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Research Paper

The passage from bone marrow niche to bloodstream triggers the metabolic impairment in Fanconi Anemia mononuclear cells

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ABSTRACT

Fanconi Anemia (FA) is a disease characterized by bone marrow (BM) failure and aplastic anemia. In addition to a defective DNA repair system, other mechanisms are involved in its pathogenesis, such as defective mitochondrial metabolism, accumulation of lipids, and increment of oxidative stress production. To better understand the role of these metabolic alterations in the process of HSC maturation in FA, we evaluated several biochemical and cellular parameters on mononuclear cells isolated from the bone marrow of FA patients or healthy donors. To mimic the cellular residence in the BM niche or their exit from the BM niche to the bloodstream, cells have been grown in hypoxic or normoxic conditions, respectively. The data show that, in normoxic conditions, a switch from anaerobic to aerobic metabolism occurs both in healthy and in pathological samples. However, in FA cells this change is associated with altered oxidative phosphorylation, the increment of oxidative stress production, no activation of the endogenous antioxidant defenses and arrest in the G2M phase of the cell cycle. By contrast, FA cells grown in hypoxic conditions do not show cell cycle and metabolic alterations in comparison to the healthy control, maintaining both an anaerobic flux.

The data reported herein suggests that the passage from the BM niche to the bloodstream represents a crucial point in the FA pathogenesis associated with mitochondrial dysfunction.

1. Introduction

Fanconi Anemia (FA) is an autosomal or X-linked recessive disorder [1], characterized by bone marrow failure and aplastic anemia, which represent the primary causes of death in FA patients [2]. Moreover, 20–25% of patients develop malignancies of myeloid origin, including acute myeloid leukemia and myelodysplastic syndrome [3], as well as head and neck squamous cell carcinomas [4]. At the present, at least 23 genes are responsible for the disease [5], determining primarily a defect in the DNA repair system and the arrest in the G2 phase of the cell cycle [6]. Despite numerous studies, the mechanisms responsible for BM failure in FA are not yet well defined, although DNA repair has long been considered the main molecular hallmark of FA cells. However, in recent years, other aberrant cellular pathways have emerged as important contributors to the pathogenesis of the disease, such as mitochondria metabolism dysfunction and the increment of the oxidative stress production, which play a pivotal role in the deranged functioning of FA cells [7,8]. In particular, FA cells are characterized by high lactate

Abbreviations: BM, bone marrow; FA, Fanconi Anemia; H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; MNCs, mononuclear cells; PB, peripheral blood; PHA, phytohemagglutinin.

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fermentation flux [9], due to the dysfunctional electron transport between Complexes I and III [10]. This determines alterations in the aerobic energy metabolism, inducing the increment of fatty acids synthesis and the accumulation of lipid droplets [11]. Moreover, the altered aerobic metabolism determines a defect in mitochondrial morphology, localization, and integrity [12], creating a vicious circle, which maintains an elevated oxidative stress production.

Therefore, to better characterize the dysfunctional aerobic metabolism in FA cells, we have investigated biochemical and cellular markers in FA BM mononucleated cells (MNCs) cultured in hypoxic and normoxic conditions that mimic the environment of the BM niche and the bloodstream, respectively.

2. Materials and methods

2.1. Reagents

Salts, substrates, and all chemicals for biochemical assays (of analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorescent probes were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ultrapure water (Milli-Q; Millipore, Billerica, MA, USA) was used throughout the study. Safety precautions were taken for chemical hazards in carrying out the experiments.

2.2. Samples and treatments

The institutional review board of the "G. Gaslini" Hospital, Genoa, Italy, approved the study, and all the subjects or their legal guardians gave written informed consent to the investigation according to the Declaration of Helsinki.

Fifteen FA patients (6 males) have been involved in the study. The median age was 8 years, with a range from 5 to 34 years. Complementation groups were 11 FANCA, 2 FANCD2, and 1 each for FANCF, FANCG and FANCC, respectively. Control samples were taken from healthy voluntary donors (HD samples; 5 males and 8 female).

Primary bone marrow and peripheral blood mononuclear cells (MNCs) were isolated using Ficoll-Hipaque Plus (GE Healthcare Biosciences, Piscataway, NJ) and cultured at 37 °C in RPMI supplemented with 10% fetal calf serum with glutamine and antibiotics. For the hypoxic condition, cells were grown at 3% of oxygen. In several experiments, to induce BM-MNCs differentiation, samples were stimulated with 10 μg/ml phytohemagglutinin (PHA) and cell cultures were analyzed every 24 h.

2.3. Cell homogenate preparation

To remove the medium, cells were centrifuged at 1000 g for 2 min. The pellet was suspended in phosphate buffer saline (PBS) plus protease inhibitors and sonicated 2 times for 10 s, with a break of 30 s, in ice. Total protein was estimated by the Bradford method [13].

2.4. Evaluation of intracellular ATP and AMP content and calculation of ATP/AMP ratio

ATP and AMP intracellular content was evaluated by the enzyme coupling method, as previously described [9]. For each assay, 50 μg of total protein was employed.

ATP was assayed, following NADP reduction, at 340 nm. The assay medium contained: 50 mM Tris-HCl pH 8.0, 1 mM NADP, 10 mM $MgCl_2$, and 5 mM glucose. Samples were analyzed spectrophotometrically before and after the addition of 4 μg of purified hexokinase plus glucose-6-phosphate dehydrogenase.

AMP was assayed following the NADH oxidation, at 340 nm. The reaction medium contained: 100 mM Tris-HCl pH 8.0, 75 mM KCl, 5 mM $MgCl_2$, 0.2 mM ATP, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 10 IU adenylate kinase, 25 IU pyruvate kinase, and 15 IU of lactate dehydrogenase.

The ATP/AMP value was calculated as the ratio between the intracellular concentration of ATP and AMP, expressed in mM.

2.5. Oxygen consumption assay

Oxygen consumption was measured at 37 °C in a closed chamber, using an amperometric electrode (Unisense-Microrespiration, Unisense A/S, Denmark) [14]. 2×10^5 cells were used for each experiment. Cells were permeabilized with 0.03 mg/ml digitonin for 1 min; then the sample was centrifuged for 8 min at 1000×*g*. 10 mM pyruvate plus 5 mM malate or 20 mM succinate were added to stimulate the pathways composed by Complexes I, III, and IV or Complexes II, III and IV, respectively.

2.6. Assay of Fo-F1 ATP synthase activity

The assay was conducted at 37 °C, over 2 min, by measuring formed ATP from added ADP. 2×10^5 cells were added to the incubation medium containing: 10 mM Tris-HCl pH 7.4, 50 mM KCl, 1 mM EGTA, 2 mM EDTA, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 0.6 mM ouabain, 0.040 mg/ml ampicillin, 0.2 mM di-adenosine-5′penta-phosphate, and the metabolic substrates: 10 mM pyruvate plus 5 mM malate or 20 mM succinate. The cells were incubated in the assay medium for 10 min at 37 °C. Afterward, ATP synthesis was induced by the addition of 0.2 mM ADP and monitored by the luciferin/luciferase ATP bioluminescence assay kit CLSII (Roche, Basel, Switzerland), on a Luminometer (GloMax® 20/20 Luminometer – Promega, Wisconsin, USA). ATP standard solutions (Roche, Basel, Switzerland) in the concentration range 10^{-10} - 10^{-7} M was used for calibration [9].

