19.
- 448 6. Wong CCY, Meaburn EL, Ronald A, Price TS, Jeffries AR, Schalkwyk LC, *et al*.
449 Methylomic analysis of monozygotic twins discordant for Autism Spectrum Disorder
450 and related behavioural traits. *Mol Psychiatry*. 2014;19:495–503.
- 451 7. Nardone S, Sams DS, Reuveni E, Getselter D, Oron O, Karpuj M, *et al*. DNA
452 methylation analysis of the autistic brain reveals multiple dysregulated biological
453 pathways. *Transl Psychiatry*. 2014;4:e433.
- 454 8. Ladd-Acosta C, Hansen KD, Briem E, Fallin MD, Kaufmann WE, Fienberg AP.
455 Common DNA methylation alterations in multiple brain regions in autism. *Mol*
456 *Psychiatry*. 2014;19(8):862–71.
- 457 9. Rylaarsdam L, Guemez-Gamboa A. Genetic causes and modifiers of Autism Spectrum
458 Disorder. *Front Cell Neurosci*. 2019;13:385.
- 459 10. Tremblay MW, Jiang YH. DNA Methylation and susceptibility to Autism Spectrum
460 Disorder. *Annu Rev Med*. 2019;70:151–66.
- 461 11. Wiśniowiecka-Kowalik B, Nowakowska BA. Genetics and epigenetics of Autism

- 462 Spectrum Disorder - current evidence in the field. *J Appl Genetics*. 2019;60:37–47.
- 463 12. Yoon SH, Choi J, Lee WJ, Do JT. Genetic and epigenetic etiology underlying Autism
464 Spectrum Disorder. *J Clin Med*. 2020;9(4):966.
- 465 13. Ayhan F, Konopka G. Regulatory genes and pathways disrupted in Autism Spectrum
466 Disorders. *Prog Neuropsychopharmacol Biol Psychiatry*. 2019;89:57–64.
- 467 14. Kumar S, Reynolds K, Ji Y, Gu R, Rai S, Zhou CJ. Impaired neurodevelopmental
468 pathways in Autism Spectrum Disorder: a review of signaling mechanisms and
469 crosstalk. *J Neurodevelop Disord*. 2019;11:10.
- 470 15. Voineagu I. Gene expression studies in autism: moving from the genome to the
471 transcriptome and beyond. *Neurobiol Dis*. 2012;45(1):69–75.
- 472 16. Frye RE, Rossignol DA. Mitochondrial dysfunction can connect the diverse medical
473 symptoms associated with Autism Spectrum Disorders. *Pediatr Res*. 2011;69(5 Pt
474 2):41–7.
- 475 17. Manzi B, Loizzo AL, Giana G, Curatolo P. Autism and metabolic diseases. *J Child*
476 Neurol. 2008;23(3):307–14.
- 477 18. Oliveira G, Diogo L, Grazina M, Garcia P, Ataíde A, Marques C, *et al*. Mitochondrial
478 dysfunction in Autism Spectrum Disorders: a population-based study. *Dev Med Child*
479 Neurol. 2005;47(3):185–9.
- 480 19. Melnik S, Fuchs GJ, Schulz E, Lopez M, Kahler SG, Fussell JJ, *et al*. Metabolic
481 imbalance associated with methylation dysregulation and oxidative damage in children
482 with autism. *J Autism Dev Disord*. 2012;42:367–77.
- 483 20. Tang G, Rios PG, Kuo SH, Akman HO, Rosoklija G, Tanji K, *et al*. Mitochondrial
484 abnormalities in temporal lobe of autistic brain. *Neurobiol Dis*. 2013;54:349–61.

