Parsing the differences in affected with LHON: genetic versus environmental triggers of disease conversion

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Sir,

Leber's hereditary optic neuropathy (LHON) is one of the best-studied mitochondrial diseases (for review see Carelli *et al*., 2004; Yu-Wai-Mann *et al*., 2011). Classically, there is a subacute loss of central vision that affects prevalently males, peaking in young adulthood. After a few months, optic atrophy becomes evident and the patient enters a chronic phase, in most cases characterized by stable blindness. Infrequently a partial recovery of visual acuity occurs. Exceptions have been reported. Concerning age of onset, childhood cases seem to represent a different version of LHON that often resembles dominant optic atrophy (Barboni *et al*., 2006), whereas very late-onset cases have also been reported, widening the spectrum (Shah *et al*., 2008; Yu-Wai-Man *et al*., 2008; Giraudet *et al*., 2011; Dimitriadis *et al*., 2014). There is also a certain degree of heterogeneity in clinical expression, ranging from mild to severe cases, and the rates of spontaneous recovery of vision vary with mutation type (Carelli *et al*., 2004, 2011; Yu-Wai-Mann *et al*., 2011).

A maternally inherited mitochondrial DNA (mtDNA) mutation is necessary but not sufficient to develop

optic neuropathy. There are three common mtDNA mutations affecting complex I (i.e. m.11778G>A/MT-ND4, m.3460G>A/MT-ND1 and m.14484T>C/MT-ND6). Most cases remain as asymptomatic carriers, with males much more likely than females to undergo conversion to the affected state. Thus, male prevalence and incomplete penetrance remain areas of active investigation.

Environmental factors have now been demonstrated as frequent triggers of the disease. The most common is tobacco smoke, in combination with or without alcohol consumption (Sadun *et al*., 2003), as corroborated by Kirkman and colleagues (2009) with their large survey of LHON affected and unaffected mutation carriers. More rarely, exposure to toxins, various forms of smoke, or certain drugs appear to trigger the disease (Mackey *et al*., 2003; Luca *et al*., 2004; Sanchez *et al*., 2006; Carelli *et al*., 2007; Seo *et al*., 2010). Tobacco and alcohol have also been implicated, together with folate and vitamin B12 deficiency, in the pathogenesis of metabolic optic neuropathies, clinically very similar to LHON (phenocopies) (Sadun, 1998; Carelli *et al*., 2002). Recently, it has become clear that a subgroup of the so-called 'tobacco-alcohol amblyopia' (TAA) patients in fact carried one of the three main mtDNA mutations predisposing to LHON (Cullom *et al*., 1993; Amaral-Fernandes *et al*., 2011).

From the genetic point of view, certain combinations of polymorphic variants characterizing mtDNA haplogroups may also be influential in determining disease penetrance (Carelli *et al*., 2006; Hudson *et al*., 2007). Furthermore, gender difference has been partly ascribed to the role played by oestrogens in regulating mitochondrial biogenesis in females (Giordano *et al*., 2011), and the compensatory activation of mitochondrial biogenesis has been shown to drive penetrance in both genders (Giordano *et al*., 2014; Bianco *et al*., 2015). A further level of complexity is the variable sensitivity of mtDNA haplogroup backgrounds to environmental exposure, for example to toxins such as hexane (Ghelli *et al*., 2009), which have been implicated as triggers in LHON (Carelli *et al*., 2007). It is also thought that, in addition to mtDNA and environmental factors, further genetic modifiers in the nuclear genome influence LHON penetrance (Carelli *et al*., 2003). However, the identification of such nuclear genetic modifiers has been elusive, possibly because of several confounding factors.

Here, we present the clinical and histological data of three brothers, all carrying the most common m.11778G>A/MT-ND4 homoplasmic LHON mutation on haplogroup J mtDNA background, who all had similar habits of smoking and drinking heavily since puberty. They all had visual loss due to LHON, but with very different clinical features and tempo. We propose that their cases were most likely modulated by defined differences in genetics and environment, allowing us to pose the problem of different forms of LHON that can each be considered as distinct. The first patient lost vision as a young adult with a classical LHON pattern. The second and third brothers did not lose vision until middle age with features of tobacco-induced LHON optic neuropathy. Remarkably, one of these two unexpectedly recovered his vision after cessation of smoking in his 60s. Briefly, we summarize the longitudinal clinical features of all three cases and significantly, eye histopathology in the two who underwent post-mortem studies. The three brothers are part of the large SOA-BR family from Brazil, which had been systematically and prospectively studied for over 10 years (Sadun *et al*., 2003).

Case 1: classical LHON

This patient was an agricultural worker who was exposed to pesticides. He was also a heavy smoker (about one pack of homemade cigarettes/day) from the age of 12. He suffered an acute loss of vision at 27 years of age. His vision loss was profound, at hand motions, and accompanied by severe bilateral optic atrophy and optic disc cupping (Fig. 1A, left panel). His vision remained stable for the following 25 years and he died at age 52 from an acute myocardial infarction. We obtained his optic nerves post-mortem and previously published the histopathology (La Morgia *et al*., 2010). Overall, we found a very severe depletion of axons in a pattern of atrophy typical of LHON with only a few fibres spared along the nasal rim (Fig. 1A, left panel). In general, his clinical case is best described as classical LHON with severe optic atrophy as confirmed histologically.