2.7. Evaluation of the electron transport between complexes I to III

The electron transfer from Complex I to Complex III was evaluated spectrophotometrically, following the reduction of cytochrome c, at 550 nm. For each assay, 50 μg of total protein was employed. The reaction medium contained: 100 mM Tris-HCl pH 7.4 and 0,03% oxidized cytochrome c. The assay started with the addition of 0.7 mM NADH [15]. If the electron transport between Complex I and Complex III is conserved, the electrons pass from NADH to Complex I, then to Complex III via coenzyme Q, and finally to cytochrome c.

2.8. Lactate dehydrogenase assay

To assay the glycolytic flux, the activity of lactate dehydrogenase (LDH; EC 1.1.1.27) was measured at room temperature on 20 μg of cell homogenate. The reaction mixtures contained: 100 mM Tris-HCl pH 7.4, 0.2 mM NADH and 5 mM pyruvate [10]. NADH molar extinction coefficient was considered 0.622 mM^{-1} cm^{-1}, at 340 nm. Enzymatic activity was expressed as mU/mg of total protein (nmol/min/mg of protein).

2.9. Evaluation of fatty acid metabolism

The activity of 3-hydroxyacyl-CoA dehydrogenase was assayed as a marker of fatty acids beta-oxidation metabolism. The assay has been performed spectrophotometrically at 340 nm, following the oxidation of NADH in the presence of acetoacetyl-CoA [16]. The reaction mixture contained: 100 mM sodium phosphate, pH 6.0, 0,2 mM NADH and 0,1 mM acetoacetyl-CoA.

The activities of β-ketoacyl-ACP reductase and Enoyl-ACP-reductase have been evaluated as markers of fatty acid synthesis metabolism.

β-ketoacyl-ACP reductase was assayed following the oxidation of NADPH in the presence of acetoacetyl-CoA [17]. The assay medium contained: 100 mM sodium phosphate, pH 7.0, 1 mM 2-mercaptoethanol, 0.2 mM NADPH and 0.1 mM acetoacetyl-CoA.

Enoyl-ACP-reductase was assayed in the presence of crotonyl-CoA,

following the reduction of NADH. Both assays were performed at 340 nm [18]. The assay solution contained: 100 mM sodium phosphate, pH 7.5, 0,2 mM NADH, 1 mM dithiothreitol and 0.8 mM crotonyl-CoA.

2.10. Evaluation of lipid content

The lipid content was evaluated by the Sulfo-Phospho-Vanillin assay [19]. Briefly, samples were incubated with 95% sulfuric acid at 95 °C for 20 min, quickly cooled and evaluated at 535 nm. Afterward, a solution of 0.2 mg/ml vanillin in 17% aqueous phosphoric acid was added to the samples, incubated for 10 min in the dark and reevaluated at 535 nm. A mix of triglycerides was used to obtain a standard curve.

2.11. Oxidative stress evaluation

To evaluate the ROS level, cells were washed and re-suspended in PBS and stained for 10 min at 37 °C with 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) at a concentration of 5 μM (Thermo Fisher Scientific, Waltham, MA, USA). H2DCFDA is a non-fluorescent dye which is cleaved inside cells to 2′,7′-dichlorofluorescein (H2DCF). In the presence of oxidants, H2DFC is converted in turn to the fluorescent compound DCF. Samples were measured on a FacsCalibur flow cytometer (Becton Dickinson, San José, CA). The analysis was confined to viable cells only, after gating based on forward- and side-scatter characteristics. Ten thousand cells per sample were analyzed [20].

H_2O_2 content was evaluated by the Fluorimetric Hydrogen Peroxide Assay Kit (Sigma-Aldrich, St Louis, MO, USA), following the manufacturer's instructions.

To assess lipid peroxidation, the malondialdehyde (MDA) concentration was evaluated, using the thiobarbituric acid reactive substances (TBARS) assay [21]. This test is based on the reaction of MDA, a breakdown product of lipid peroxides, with thiobarbituric acid. The TBARS solution containing: 15% trichloroacetic acid in 0.25 N HCl and 26 mM thiobarbituric acid. To evaluate the basal concentration of MDA, 600 μl of TBARS solution was added to 50 μg of total protein dissolved in 300 μl of milliQ water. The mix was incubated for 40 min at 95 °C. After the sample was centrifuged at 14000 rpm for 2 min and the supernatant was analyzed spectrophotometrically, at 532 nm.

2.12. Antioxidant defenses assay

The activity of superoxide dismutase (SOD) was evaluated by the SOD Assay Kit (Sigma-Aldrich, St Louis, MO, USA), following the manufacturer's instructions.

Catalase activity was assayed spectrophotometrically, following the decomposition of H_2O_2 at 240 nm. The assay mix contained: 50 mM phosphate buffer pH 7.0 and 5 mM H_2O_2 [22].

Glutathione peroxidase activity was assayed by the Glutathione Peroxidase Cellular Activity Assay Kit (Sigma-Aldrich, St Louis, MO, USA), following the manufacturer's instructions.

The general antioxidant defenses and the relative level of scavengers were evaluated by the Antioxidant Assay Kit (Sigma-Aldrich, St Louis, MO, USA), following the manufacturer's instructions.

2.13. Evaluation of cell cycle and cellular activation

Flow-cytometric analysis of DNA content was performed on cells permeabilized with 0.05% Triton X-100 and stained with 30 mg/ml PI plus 0.5 mg/ml RNase for 30 min. Samples were measured on a FacsCalibur flow cytometer (Becton Dickinson, San José, CA) and cell cycle phase distributions were achieved from the DNA content histograms of at least 10 thousand cells [20].

2.14. Statistical analysis

Statistical analyses were performed with GraphPad Prism software version 7 (GraphPad Software). All parameters were tested by one-way ANOVA followed by Bonferroni test. Data are expressed as mean ± standard deviation (SD) from 3 to 5 independent determinations performed in duplicate. In the figures SD are shown as error bars. An error probability with $p < 0.05$ was selected as significant.

3. Results

FA cells display a metabolic impairment associated with the unbalanced oxidative stress in peripheral blood but not in the BM niche.

Fig. 1 shows the analysis of glucose and lipid metabolism, and markers of oxidative stress, in MNCs isolated from the BM or the peripheral blood (PB). Samples from FA patients are compared with healthy donor cells. MNCs isolated from PB of FA patients display the impairment of the ATP/AMP ratio in comparison to the control (Fig. 1A), associated with a decrement of oxygen consumption and aerobic ATP synthesis (Fig. 1B and C, respectively) induced by the addition of pyruvate/malate, but not of succinate. This impairment depends on the defect in the electron transport between respiratory Complexes I and III (Fig. 1D). As a consequence of this mitochondrial impairment, PB MNCs from FA patients show an enhancement of lactate dehydrogenase (LDH) activity (Fig. 1E) and a lipid metabolism alteration (Fig. 1F–H), which determines the increment of lipid content (Fig. 1I). In FA PB-MNCs, the impairment of mitochondrial aerobic metabolism determines also the increase of ROS production in comparison to the healthy control samples (Fig. 1J), with a consequent accumulation of lipid peroxidation (Fig. 1K). Moreover, the very low level of catalase activity suggests that FA PB-MNCs are unable to activate the endogenous antioxidant defenses in response to the increment of oxidative stress (Figure 1L). By contrast, the same biochemical parameters assayed in the MNCs isolated from the BM do not show significant differences between HD and FA patients, suggesting that the metabolic alteration in FA cells only occurs out of the BM niche.