- 485 21. Dhillon S, Hellings JA, Butler MG. Genetics and mitochondrial abnormalities in
486 Autism Spectrum Disorders: a review. *Curr Genomics*. 2011;12(5):322–32.
- 487 22. Stathopoulos S, Gaujoux R, Lindeque Z, Mahony C, Van Der Colff R, Van Der
488 Westhuizen F, *et al.* DNA Methylation associated with mitochondrial dysfunction in a
489 South African Autism Spectrum Disorder cohort. *Autism Res*. 2020;13(7):1079–93.
- 490 23. Citrigno L, Muglia M, Qualtieri A, Spadafora P, Cavalcanti F, Pioggia G, *et al.* The
491 mitochondrial dysfunction hypothesis in Autism Spectrum Disorders: current Status
492 and Future Perspectives. *Int J Mol Sci*. 2020;21(16):5785.
- 493 24. Ploumi C, Daskalaki I, Tavernarakis N. Mitochondrial biogenesis and clearance: a
494 balancing act. *FEBS J*. 2017;284(2):183–95.
- 495 25. Gaziev AI, Abdullaev S, Podlitsky A. Mitochondrial function and mitochondrial DNA
496 maintenance with advancing age. *Biogerontology*. 2014;15:417–38.
- 497 26. Santos RX, Correia SC, Alves MG, Oliveira PF, Cardoso S, Carvalho C, *et al.* Insulin
498 therapy modulates mitochondrial dynamics and biogenesis, autophagy and tau protein
499 phosphorylation in the brain of type 1 diabetic rats. *Biochim Biophys Acta*.
500 2014;1842(7):1154–66.
- 501 27. Malik AN, Czajka A. Is mitochondrial DNA content a potential biomarker of
502 mitochondrial dysfunction? *Mitochondrion*. 2013;13(5):481–92.
- 503 28. Sun J, Longchamps RJ, Piggott DA, Castellani CA, Sumpter JA, Brown TT, *et al.*
504 Association between HIV infection and mitochondrial DNA copy number in peripheral
505 blood: a population-based, prospective cohort study. *J Infect Dis*. 2019;219(8):1285–
506 93.
- 507 29. Barone R, Rizzo R, Tabbí G, Malaguarnera M, Frye RE, Bastin J. Nuclear Peroxisome

- 508 Proliferator-Activated Receptors (PPARs) as therapeutic targets of resveratrol for
509 Autism Spectrum Disorder. *Int J Mol Sci.* 2019;20(8):1878.
- 510 30. Barrès R, Osler ME, Yan J, Rune A, Fritz T, Caidahl K, *et al.* Non-CpG methylation
511 of the PGC-1 α promoter through DNMT3B controls mitochondrial density. *Cell*
512 *Metab.* 2009;10(3):189–98.
- 513 31. Sookoian S, Rosselli MS, Gemma C, Burgueño AL, Gianotti TF, Castaño GO, *et al.*
514 Epigenetic regulation of insulin resistance in nonalcoholic fatty liver disease: impact of
515 liver methylation of the Peroxisome Proliferator-Activated Receptor γ Coactivator 1 α
516 promoter. *Hepatology.* 2010;52(6):1992–2000.
- 517 32. Heinonen S, Buzkova J, Muniandy M, Kaksonen R, Ollikainen M, Ismail K, *et al.*
518 Impaired mitochondrial biogenesis in adipose tissue in acquired obesity. *Diabetes.*
519 2015;64(9):3135–45.
- 520 33. Kresovich JK, Joyce BT, Gao T, Zheng Y, Zhang Z, Achenbach CJ, *et al.* Promoter
521 methylation of PGC1A and PGC1B predicts cancer incidence in a veteran cohort.
522 *Epigenomics.* 2018;10(6):733–43.
- 523 34. Karbowski M, Youle RJ. Dynamics of mitochondrial morphology in healthy cells and
524 during apoptosis. *Cell Death Differ.* 2003;10:870–80.
- 525 35. Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC. Mitofusins Mfn1 and
526 Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic
527 development. *J Cell Biol.* 2003;160(2):189–200.
- 528 36. Duvezin-Caubet S, Jagasia R, Wagener J, Hofmann S, Trifunovic A, Hansson A, *et al.*
529 Proteolytic processing of OPA1 links mitochondrial dysfunction to alterations in
530 mitochondrial morphology. *J Biol Chem.* 2006;281(49):37972–9.

