**Supplementary Material to the manuscript by Loesch et al entitled:**

**Novel blood biomarkers are associated with the white matter lesion in Fragile X Tremor/Ataxia Syndrome.**

**Isolation of PBMC and transformation with EBV 30**.

Participant blood samples were diluted 1:1 with wash buffer (RPMI media, 1% Glutamax, 1% Penicillin/Streptomycin) (Gibco) and peripheral blood monocytic cells (PBMCs) isolated from them by centrifugation (1,800 rpm/40 min with no brake) onto a Ficoll-paque Plus (Sigma-Aldrich) cushion. The PBMCs were washed by two-fold serial centrifugation in wash buffer to remove any residual Ficoll, then resuspended in 5ml RPMI medium containing 1x Glutamax, 1% Penicillin/Streptomycin and 10% Foetal Bovine Serum (FBS) (Gibco). The viable cell density was estimated by microscopy after staining with Trypan Blue. An aliquot of 1 x 106 viable PBMCs was transformed with EBV prepared from the cell line B95 in the presence of cyclosporin A8 (Sigma-Aldrich) to reduce the innate immune response and rejection of the virus.

**Cell Culture.**

Lymphoblastoid cell lines were maintained in MEMAlpha (Gibco) supplemented with 10% Foetal Bovine Serum (FBS) (Gibco) and 1% Penicillin/Streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO2. For the reactive oxygen species (ROS) assays lymphoblastoid cell lines were grown at 37°C with 5% CO2 in 1640 RPMI medium (Gibco) supplemented with 100 UNITS/ml Penicillin and 100 μg/ml Streptomycin [PenStrep (Gibco)], 5% GlutaMax (Gibco) and 10% Fetal Bovine Serum (Gibco).

**Mitochondrial membrane potential and mitochondrial mass.**

Two fluorescent mitochondrial stains were used to estimate mitochondrial mass and mitochondrial membrane potential - MitoTracker Red CMXRos and MitoTracker Green FM (ThermFisher Scientific) [1]. For each sample, three aliquots of 1x 106 cells were each rested in 1 mL Dulbecco’s phosphate buffered saline (Sigma-Aldrich) (PBS) for 1 hr at 37ºC in 5% CO2, harvested and transferred for vital staining to 1) fresh PBS (for background fluorescence measurement), 2) PBS containing 200 nM MitoTracker Red or 3) PBS containing 200 nM MitoTracker Green. After 1h incubation in the dark at 37ºC/5% CO2, the cells were washed and resuspended in 2 ml PBS for fluorescence measurements in a Modulus Fluorometer (Turner Biosystems, Sunnyvale, CA). Mitochondrial mass was assayed as the background-subtracted fluorescence of Mitotracker Green using the Blue Module (Excitation 460 nm, Emission 515 - 570 nm). Mitochondrial membrane potential was assayed as the ratio of the background-subtracted Mitotracker Red fluorescence (measured with the Green Module - excitation 525 nm, emission 580 - 640 nm) to the Mitotracker Green fluorescence (thus normalizing against mitochondrial mass).

**Seahorse respirometry.**

The Seahorse XFe24 Extracellular Flux Analyzer (Seahorse Bioscience) was used to measure the oxygen consumption rate (OCR), a measure of mitochondrial respiration in real-time in live intact lymphoblastoid cells. Prior to inoculating cells, a 24-well PS cell culture plate was precoated with 4.5μl Matrigel® growth factor-reduced (GFR) basement membrane matrix, (phenol-red free, LDEV-free, Corning, MA, USA, cat. no. 356231). Ten mL of Matrigel® was diluted 1:2 in XF assay medium (unbuffered DMEM supplemented with 2.5 mM glucose and 1 mM sodium pyruvate) and then allowed to dry completely. One hour prior to the assay 8 x 105 cells in XF assay medium were inoculated and allowed to attach to the Matrigel® coated wells.