Moreover, it is possible to note that the mitochondrial aerobic activities (electron transport between Complexes I and III, oxygen consumption and ATP synthesis) and the enzymatic activities involved in lipid metabolism are significantly higher in healthy MNCs derived from the PB if compared to those evaluated in healthy MNCs derived from the BM. Conversely, LDH activity, a marker of lactate fermentation, appeared lower in PB-derived samples with respect to the BM MNCs. These results could depend on the different oxygen tension in BM and PB niches. BM niches display a very low oxygen tension, determining the prevalence of anaerobic glycolytic metabolism, while in PB the oxygen tension is around 20%, promoting the aerobic mitochondrial metabolism [23].

Oxygen concentration plays a major role in metabolic alterations in FA-BM MNCs.

To verify the hypothesis that the metabolic defects in FA MNCs are evident only when the cells leave the hypoxic condition of the BM niche, we activated BM-MNCs from HD and FA subjects with PHA to mimic proliferation/differentiation stimuli required for exiting the BM niche and cultured them in normoxic condition (the same as in the PB, i.e. 20% O_2 tension) (Fig. 2).

In HD BM-MNCs, the oxygen consumption and the ATP synthesis (both after pyruvate/malate or succinate induction) (Fig. 2A–B, D-E), the electron transfer between Complexes I and III (Fig. 2C), and the mitochondrial membrane potential (Fig. 2F) gradually increase over time, inducing a little, but significant, increment of ATP/AMP ratio (Fig. 2G) and maintaining low and stable the LDH activity and the lipid content (Fig. 2H and I).

By contrast, stimulated FA BM-MNCs show an attempt to activate the aerobic metabolism in the first 24 h of normoxic condition, which failed after 48 h. In particular, in the first 24 h, FA BM-MNCs display a weak activation of the aerobic metabolism activities (Fig. 2A–F), even if the LDH activity appears already enhanced (Fig. 2H) and the ATP/AMP ratio is reduced compared to the control (Fig. 2G). Afterward, the

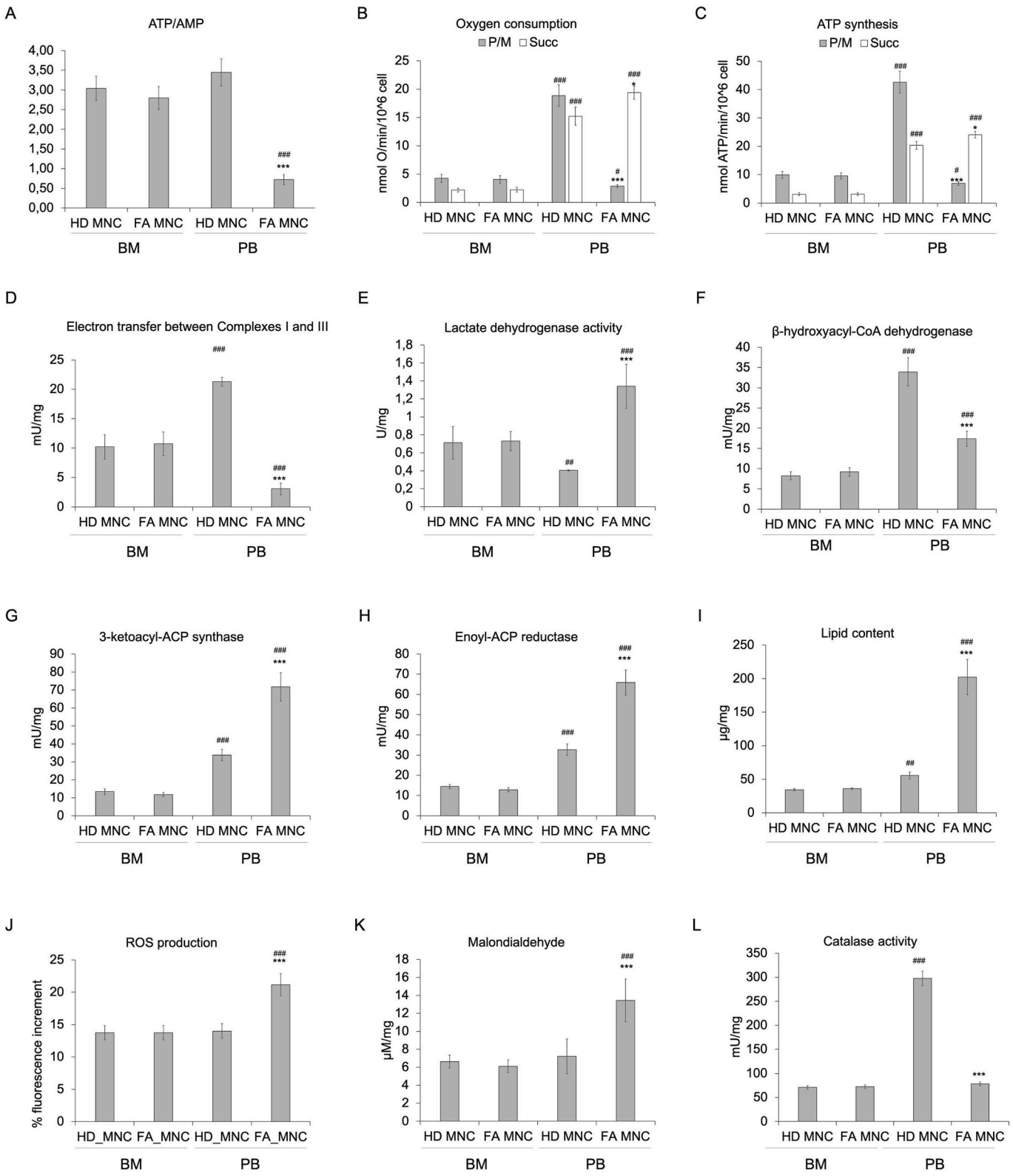

Fig. 1. Comparison of glucose and fatty acid metabolism, and oxidative stress levels in MNCs isolated from BM and PB of FA patients and healthy donors. All experiments were performed on MNCs isolated from BM and PB from FA patients (n = 8) and healthy donors (n = 7). **(A)** ATP/AMP ratio, marker of cellular energy status; **(B, C)** oxygen consumption and ATP synthesis trough F_o-F_1 ATP synthase, respectively, in the presence of pyruvate + malate (grey columns) or succinate (white columns), markers of aerobic metabolism; **(D)** activity of electron transfer between respiratory complexes I and III; **(E)** lactate dehydrogenase activity, marker of anaerobic metabolism; **(F)** β-hydroxyacyl-CoA dehydrogenase activity, marker of fatty acids beta-oxidation; **(G, H)** 3-ketoacyl-ACP synthase and enoyl-ACP reductase activity, respectively, markers of fatty acids synthesis; **(I)** lipid content in MNCs; **(J, K)** ROS and MDA levels, markers of oxidative stress production; **(L)** catalase activity, marker of endogenous antioxidant defenses. All data are expressed as mean ± SD. *** indicates a significant difference for $p < 0.001$ between FA-MNCs and HD-MNCs, isolated from the same site. #, ## or ### indicate a significant difference for $p < 0.05$, 0.01 or 0.001, respectively, between BM-MNCs and PB-MNCs in the same clinical condition.