- 531 37. Ishihara N, Fujita Y, Oka T, Mihara K. Regulation of mitochondrial morphology
532 through proteolytic cleavage of OPA1. *EMBO J.* 2006;25(13):2966–77.
- 533 38. Tondera D, Grandemange S, Jourdain A, Karbowski M, Mattenberger Y, Herzig S, *et*
534 *al.* SLP-2 is required for stress-induced mitochondrial hyperfusion. *EMBO J.*
535 2009;28:1589–600.
- 536 39. Smirnova E, Griparic L, Shurland DL, Van Der Bliek AM. Dynamin-related protein
537 Drp1 is required for mitochondrial division in mammalian cells. *Mol Biol Cell.*
538 2001;12(8):2245–56.
- 539 40. Stojanovski D, Koutsopoulos OS, Okamoto K, Ryan MT. Levels of human Fis1 at the
540 mitochondrial outer membrane regulate mitochondrial morphology. *J Cell Sci.*
541 2004;117(7):1201–10.
- 542 41. Bu X, Wu D, Lu X, Yang L, Xu X, Wang J, *et al.* Role of SIRT1/PGC-1 α in
543 mitochondrial oxidative stress in autistic spectrum disorder. *Neuropsychiatr Dis Treat.*
544 2017;13:1633–45.
- 545 42. Carrasco M, Salazar C, Tiznado W, Ruiz LM. Alterations of mitochondrial biology in
546 the oral mucosa of Chilean children with Autism Spectrum Disorder (ASD). *Cells.*
547 2019;8(4):367.
- 548 43. Pecorelli A, Ferrara F, Messano N, Cordone V, Schiavone ML, Cervellati F, *et al.*
549 Alterations of mitochondrial bioenergetics, dynamics, and morphology support the
550 theory of oxidative damage involvement in Autism Spectrum Disorder. *FASEB J.*
551 2020;34(5):6521–38.
- 552 44. Ziller MJ, Gu H, Müller F, Donaghey J, Tsai LTY, Kohlbacher O, *et al.* Charting a
553 dynamic DNA methylation landscape of the human genome. *Nature.*

- 554 2013;500(7463):477–81.
- 555 45. D’Aquila P, De Rango F, Guarasci F, Mandalà M, Corsonello A, Bellizzi D, *et al.*
556 Erratum: multi-tissue DNA methylation remodeling at mitochondrial quality control
557 genes according to diet in rat aging models. *Nutrients*. 2020;12(5):1494.
- 558 46. Castellani CA, Longchamps RJ, Sun J, Guallar E, Arking DE. Thinking outside the
559 nucleus: mitochondrial DNA copy number in health and disease. *Mitochondrion*.
560 2020;53:214–23.
- 561 47. Yu X, Qiao S, Wang D, Dai J, Wang J, Zhang R, *et al.* A metabolomics-based
562 approach for ranking the depressive level in a chronic unpredictable mild stress rat
563 model. *RSC Adv*. 2016;6:25751–65.
- 564 48. Nardone S, Sams DS, Zito A, Reuveni E, Elliott E. Dysregulation of cortical neuron
565 DNA methylation profile in Autism Spectrum Disorder. *Cereb Cortex*.
566 2017;27(12):5739–54.
- 567 49. Andrews SV, Sheppard B, Windham GC, Schieve LA, Schendel DE, Croen LA, *et al.*
568 Case-control meta-analysis of blood DNA methylation and Autism Spectrum Disorder.
569 *Mol Autism*. 2018;9:40.
- 570 50. Wong CCY, Smith RG, Hannon E, Ramaswami G, Parikshak NN, Assary E, *et al.*
571 Genome-wide DNA methylation profiling identifies convergent molecular signatures
572 associated with idiopathic and syndromic autism in post-mortem human brain tissue.
573 *Hum Mol Genet*. 2019;28(13):2201–11.
- 574 51. Wu Z, Huang X, Feng Y, Handschin C, Feng Y, Gullicksen PS, *et al.* Transducer of
575 regulated CREB-binding proteins (TORCs) induce PGC-1 α transcription and
576 mitochondrial biogenesis in muscle cells. *Proc Natl Acad Sci U S A*.