Case 2: TAA/LHON

This is the older brother of Case 1 who also was an agricultural worker exposed to pesticides. Like his brother, Case 2 smoked heavily (homemade cigarettes about two packs/day) from the age of 13. However, his loss of vision occurred insidiously and progressively over ~2 years and not beginning until the age of 51 and then progressing to light perception bilaterally at last evaluation (59 years). Despite the severe visual loss, his fundus examination showed only mild optic atrophy limited to the temporal side (Fig. 1A, middle panel). This patient also died of acute myocardial infarction, at 59 years of age. Post-mortem evaluation showed, as previously described (La Morgia *et al*., 2010), a regional loss of axons limited to the temporal sector, thus severely affecting only the axons belonging to the papillomacular bundle (Fig. 1A, middle panel). The mild extent of his axonal loss was not congruent with the very severe visual defect. This late-onset LHON case, associated with a life-long heavy exposure to tobacco smoking, had clinical features resembling nutritional, or so called TAA, both in terms of the insidious onset and also reflected by the axonal loss strictly limited to the papillo-macular bundle with good preservation of the remaining fibres of the optic nerve at histopathology. In general, this case is best described as LHON but with late onset and associated with heavy tobacco use.

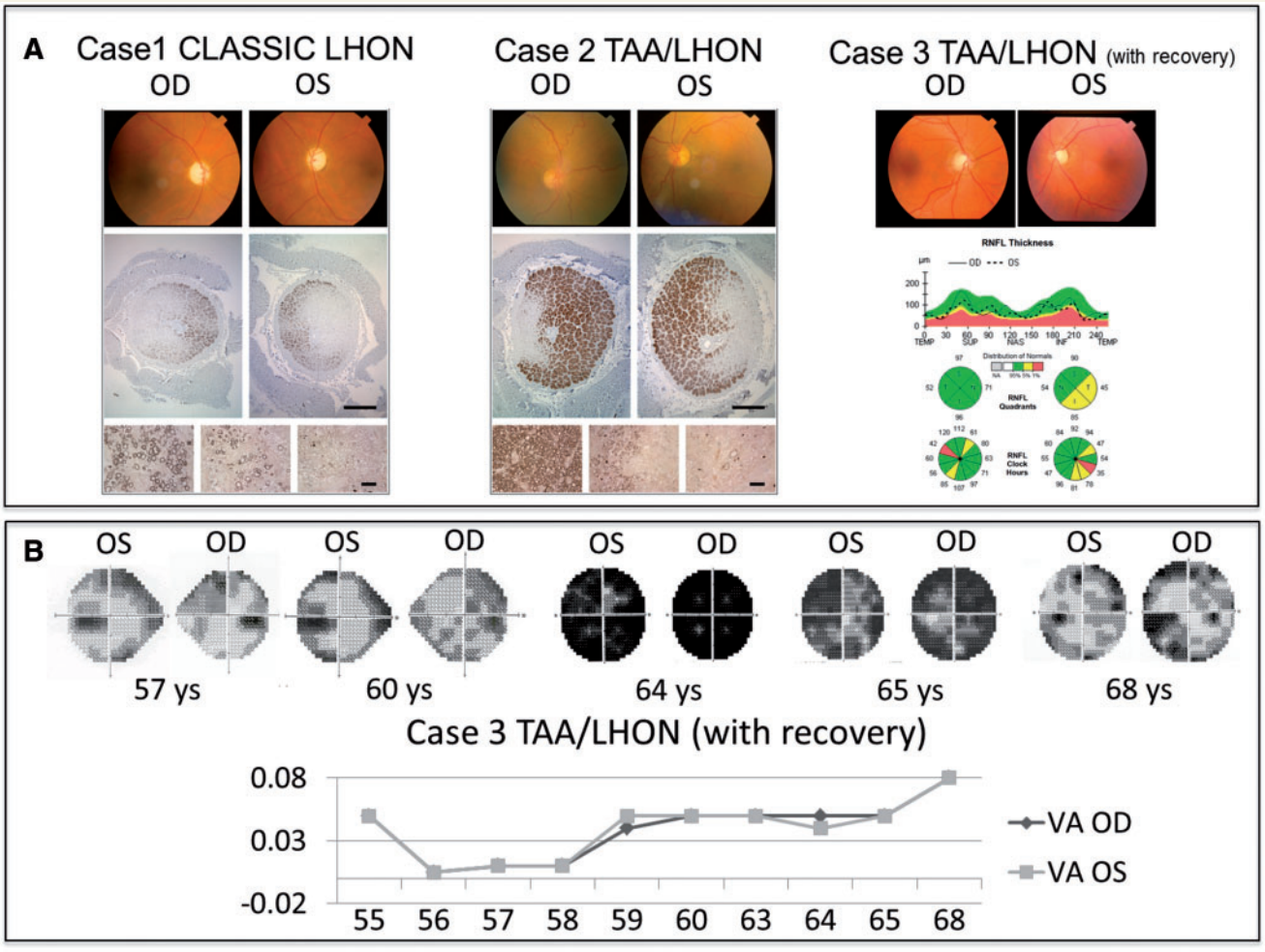


Figure 1 Three brothers with different LHON types. (**A**) *Left* panel shows fundus picture and cross-sectional optic nerve histopathology (low and high magnification) of Case 1 (classic LHON, type I), demonstrating optic disc atrophy and profound axonal depletion with residual axons only in the far nasal periphery. *Middle* panel shows fundus picture and cross-sectional optic nerve histopathology (low and high magnification) of Case 2 (TAA-like LHON, type II), demonstrating sectorial pallor of the temporal sector of the optic disc with selective loss of axons limited to the temporal side of the optic disc, corresponding to the papillo-macular bundle. *Right* panel shows fundus picture and stratus OCT assessment of retinal nerve fibre layer (RNFL) thickness of Case 3 (TAA-like LHON, type II), demonstrating sectorial pallor of the temporal side of the optic disc, with significant reduction of RNFL limited to the temporal sector, corresponding to the papillo-macular bundle. (**B**) Humphrey visual field (*upper* panel) and visual acuity (*lower* panel) follow up of Case 3, showing a recovery of visual function after he quit smoking at age 61. OD = oculus destrum (right eye); OS = oculus sinistrum (left eye); VA = visual acuity.