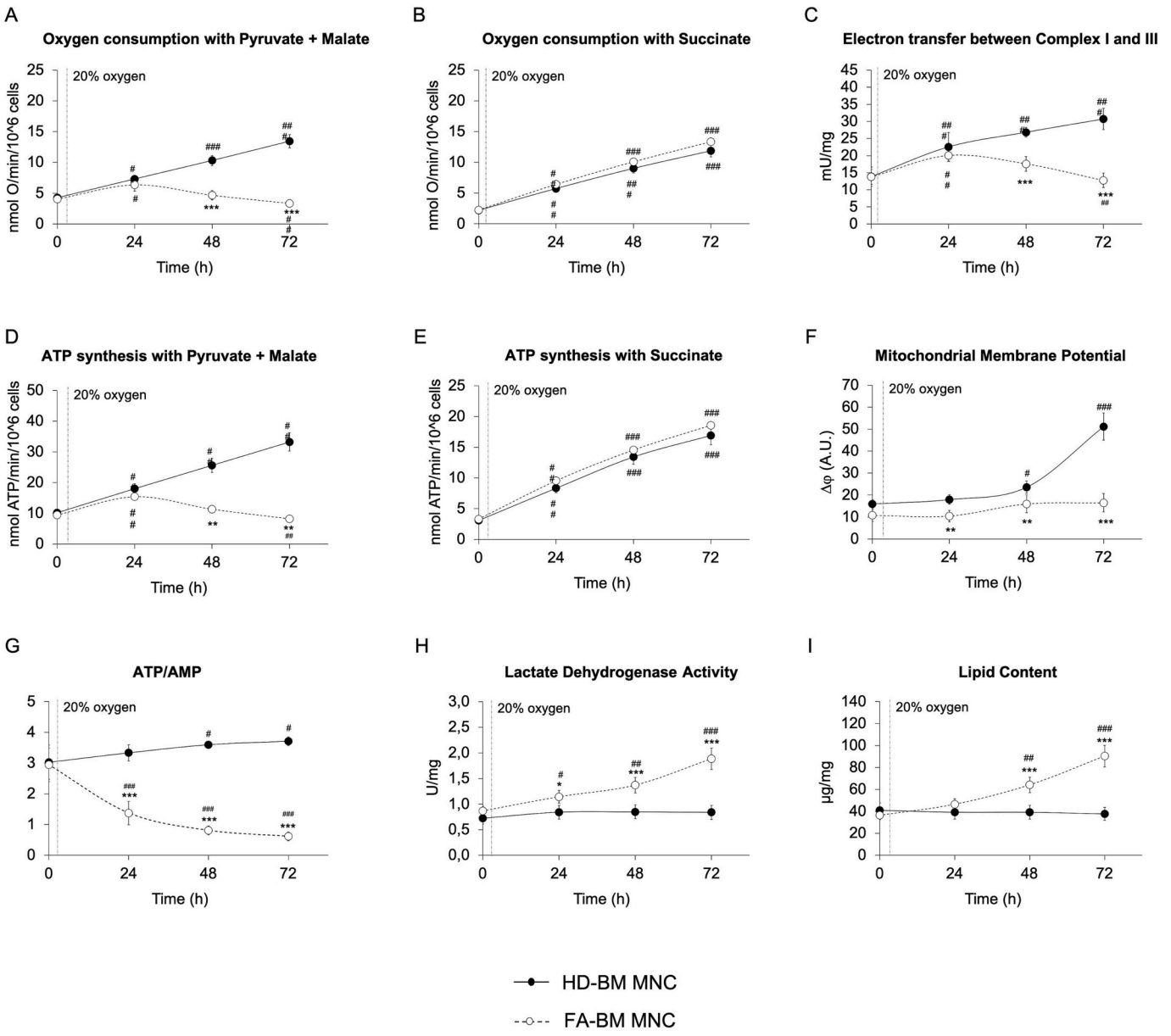

Fig. 2. Modifications of energy metabolism in HD and FA BM-MNCs cultured in normoxic conditions. All data reported in this figure are obtained on BM-MNCs stimulated with PHA and grown in 20% oxygen for 72 h. Black circles represent HD BM-MNCs (n =) and white circles represent FA BM-MNCs (n = 6). **(A, B)** oxygen consumption in the presence of pyruvate + malate or succinate, respectively; **(C)** activity of electron transfer between respiratory complexes I and III; **(D, E)** ATP synthesis trough F_o-F_1 ATP synthase in the presence of pyruvate + malate or succinate, respectively; **(F)** mitochondrial membrane potential; **(G)** ATP/AMP ratio; **(H)** lactate dehydrogenase activity; **(I)** lipid content. All data are expressed as mean ± SD. *, ** or *** indicate a significant difference for $p < 0.05$, 0.01 or 0.001, respectively, between HD BM-MNCs and FA-MNCs at the same time-point. #, ## or ### indicate a significant difference for $p < 0.05$, 0.01 or 0.001, respectively, between the BM-MNCs at time 0 and the same sample at the subsequent time points.

electron transport between Complexes I and III begins to decrease (Fig. 2C), determining a decrement of oxygen consumption and ATP synthesis induced by pyruvate/malate, which becomes more evident after 48h and 72h (Fig. 2A, D, respectively). Notably, the aerobic metabolism induced by succinate increases (Fig. 2B, E), confirming that OxPhos alteration involves only the pathway triggered by Complex I. This impairment of aerobic metabolism determines a very low increment of mitochondrial membrane potential (Fig. 2F), since only Complex II drives the OxPhos, while the lactate fermentation (Fig. 2H) and lipid accumulation (Fig. 2I) increase remarkably. After 72 h, FA BM-MNCs reach a new energetic balance, in which the lactate fermentation appears to substitute the aerobic metabolism. Of note, incubation of FA BM-MNCs for 72h to 20% oxygen without proliferation stimuli induces the same metabolic changes characterized by an altered ATP/AMP ratio, an increment of LDH activity and a reduction of electron transport between complexes I to III and OxPhos metabolism (Supplementary Fig. 1), indicating that FA cells are subject to metabolic stress after the passage to normal oxygen concentrations regardless of the proliferative stimulus.

Interestingly, the alteration of the aerobic metabolism is associated with decreased proliferative response to the PHA stimulus, as assessed by the lower S-phase percentage in FA BM-MNCs cells as compared to HD BM-MNCs samples (Fig. 3A and Supplementary Fig. 2).

Also, an accumulation of FA BM-MNCs cells, but not of HD-derived cells, in the G2M-phase of the cell cycle was observed (Fig. 3B and Supplementary Fig. 2), suggesting a possible cytodieresis inhibition.

FA-BM MNCs, grown in normoxic conditions, display the unbalance between oxidative stress production and antioxidant defenses.