- 577 2006;103(39):14379–84.
- 578 52. Wai T, Langer T. Mitochondrial dynamics and metabolic regulation. Trends
579 Endocrinol Metab. 2016;27(2):105–17.
- 580 53. Yang X, Xu S, Qian Y, He X, Chen S, Xiao Q. Hypermethylation of the gene coding
581 for PGC-1 α in peripheral blood leukocytes of patients with Parkinson’s disease. Front
582 Neurosci. 2020;14:97.
- 583 54. Gu F, Chauhan V, Kaur K, Brown WT, LaFauci G, Wegiel J, *et al.* Alterations in
584 mitochondrial DNA copy number and the activities of electron transport chain
585 complexes and pyruvate dehydrogenase in the frontal cortex from subjects with
586 autism. Transl Psychiatry. 2013;3(9):e299.
- 587 55. Chen S, Li Z, He Y, Zhang F, Li H, Liao Y, *et al.* Elevated mitochondrial DNA copy
588 number in peripheral blood cells is associated with childhood autism. BMC Psychiatry.
589 2015;15:50.
- 590 56. Yoo HJ, Park M, Kim SA. Difference in mitochondrial DNA copy number in
591 peripheral blood cells between probands with Autism Spectrum Disorders and their
592 unaffected siblings. World J Biol Psychiatry. 2017;18(2):151–6.
- 593 57. Valiente-Pallejà A, Torrell H, Muntané G, Cortés MJ, Martínez-Leal R, Abasolo N, *et*
594 *al.* Genetic and clinical evidence of mitochondrial dysfunction in Autism Spectrum
595 Disorder and intellectual disability. Hum Mol Genet. 2018;27(5):891–900.
- 596 58. Singh K, Singh IN, Diggins E, Connors SL, Karim MA, Lee D, *et al.* Developmental
597 regression and mitochondrial function in children with autism. Ann Clin Transl
598 Neurol. 2020;7(5):683–94.
- 599 59. Al-Kafaji G, Golbahar J. High glucose-induced oxidative stress increases the copy

- 600 number of mitochondrial DNA in human mesangial cells. Biomed Res Int.
601 2013;2013:Article ID 754946.
- 602 60. Masser DR, Clark NW, Van Remmen H, Freeman WM. Loss of the antioxidant
603 enzyme CuZnSOD (Sod1) mimics an age-related increase in absolute mitochondrial
604 DNA copy number in the skeletal muscle. Age Dordr. 2016;38(4):323–33.
- 605 61. Zeng Y, Pan Q, Wang X, Li D, Lin Y, Man F, *et al.* Impaired Mitochondrial fusion
606 and oxidative phosphorylation triggered by high glucose is mediated by Tom22 in
607 endothelial cells. Oxid Med Cell Longev. 2019;2019:Article ID 4508762.
- 608 62. Kolbasina NA, Gureev AP, Serzhantova OV, Mikhailov AA, Moshurov IP, Starkov
609 AA, *et al.* Lung cancer increases H₂O₂ concentration in the exhaled breath condensate,
610 extent of mtDNA damage, and mtDNA copy number in buccal mucosa. Heliyon.
611 2020;6(6):e04303.
- 612 63. Bai RK, Perng CL, Hsu CH, Wong LJC. Quantitative PCR analysis of mitochondrial
613 DNA content in patients with mitochondrial disease. In: Lee HK, DiMauro S, Tanaka
614 M, Wei YH (eds) *Mitochondrial Pathogenesis*. Berlin, Heidelberg: Springer; 2004. p.
615 304–9.
- 616 64. Grady JP, Murphy JL, Blakely EL, Haller RG, Taylor RW, Turnbull DM, *et al.*
617 Accurate measurement of mitochondrial DNA deletion level and copy number
618 differences in human skeletal muscle. PLoS One. 2014;9(12):e114462.
- 619 65. Thompson K, Majd H, Dallabona C, Reinson K, King MS, Alston CL, *et al.* Recurrent
620 de novo dominant mutations in SLC25A4 cause severe early-onset mitochondrial
621 disease and loss of mitochondrial DNA copy number. Am J Hum Genet.
622 2016;99(4):860–76.

