Response to: "Heterogeneous phenotypic expression of C1QBP variants is attributable to variable heteroplasmy of secondary mtDNA deletions and mtDNA copy number"

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To the Editor,

This letter is a response to the commentary by Dr. Finsterer (Finsterer, 2020) on our paper entitled "Homozygous mutations in *C1QBP* as cause of progressive external ophthalmoplegia (PEO) and mitochondrial myopathy with multiple mtDNA deletions" (Marchet et al. 2020). Here we try to address the key concerns raised by him.

We did not make any distinction between pure PEO and PEO plus, but just considered PEO as a clinical sign which is extremely useful to suggest the presence of a mitochondrial disorder, in particular of primary mitochondrial myopathy (PMM). We stressed the use of the term PEO because it is the one used in OMIM to describe these genetic diseases, with 10 entry genes classified as "Progressive external ophthalmoplegia with mtDNA deletions" (Phenotypic Series - PS157640). Moreover, we stated that PEO usually (and not necessarily) starts with ptosis; indeed, the two patients with *C1QBP* mutations we described (P1 and P2) presented with ptosis at disease onset. Accordingly, the consortium on Trial Readiness in Mitochondrial Myopathies confirmed that "the most common presentation of PMM is chronic PEO" and that "PEO is usually accompanied by bilateral eyelid ptosis, which is often the presenting symptom" (Mancuso et al. 2017).

In the first paper about *C1QBP* mutations (Feichtinger et al. 2017), all the four reported patients presented with cardiac involvement leading the authors to sustain in the title that biallelic *C1QBP*mutations cause severe neonatal-, childhood-, or later-onset cardiomyopathy. Although we cannot exclude subtle cardiac dysfunction in our patients (since they did not undergo long-term ElectroCardioGram recordings, transesophageal echocardiography, or cardiac MRI), their standard ECGs and echoCGs were normal and thus we still consider valid the main message of our paper: subjects with *C1QBP* mutations may present with adult-onset PEO/PMM phenotype, without overt cardiomyopathy.

We obviously agree that mtDNA genetics is peculiar, and that different level of heteroplasmy may explain the variable phenotypic expression of mtDNA mutations, but we are talking here about mutations in a nuclear gene with an autosomal recessive inheritance. No DNA from any family members was available for segregation studies but we expect that the parents of P1 and P2 (reported to be second-grade and third-grade cousins, respectively) would have tested as heterozygous carrier. Both patients have no siblings. Anyway, detailed clinical investigations of first-degree relatives, not harboring the homozygous *C1QBP*mutation, would be not informative and hence, in our opinion, useless.

The presence of multiple mtDNA deletions is a secondary effect of the mutations in *C1QBP* , although the exact mechanism linking C1QBP with mtDNA maintenance and stability is not known. The assertion

that residual protein amount and different localization of the *C1QBP*mutations could explain the variable observed phenotypes, including both clinical symptoms and molecular/biochemical defects (mtDNA deletions, mitochondrial respiratory chain - MRC - complex activities, histochemical staining) remains plausible. As suggested by Dr. Finsterer, it is possible that the phenotypic variability of*C1QBP* variants is fairly attributable to variable heteroplasmy of secondary mtDNA deletions and/or mtDNA copy number, but it is not possible to test this hypothesis in detail (e.g. throughout assessment of heteroplasmy in different muscle types, including extraocular muscle, and at different time points during disease progression). All the experiments reported in our paper (Marchet et al. 2020) were performed on a single muscle biopsy from quadriceps of the two patients. Densitometry analysis of the Southern blot (Fig. 1C reported in Marchet et al. 2020) revealed 58% and 48% mtDNA deleted species in P1 and P2, respectively. Nevertheless, an exponential accumulation of multiple mtDNA deletions has been reported in post-mitotic tissues during aging (Cortopassi et al. 1992), and thus we cannot exclude an influence of the age at biopsy and of duration from onset disease on this result.

Another important issue is related to the presence of mosaic of cells in the same tissue, which is expected also in our patients based on histological analyses showing fibers with different features likely related to different levels of mtDNA deletions. Accordingly, previous single-cell analysis has revealed that mtDNA deletions are distributed as a mosaic of affected and non-affected cells (He et al. 2002). While the link between heteroplasmy level and biochemical/clinical phenotype is well established in patients with single large-scale mtDNA deletion, it is more complex in patients with multiple mtDNA deletions, where each muscle fiber may contain different, and more than one, mtDNA deleted species (Lehman et al. 2019).

Regarding mtDNA copy number, we did not assess it directly but we expect the same limitations reported above, because of experimental data from a single specimen characterized by intercellular heterogeneity. Nevertheless, some indirect indications can be obtained by already reported histological and biochemical findings. Muscle cells with high levels of mtDNA deletions typically show mitochondrial proliferation as compensatory mechanism, which is reflected by the presence of Ragged Red Fibers (RRF). Moreover, the activity of citrate synthase (CS) is often used as a quantitative marker for mitochondrial mass. In both P1 and P2, we observed the presence of many RRF but the CS activity in total muscle homogenate was in the normal range (118% and 100% of the controls' mean for P1 and P2, respectively) again confirming variable heteroplasmy in different fibers but indicating an overall normal amount of mitochondria and, roughly, of mtDNA copy number.

All the above considerations are useful to explain also the last concern by Dr. Finsterer, i.e. why biochemical investigations of P2 were normal. Notably, the histochemical staining for cytochrome c oxidase (i.e. complex IV) was decreased in scattered fibers from P2, despite biochemical assay showed normal values for MRC complexes. It has already been reported that the activities of respiratory complexes in muscle from PEO patients range from normal to about 50% of the controls' mean (Viscomi & Zeviani, 2017). Likewise, normal MRC activity has been observed in several patients presenting with mtDNA deletions caused by mutations in nuclear genes (e.g. *POLG, POLG2, RNASEH1* ...). More recently, by single cell studies some authors demonstrated that genetic defects do not strictly correlate with MRC deficiency in fibers with multiple mtDNA deletions (Lehman et al. 2019).

In conclusion, the very limited number of *C1QBP* cases reported up to now and their allelic heterogeneity hamper to define any genotype–phenotype correlations, but nevertheless indicate a huge clinical spectrum associated with *C1QBP* mutations, ranging from early-onset severe cardiomyopathy to adult-onset PEO/PMM.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests.

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