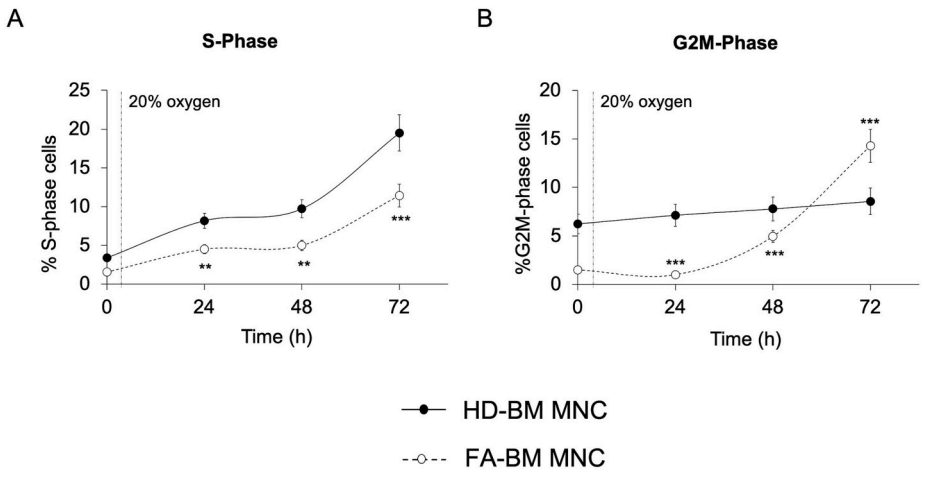

Fig. 3. Proliferative response of HD and FA BM-MNCs cultured in normoxic conditions. All data reported in this figure are obtained on BM-MNCs stimulated with PHA and grown in 20% oxygen for 72 h. Black circles represent HD BM-MNCs (n = 8) and white circles represent FA BM-MNCs (n = 6). **(A)** mean value of S-phase cell percentage; **(B)** mean value of G2/M-phase percentage. All data are expressed as mean ± SD. ** or *** indicate a significant difference for $p < 0.01$ or 0.001, respectively, between HD BM-MNCs and FA-MNCs at the same time-point.

In healthy conditions, the oxidative stress production is balanced by several antioxidant (AO) defenses, which can be induced according to the needs of the cells. Only when this balance is compromised, the oxidative stress triggers several damages to proteins, membranes and nucleic acids.

Since mitochondria activity is one of the main sources of oxidative stress [24] and FA cells are characterized by an altered aerobic metabolism [7,8], we have evaluated the oxidative stress production and the antioxidant response in stimulated HD and FA BM-MNCs grown under normoxic conditions (Fig. 4). FA BM-MNCs show an increment over time of ROS and H_2O_2 production (Fig. 4A and B), which, in turn, causes a high level of lipid peroxidation (Fig. 4C). By contrast, the activity of SOD, catalase and glutathione reductase do not increase in response to oxidative damage (Fig. 4D–F, respectively), resulting in the impairment of the antioxidant capacity of the cell and its scavenger content (Fig. 4G and H). Interestingly, also HD BM-MNCs are characterized by an increment of ROS production and H_2O_2, probably due to the enhancement of aerobic metabolism over time. However, the increased oxidative stress production is associated with a significant increment of SOD, catalase and glutathione peroxidase activity, which determines the positive maintenance of the AO defenses and scavenger in HD BM-MNCs (Fig. 4).

Maintaining the hypoxic condition during the proliferative stimulus preserves FA cells.

The above findings suggest that the metabolic deficiency of FA BM-MNCs and the consequent proliferation impairment may become evident only in normoxic conditions. To confirm this hypothesis, we have evaluated the biochemical markers on FA and HD BM-MNCs stimulated with PHA but maintained at 3% of oxygen (Fig. 5). As expected, hypoxia determines a slow-down of the OxPhos activity (Fig. 5A–E), improving the lactate fermentation (Fig. 5G) and stabilizing the lipid accumulation (Fig. 5H), equally in FA and HD cells. However, the proliferative energy expenditure causes a reduction of ATP/AMP ratio in both samples (Fig. 5F), although more evident in FA cells, at least in the first 48h.

The hypoxic condition inhibits the production of ROS and lipid peroxidation. Likewise, the antioxidant response does not show the increment observed in normoxic conditions, even though FA BM-MNCs show a negative trend, but not significant, of the level of antioxidant capacity and scavengers with respect to HD BM-MNCs (Fig. 6).

Moreover, cell cycle analysis suggests a very similar cell growth behavior of FA and HD BM-MNCs when they are cultured in hypoxia for 72h (Fig. 7 and Supplementary Fig. 3). Interestingly, the aberrant accumulation of FA cells in G2M observed when they are cultured in normoxic conditions does not occur when FA cells are grown at the low oxygen tension present in the BM.

4. Discussion

Data reported herein suggest that the mitochondrial defect, which characterizes FA cells, is evident only in hematopoietic bloodstream cells while the hematopoietic cells stored in BM niche appear normal. In detail, BM-MNCs from FA patients display an energetic metabolism similar to that observed in HD subjects (Fig. 1), characterized by a high flux of anaerobic lactate fermentation, which represents the principal source of energy production, and by a low level of oxidative stress.

Conversely, the PB-MNCs from FA patients are characterized by an impairment of mitochondria aerobic metabolism associated with decrement of energy balance, dysregulation of fatty acid metabolism, accumulation of lipid and increment of oxidative stress (Fig. 1). By contrast, the PB MNCs from HD subjects display an efficient mitochondrial aerobic metabolism and antioxidant defenses.

This discrepancy can be explained considering the different microenvironment that characterize the BM niche compared to the bloodstream. BM niche is a hypoxic environment with a prevalence of anaerobic/fermentative metabolism, due to a limited oxygen supply [23,25]. This condition is necessary to minimize the oxidative damage derived from the mitochondrial oxidative phosphorylation and to limit the accumulation of genomic damage in HSC [23]. Conversely, the bloodstream is characterized by a normoxic environment, which determines the shift to aerobic metabolism. However, the energy advance of the oxidative phosphorylation activation is associated with the increment of oxidative stress production, which, in healthy conditions, is counterbalanced by the endogenous antioxidant defenses [26]. Therefore, it is possible to speculate that the transition from BM to PB represents a moment of stress for hematopoietic cells, especially in the presence of a defect in aerobic metabolism. When BM-MNCs are grown in normoxic conditions (20% of oxygen), HD cells increase the OxPhos metabolism, producing more ATP and ameliorating the ATP/AMP ratio. By contrast, FA cells show impaired mitochondrial activity. In particular, during the first 24 h, FA BM-MNCs try to activate the aerobic metabolism, increasing the oxygen consumption and the relative ATP synthesis. However, the defect in the electron transport between Complexes I and III does not allow the metabolic switch and, after 48 h of proliferative stimulation, FA cells are obliged to restore the lactate fermentation as principal energy metabolism (Fig. 2) [9]. Moreover, despite the limited aerobic metabolism, the high oxygen concentration determines an increment of oxidative stress due to both the impairment of mitochondrial activity and the incapacity to activate the AO responses (Fig. 4). In fact, faced with an increase in the production of ROS, hydrogen peroxide, and MDA levels, FA cells do not activate the enzymes responsible for the antioxidant response, such as SOD, catalase and glutathione peroxidase. This causes an imbalance in the oxidative