- 623 66. Montier LLC, Deng J, Bai Y. Numbers matters: control of mammalian mitochondrial
624 DNA copy number. *J Genet Genomics*. 2009;36(3):125–31.
- 625 67. Chung JK, Lee SY, Park M, Joo EJ, Kim SA. Investigation of mitochondrial DNA
626 copy number in patients with major depressive disorder. *Psychiatry Res*.
627 2019;282:112616.
- 628 68. Chen C, Vincent AE, Blain AP, Smith AL, Turnbull DM, Reeve AK. Investigation of
629 mitochondrial biogenesis defects in single substantia nigra neurons using post-mortem
630 human tissues. *Neurobiol Dis*. 2020;134:104631.
- 631 69. Ridout KK, Coe JL, Parade SH, Marsit CJ, Kao HT, Porton B, *et al*. Molecular
632 markers of neuroendocrine function and mitochondrial biogenesis associated with
633 early life stress. *Psychoneuroendocrinology*. 2020;116:104632.
- 634 70. Zhao H, Zhao Y, Ren Y, Li M, Li T, Li R, *et al*. Epigenetic regulation of an adverse
635 metabolic phenotype in polycystic ovary syndrome: the impact of the leukocyte
636 methylation of PPARGC1A promoter. *Fertil Steril*. 2017;107(2):467-474.e5.
- 637 71. St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jäger S, *et al*. Suppression of
638 reactive oxygen species and neurodegeneration by the PGC-1 transcriptional
639 coactivators. *Cell*. 2006;127(2):397–408.
- 640 72. Bjørklund G, Meguid NA, El-Bana MA, Tinkov AA, Saad K, Dadar M, *et al*.
641 Oxidative stress in Autism Spectrum Disorder. *Mol Neurobiol*. 2020;57:2314–32.
- 642 73. Erlank H, Elmann A, Kohen R, Kanner J. Polyphenols activate Nrf2 in astrocytes via
643 H2O2, semiquinones, and quinones. *Free Radic Biol Med*. 2011;51(12):2319–27.
- 644 74. Glinton KE, Elsea SH. Untargeted metabolomics for Autism Spectrum Disorders:
645 current status and future directions. *Front Psychiatry*. 2019;10:647.

- 646 75. Reinecke CJ, Koekemoer G, van der Westhuizen FH, Louw R, Lindeque JZ, Mienie
647 LJ, *et al.* Metabolomics of urinary organic acids in respiratory chain deficiencies in
648 children. *Metabolomics*. 2012;8:264–83.
- 649 76. Marotta R, Risoleo MC, Messina G, Parisi L, Carotenuto M, Vetri L, *et al.* The
650 neurochemistry of Autism. *Brain Sci*. 2020;10(3):163.
- 651 77. Ben-Shachar D. The bimodal mechanism of interaction between dopamine and
652 mitochondria as reflected in Parkinson’s disease and in schizophrenia. *J Neural*
653 *Transm*. 2020;127:159–68.
- 654 78. Needham BD, Adame MD, Serena G, Rose DR, Preston GM, Conrad MC, *et al.*
655 Plasma and fecal metabolite profiles in Autism Spectrum Disorder. *Biol Psychiatry*.
656 2020.
- 657 79. Smith AM, Natowicz MR, Braas D, Ludwig MA, Ney DM, Donley ELR, *et al.* A
658 metabolomics approach to screening for autism risk in the Children’s Autism
659 Metabolome Project. *Autism Res*. 2020;13(8):1270–85.
- 660 80. Maddocks ODK, Labuschagne CF, Adams PD, Vousden KH. Serine metabolism
661 supports the methionine cycle and DNA/RNA methylation through de novo ATP
662 synthesis in cancer cells. *Mol Cell*. 2016;61(2):210–21.
- 663 81. Shen W, Gao C, Cueto R, Liu L, Fu H, Shao Y, *et al.* Homocysteine-methionine cycle
664 is a metabolic sensor system controlling methylation-regulated pathological signaling.
665 *Redox Biol*. 2020;28:101322.
- 666 82. Yudkoff M. Interactions in the metabolism of glutamate and the branched-chain amino
667 acids and ketoacids in the CNS. *Neurochem Res*. 2017;42(1):10–18.
- 668 83. Essa MM, Braidly N, Vijayan KR, Subash S, Guillemin GJ. Excitotoxicity in the

669 pathogenesis of autism. *Neurotox Res.* 2013;23(4):393–400.

670 84. Smuts I, van der Westhuizen FH, Louw R, Mienie LJ, Engelke UFH, Wevers RA, *et*
671 *al.* Disclosure of a putative biosignature for respiratory chain disorders through a
672 metabolomics approach. *Metabolomics.* 2013;9:379–91.

673 85. Esterhuizen K, van der Westhuizen FH, Louw R. Metabolomics of mitochondrial
674 disease. *Mitochondrion.* 2017;35:97–110.