**ATP assay**.

Steady state ATP levels were measured using the ATP Determination Kit (Molecular Probes) according to the manufacturer’s instructions using 1 ml Tricine-buffered lysate from 5 x 105 cells. Duplicate aliquots containing 5 μl of lysate were each added to 45 μl of standard reaction solution and measured using a Modulus Fluorometer (Turner Biosystems, Sunnyvale, CA) and the chemiluminescence module. Background luminescence was measured immediately before each individual assay (conducted in duplicate) and the average background-subtracted luminescence used to determine ATP concentrations from a standard curve constructed using 10-fold serial dilutions of the ATP standard (100 nM - 1μM).

**Reactive Oxygen Species (ROS).**

Intracellular ROS were detected in live cells using the Fluorometric Intracellular ROS kit (Sigma-Aldrich) according to manufacturer’s instructions. At a density of 1-4 × 106 cells/ml the cells were centrifuged at 500 *x g* for 5 min and resuspended in Dulbecco’s phosphate buffered saline (Sigma) at 5 × 105 cells/ml. A 500 μl aliquot of cells was mixed with 600 μl of Master Reaction Mix and incubated at 37 °C/5% CO2 for one hour. An unstained aliquot of cells was used to measure background autofluorescence. The background-subtracted fluorescence (a measure of ROS production) was measured using the Green Module (λex = 520 nm, λem = 605 nm) in a Modulus Fluorometer (Turner Biosystems, Sunnyvale, CA).

**CGG repeat sizing and F*MR1* mRNA expression**.

Genomic DNA was isolated from peripheral blood lymphocytes using standard methods (Purygene Kit; Gentra,Inc., Minneapolis, MN). For Southern blot analysis, 5 micrograms of isolated DNA was digested with *PstI*. Hybridization was performed using the *FMR1* genomic dCTP-P32 labelled pfxa3 probe. Genomic DNA was also amplified by PCR primers A and 571R [3]. PCR was performed using the previously described method [2], with all assays fully validated and internal/ external quality assessed to provide precision of +/- one repeat. In a subset of individuals (9 of 15) this test was also performed on genomic DNA from cultured lymphoblasts, with consistent results (except for one individual where there was a discrepancy of 15 repeats).

RNA was obtained from fresh blood collected directly into Tempus Blood RNA Tubes (Applied Biosystems, Foster City, CA) and RNA was isolated using the ABI PRISM™ 6100 Nucleic AcidPrepStation as per the manufacturer’s protocol. Total RNA was extracted, purified and reverse transcribed. qRT-PCR was be used to quantify *FMR1* and internal control genes GUS, EIF4A2 and SDHA using the relative standard curve method, performed on each plate, standardized to the mean of the 3 internal control genes [4]. Previously published sequences were used for primers and probes for *FMR1* and the internal control genes. All of the above assays were single-plexed, using PCR conditions as described previously [5].

**References**

**1.** Pendergrass W, Wolf N, Poot M. Efficacy of MitoTracker Green TM and CMXrosamine to measure changes in mitochondrial membrane potentials in living cells and tissues. *Cytometry* 2004; 61A: 162-169.

**2.** Khaniani MS, Kalitsis P, Burgess T, Slater HR. An improveddiagnostic PCR assay for identification of cryptic heterozygosity for CGG triplet repeat alleles in the Fragile X gene (FMR1). *Mol Cytogen* 2008; 1:5.

**3.**Fu YH, Kuhl DP, Pizzuti A, et al. Variation of the CGG repeat X site results in genetic instability. Resolution of Sherman paradox. Cell 1991; 67:1047-1058.

4**.** Godler DE, Loesch DZ, Huggins R, et al. Improved methodology for assessment of mRNA levels in blood of patients with FMR1 related disorders. *BMC Clin Pathol* 2009; 9:5. PMCID2708186.

**5.** Tassone F, Hagerman RJ, Taylor AK, et al. Elevated levels of FMR1 mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome*. Am J Hum Genet* 2000; 66(1):6-15.