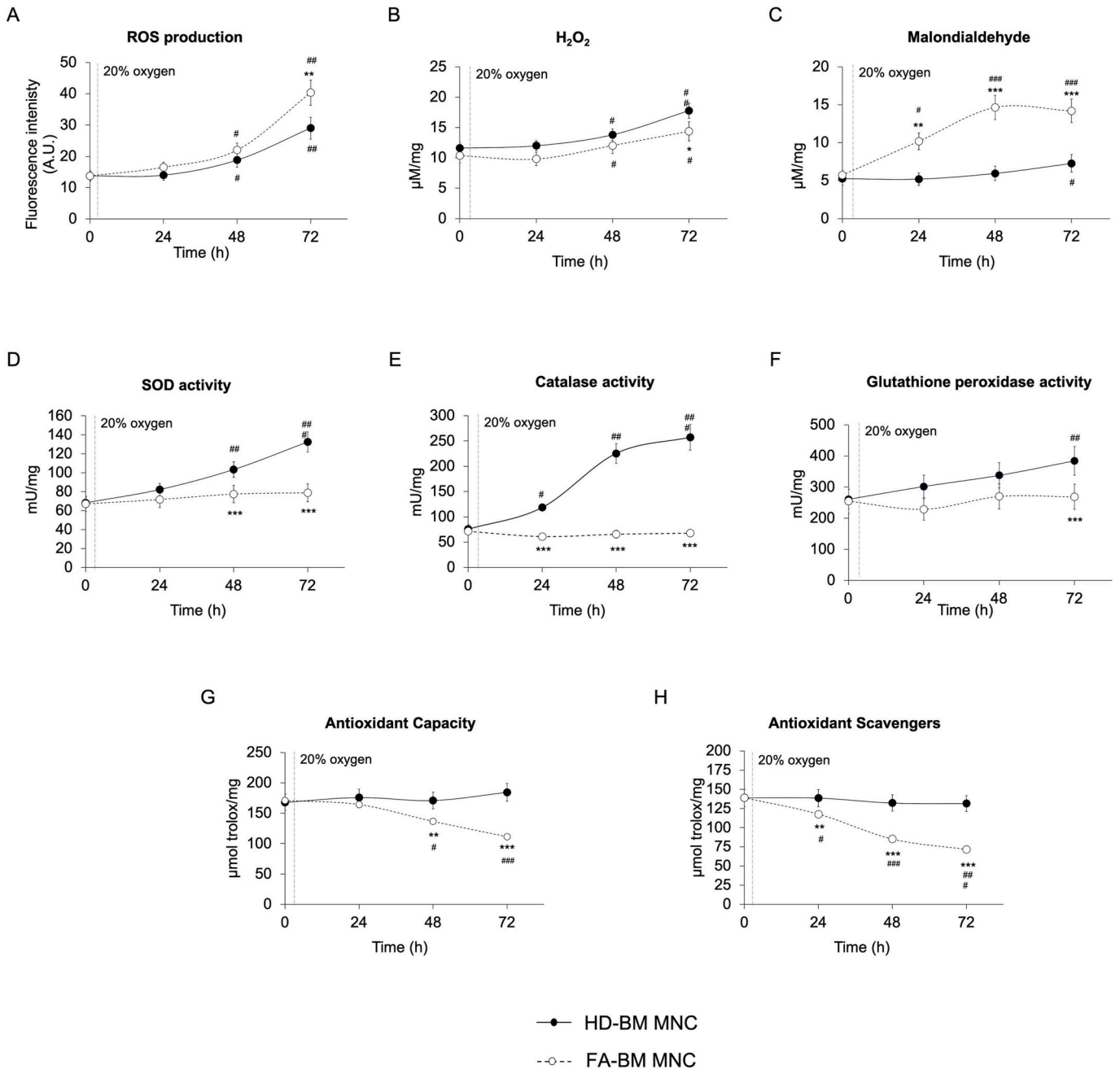

Fig. 4. Oxidative stress production and antioxidant defenses in HD and FA BM-MNCs exposed to normoxic conditions. All data reported in this figure are obtained on BM-MNCs stimulated with PHA and grown in 20% oxygen for 72 h. Black circles represent HD BM-MNCs (n = 8) and white circles represent FA BM-MNCs (n = 6). **(A)** ROS production; **(B)** intracellular content of hydrogen peroxide; **(C)** intracellular level of malondialdehyde, marker of lipid peroxidation; **(D–F)** SOD, catalase and glutathione peroxidase activity assay, respectively, markers of endogenous antioxidant enzymatic defenses; **(G–H)** general antioxidant capacity and level of intracellular antioxidant scavengers, respectively. All data are expressed as mean ± SD. *, ** or *** #, ## or ### indicate a significant difference for $p < 0.05$, 0.01 or 0.001, respectively, between HD BM-MNCs and FA-MNCs at the same time-point. #, ## or ### indicate a significant difference for $p < 0.05$, 0.01 or 0.001, respectively, between the BM-MNCs at time 0 and the same sample at the subsequent time points.

state since the increased production of oxidative stress is not balanced by an enhancement of the antioxidant response, as instead observed in HD. The inability to balance the accumulation of oxidative stress can favor the damage to DNA, that FA cells are not able to repair [27]. This could trigger a vicious circle, in which DNA damage accumulation enhances the oxidative stress and *vice versa*, determining the cancerogenic fate in FA cells [7]. This condition could be exasperated during infection when lymphocytes increase ROS production. Accordingly, the consequently increased exposition of FA cells to DNA damage may explain their arrest in the G2M phase (Fig. 3 and Supplementary Fig. 2).

These alterations are all not detectable when BM-MNCs are stimulated and grow in hypoxic conditions (3% of oxygen) (Figs. 5–7), confirming that only the passage from hypoxic to normoxic conditions unmasks the aerobic metabolism defect of FA cells. In fact, in the hypoxic environment, such as the BM niche, both FA and HD BM-MNCs display anaerobic metabolism, with a low production od oxidative stress, suggesting that the BM niche is a safe environment for FA cells. Moreover, the arrest in the G2/M phase is reverted, although the entire cell cycle is expectedly slower than under normoxic conditions (Fig. 7 and Supplementary Fig. 3).