675 86. Notarangelo FM, Pocivavsek A. Elevated kynurenine pathway metabolism during
676 neurodevelopment: implications for brain and behavior. *Neuropharmacology.*
677 2017;112(Pt B):275–85.

678 87. Savino R, Carotenuto M, Polito AN, Di Noia S, Albenzio M, Scarinci A, *et al.*
679 Analyzing the potential biological determinants of Autism Spectrum Disorder: from
680 neuroinflammation to the kynurenine pathway. *Brain Sci.* 2020;10(9):631.

681 88. Lozoya OA, Martinez-Reyes I, Wang T, Grenet D, Bushel P, Li J, *et al.* Mitochondrial
682 nicotinamide adenine dinucleotide reduced (NADH) oxidation links the tricarboxylic
683 acid (TCA) cycle with methionine metabolism and nuclear DNA methylation. *PLoS*
684 *Biol.* 2018;16(4):e2005707.

685 89. Berko ER, Suzuki M, Beren F, Lemetre C, Alaimo CM, Calder RB, *et al.* Mosaic
686 epigenetic dysregulation of ectodermal cells in Autism Spectrum Disorder. *PLoS*
687 *Genet.* 2014;10(5):e1004402.

688 90. Lowe R, Gemma C, Beyan H, Hawa MI, Bazeos A, David Leslie R, *et al.* Buccals are
689 likely to be a more informative surrogate tissue than blood for epigenome-wide
690 association studies. *Epigenetics.* 2013;8(4):445–54.

LIST OF FIGURES

Fig. 1: The PGC-1 α promoter is hypermethylated in ASD. a) Diagrammatic representation of the PGC-1 α gene promoter region (Chr4:23889974) showing the location of the eight hypermethylated CpG sites (black circles) relative to the transcription start site (TSS) and the sequence of the binding site for the transcription factor, CREB1. b) Box plot showing the percentage methylation of the eight differentially methylated PGC-1 α promoter CpG sites measured using Targeted Next-Generation Bisulfite Sequencing (n = 55 ASD, n = 44 controls) (UTR = untranslated region).

Fig. 2: Differentially methylated genes in ASD converge on pathways regulating mitochondrial homeostasis. a) Targeted Next-Generation Bisulfite Sequencing of STOML2 (n = 55 ASD, 44 controls) and FIS, MFN2, OPA1 and NRF2A (n = 22 ASD, n = 22 controls) with differential methylation at two CpG sites in STOML2, MFN2 and OPA1 and one site in FIS1 and NRF2A. Data represent mean percentage methylation in ASD relative to controls and error bars represent sample standard deviation as a fraction of average methylation at each site. Significant differential methylation was identified using a two-tailed unpaired t-test with unequal variance (p<0.05). b) Mitochondrial homeostasis is maintained by differentially methylated genes (in red) involved in mitochondrial biogenesis, fission and fusion that converge on the regulation of mitochondrial copy number in response to metabolic and oxidative stress. Metabolic stress (decrease in ATP production) activates cAMP and AMPK signalling, leading to the transcription and activation of PGC-1 α . PGC-1 α upregulates the expression of Nrf1 and NRF2A which induce the transcription of the mitochondrial transcription factors to facilitate mitogenesis. PGC-1 α also activates AREs to modulate oxidative stress or ROS levels. Oxidative stress activates the redox sensitive Nfe2l2 pathway, which upregulates both NRF2A and the Pink-Parkin pathway that controls mitophagy, fission

and fusion. (AREs = antioxidant response elements; mtDNA = mitochondrial DNA; ROS = reactive oxygen species).

Fig. 3: Relationship between mitochondrial DNA (mtDNA) copy number and mtDNA deletion in ASD. a) mtDNA copy number is significantly elevated in ASD. Relative quantification of mtDNA copy number was performed by multiplex real-time qPCR of ND1 and B2M (n = 59 ASD, n = 40 control). b) A non-significant increase in mtDNA deletions is observed in ASD. Relative quantification of mtDNA deletions was performed by multiplex real-time qPCR of ND1 and ND4 (n = 59 ASD, n = 40 control). Significance was established using Student t-tests where ($p < 0.05$). c) mtDNA copy number correlates significantly with mtDNA deletions in ASD and controls (n=99), where Spearman's $\rho = 0.882$, $p < 0.001$.