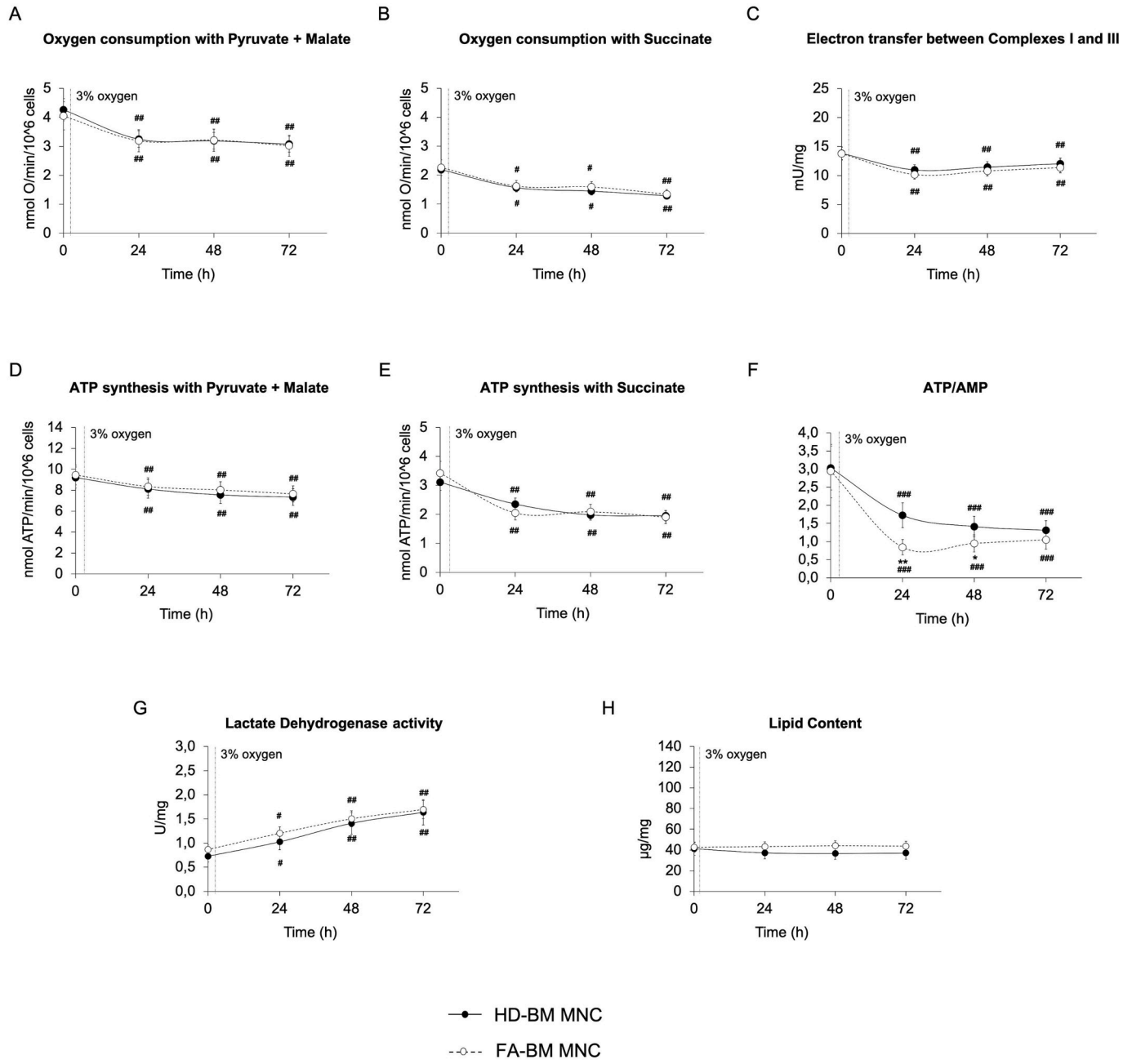

Fig. 5. Modifications of energy metabolism in HD and FA BM-MNCs exposed to hypoxic conditions. All data reported in this figure are obtained on BM-MNCs stimulated with PHA and grown in 3% oxygen for 72 h. Black circles represent HD BM-MNCs (n = 8) and white circles represent FA BM-MNCs (n = 8). **(A, B)** oxygen consumption in the presence of pyruvate + malate or succinate, respectively; **(C)** activity of electron transfer between respiratory complexes I and III; **(D, E)** ATP synthesis trough F_o-F_1 ATP synthase in the presence of pyruvate + malate or succinate, respectively; **(F)** ATP/AMP ratio; **(G)** lactate dehydrogenase activity; **(H)** lipid content. All data are expressed as mean ± SD. * or ** indicate a significant difference for $p < 0.05$ or 0.01, respectively, between HD BM-MNCs and FA-MNCs at the same time-point. #, ## or ### indicate a significant difference for $p < 0.05$, 0.01 or 0.001, respectively, between the BM-MNCs at time 0 and the same sample at the subsequent time points.

The unmasking of the mitochondrial impairment when the MNCs are exposed to 20% O_2 levels could represent one of the causes of bone marrow failure (BMF), which is considered the main clinical defect and cause of death in Fanconi Anemia. Generally, cells are exposed to increased microenvironment oxygen levels when the local cell density decreases [28], which occurs in FA-BM, or when they leave the BM niche to enter the bloodstream. Accordingly, it could be envisaged that chronic recruitment of MNCs from the BM into blood, needed to replenish loss of PB MNCs caused by the new normoxic environment conditions, may lead to a progressive impoverishment of HSC and pancytopenia, which in turn may increase BM O_2 levels thus providing also BM-MNCs with increased local stress [28]. Since the activation of mitochondrial metabolism is considered one of the principal stimuli to the cell differentiation, it is also possible to speculate that the increment of local O_2 level in the BM niche could induce the HSC differentiation by the activation of the mitochondrial metabolism [23]. This could favor the accumulation of DNA damage, as suggested by Walter et al. which have observed that the genomic damage in HSC is a direct consequence of the homeostatic quiescent exit HSC [25]. Moreover, repeated activation of HSCs out of their dormant state provokes the attrition of normal HSCs, which in cells with an altered DNA repair pathway, as in FA cells, could trigger a complete collapse of the hematopoietic system,