Fig. 4: Urinary metabolite, 3-hydroxy-3-methylglutaric acid (3-H-3-MGA), correlates with PGC-1 α methylation and mitochondrial DNA (mtDNA) copy number. Normal linear regression analysis shows that a) DNA methylation at PGC-1 α CpG#1 correlates with 3-H-3-MGA levels, $RSQ = 0.2163$, Spearman $Rho = 0.386$, $p = 0.043$ and b) mtDNA copy number correlates with 3-H-3-MGA, $RSQ = 0.2282$, Spearman $Rho = 0.355$, $p = 0.004$; (n= 20 ASD, n= 13 controls).

Fig. 5: Mitochondrial DNA (mtDNA) copy number correlates with urinary metabolites implicating mitochondrial metabolism and neurotransmitter synthesis. Urinary metabolites that most significantly correlate with mitochondrial DNA copy number ($p < 0.01$) converge on pathways involved in the metabolism of Fatty Acids (Ethylmalonic Acid); Branched Chain Amino Acids (BCAAS) (Hydroxy-isovaleric acid, 3-hydroxy-3-methylglutaric acid); and neurotransmitters including Dopamine (2-Hydroxyphenylacetic acid, 4-Hydroxyhippuric acid, Vanillylmandelic acid and Homovanillic acid), Serotonin (Quinolinic acid), Glutamate (2-ketoglutaric acid, D-L-Hydroxyglutaric acid; 2,5-

Furandicarboxylic acid, Hydroxymethyl-2-furoic acid) and Glycine (N-2Furanylcarbonylglycine, oxalic acid). Metabolites that are associated with elevated mtDNA copy number are shown in red; pathways involved in neurotransmitter metabolism are highlighted in blue.

LIST OF ADDITIONAL FILES

Additional File 1.xls: Supplementary Tables

Table S1: Summary of demographic and phenotypic characteristics of participants used in this study. ASD = Autism Spectrum Disorder; ADOS-2 = Autism Diagnostic Observation Schedule, Second Edition.

Table S2: Differentially methylated CpG sites ($p < 0.05$, methylation range $> 5\%$) of PGC-1 α and STOML2 ($n = 55$ ASD, 44 controls) and NRF2A, MFN2, FIS1 and OPA1 ($n = 22$ ASD, $n = 22$ controls).

Table S3A: Spearman correlation coefficients for urinary organic acids that correlate significantly with mtDNA copy number ($n = 20$ ASD, $n = 13$ controls). The most significant ($p < 0.01$) correlations with $r < -0.3$ or $r > 0.3$ are shown in bold, non-significant correlations are not shown. BCAA- Branched Chain Amino Acids, TCA - Tricarboxylic acid cycle.

Table S3B: Spearman correlation coefficients for urinary organic acids ($n = 20$ ASD, $n = 13$ controls) that correlate significantly with methylation at promoter of PGC-1 α at the CpG#1 site.

761 **Additional File 2.png : Fig. S1:** PGC-1 α is differentially methylated in ASD. Targeted
762 Next-Generation Bisulfite Sequencing (n = 55 ASD, n= 44 controls) identified differential
763 methylation 12 CpG sites on PGC-1 α . Differential methylation is shown as fold change in
764 percentage methylation in ASD relative to Controls.

765 **Additional File 3.png: Fig. S2:** The relationship between PGC-1 α promoter methylation and
766 mitochondrial DNA (mtDNA) copy number and deletions is altered in ASD. Heatmap shows
767 significant Spearman rank correlations between mtDNA deletions, mtDNA copy number and
768 percentage methylation at differential methylated CpG sites in the PGC-1 α promoter (n = 49
769 ASD, n = 42 controls); * indicates $p < 0.05$. PGC-1 α CpG#1 correlated positively with
770 mtDNA copy number in ASD (Spearman's $r = 0.9$, $p = 8.814 \times 10^{-10}$) while CpG#4 correlated
771 negatively with both mtDNA copy number and (Spearman's $r = -0.4$, $p = 0.045$) and mtDNA
772 deletions (Spearman's $r = -0.4$, $p = 0.032$) in controls.

a

b

a

Methylation (%)

b

a **b** **c**