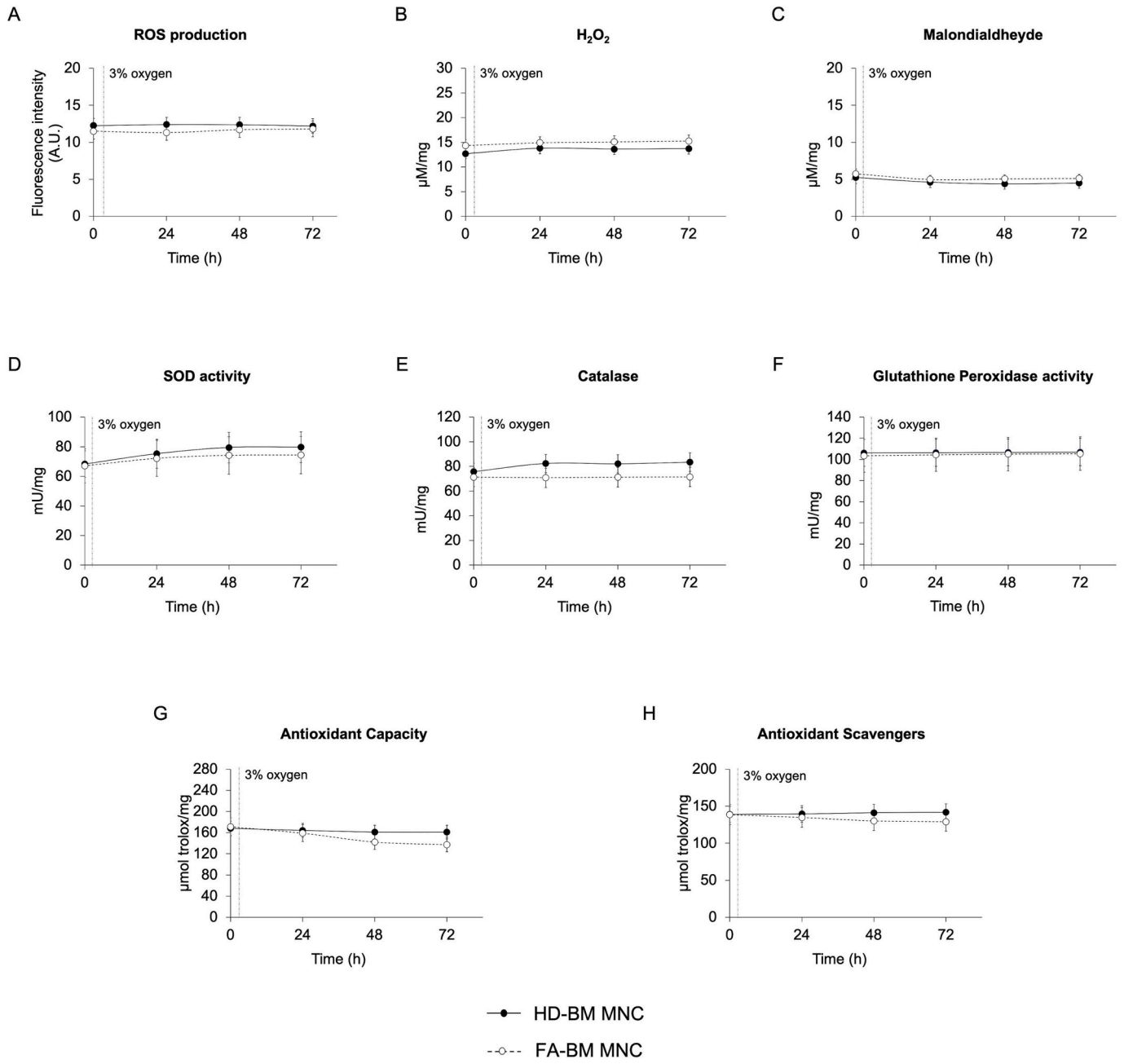

Fig. 6. Oxidative stress production and antioxidant defenses in HD and FA BM-MNCs exposed to hypoxic conditions. All data reported in this figure are obtained on BM-MNCs stimulated with PHA and grown in 3% oxygen for 72 h. Black circles represent HD BM-MNCs (n = 8) and white circles represent FA BM-MNCs (n = 8). **(A)** ROS production; **(B)** intracellular content of hydrogen peroxide; **(C)** intracellular level of malondialdehyde, marker of lipid peroxidation; **(D–F)** SOD, catalase and glutathione peroxidase activity assay, respectively, markers of endogenous antioxidant enzymatic defenses; **(G–H)** general antioxidant capacity and level of intracellular antioxidant scavengers, respectively. All data are expressed as mean ± SD. No significant differences have been observed.

causing a bone marrow failure [25].

However, we are aware that the hypothesis prosed above could represent only one of the aspects involved in BMF and that the experimental linkage of our data with BMF is beyond the scope of this manuscript and our observations may give some information useful in future experiments.

We chose MNC as the most suitable cellular model to evaluate the metabolic changes occurring during the passage from the bone marrow niche to the peripheral circulation, i.e. from hypoxic to normoxic conditions, because this event mainly involves cells that are finishing their differentiation process. Stem cells, instead, do not really represent the best cellular compartment that transits from the BM to the periphery because by definition they are far more oriented towards the self-renewal than to the differentiation function. In addition although MNC represents a heterogeneous cell population: a) they have a quite homogeneous anaerobic/aerobic metabolism [29] and b) the potential metabolic differences within the MNC of FA patients, all carrying the pathogenic genetic abnormalities, are expected to be remarkably lower than the overall differences between the FA and the normal donor groups. Therefore, the difference in energy metabolism we found in our study between healthy and FA MNCs cannot be attributed to sample heterogeneity.

We understand that cell activation with PHA might have skewed the distribution of MNC sub-types, by increasing the T cell pool. However, the biochemical experiments conducted without proliferation stimuli provided results similar to those carried out in the PHA-treated samples.

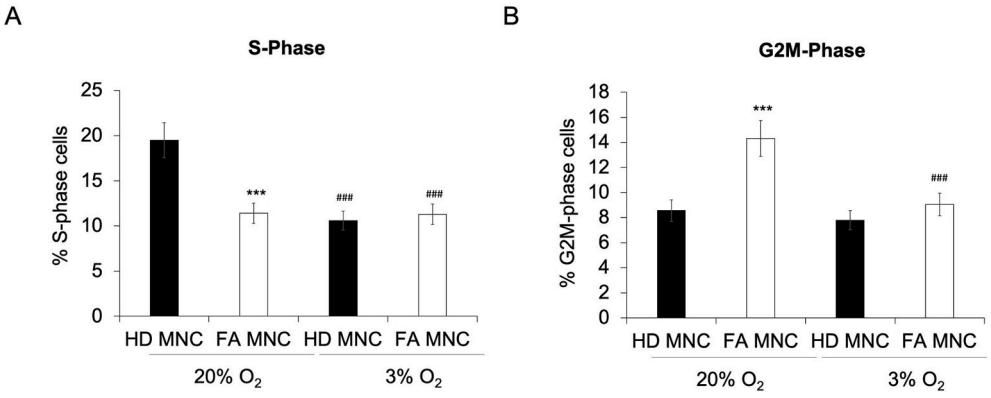

Fig. 7. Comparison of cellular activation and cell cycle between HD and FA BM-MNCs exposed to normoxic or hypoxic conditions. All data reported in this figure are obtained on BM-MNCs stimulated with PHA and grown in 20% oxygen (normoxic) or in 3% oxygen (hypoxic) for72 h. Black columns represent HD BM-MNCs (n = 8 for 20% and n = 5 for 3% oxygen) and white columns represent FA BM-MNCs (n = 6 for 20% and n = 4 for 3% oxygen). **(A)** percentage of cells in S-phase; **(B)** percentage of cells in G2/M-phase. All data are expressed as mean ± SD. ** or *** indicate a significant difference for $p < 0.01$ or 0.001, respectively, between the HD BM-MNCs and FA BM-MNCs in the same oxygen condition. ### indicates a significant difference for $p < 0.001$ between the same sample grown in normoxic or hypoxic condition.

Altogether, these considerations indicate that it is the mere passage of FA cells to normal oxygen concentrations that induces a defect in mitochondrial metabolism, regardless of the MNC cell type distribution and the proliferative status.

Aiming to reach a reasonably sized study sample, we collected FA different by age, gender, complementation group, and hematological disease severity. Interestingly, the different FA samples displayed the same trend of the analyzed parameters, as revealed by the low inter-sample experimental variability shown by the low standard deviation values reported in the graphs. This indicates that the alteration of energy metabolism in FA patients occurring when MNC exit the BM niche is a crucial point inherent to the disease, that goes beyond the different patients' characteristics.

5. Conclusions

Data reported in this manuscript indicate that the transition from BM to PB might be crucial in the context of the metabolic/mitochondrial defect highlighted in FA, and provide the basis for further investigating the hypothesis that, during the passage from BM to PB, FA cells face the simultaneous activation of the mitochondrial metabolism and the increment of ROS generation. The significant presence of DNA damages and the lack of activation of the antioxidant response may thus determine the inability of FA cells to correctly handle the chain of events necessary for the maturation process, thus accelerating the hematopoietic system failure.

Authors contributions

E.C, P.D., S.B., F.R. and S.R. performed experiments; F.P., P.F. and B. C. provided the samples; E.C., P.D., S.B., V.N., C.D. and S.R. analyzed results; S.B. and S.R made the figures; E.C., P.D., V.N. and S.R. designed the research and wrote the paper.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101618.

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