Case 3: TAA/LHON with recovery

The third brother is a 68-year-old male who experienced bilateral progressive visual loss beginning about age 42. He was a heavy smoker (at least one pack/day of homemade cigarettes) from the age of 10 but quit at age 61 after a first acute myocardial infarction and advice to modify his lifestyle. His visual acuity was counting fingers OU (*oculi uterque*) in his late 50's. Surprisingly, 4 years after quitting smoking his vision began improving, at the age 65, reaching 20/250 OU at last evaluation (68 years). This recovery of visual acuity was paralleled by improvement of visual fields (for both visual acuity and fields see Fig. 1B). At last evaluation, he had mild temporal pallor and his optical coherence tomography (OCT) showed thinning of RNFL thickness only in the temporal sector (Fig. 1A, right panel). He recently had a second fatal myocardial infarction, but his eyes were not available post-mortem. Overall, as his brother with late-onset LHON, this patient also displayed clinical features resembling TAA, with progressive loss of vision followed by more recent recovery associated with the cessation of cigarette smoking. In general his case is best described as LHON with late onset, associated with heavy smoking and recovery of vision after discontinuation of tobacco use.

It must be noted that both brothers with TAA-like LHON had discordance between structure and function. There was severe loss of vision compared with the anatomical evidence of good preservation of axons in the optic nerve demonstrated by histopathology in one case, and by *in vivo* OCT in the other brother. We think that the preservation of axons represented a reservoir for the unusual late recovery of vision experienced by the third brother after he quit smoking.

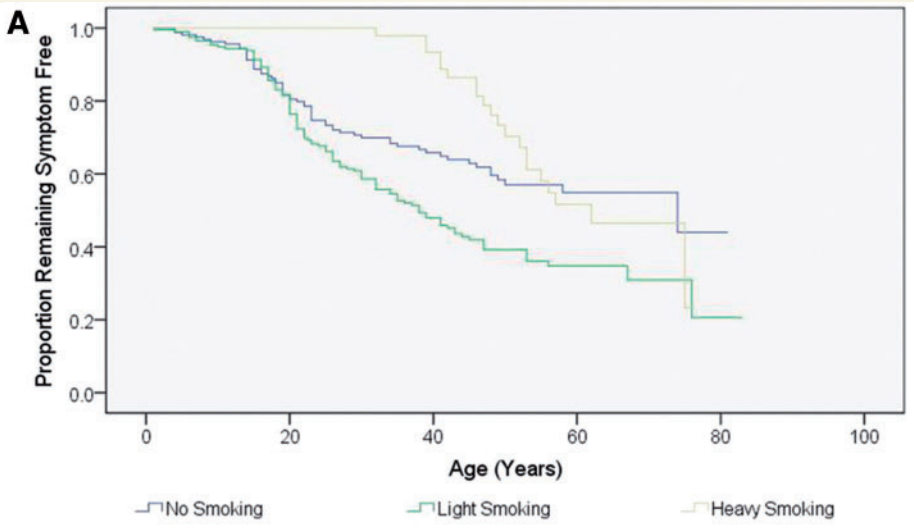
It is well accepted that the primary mtDNA mutation is necessary but not sufficient for visual loss in LHON (Carelli *et al*., 2003, 2014). Hence, there must be a second hit that triggers visual loss, which could be either genetic (a nuclear modifier) or environmental, or a combination of both. As mentioned above, one major environmental trigger is smoking (Sadun *et al*., 2003; Kirkman *et al*., 2009). In the attempt to parse these two elements apart, we re-examined the relationship between age of onset and environmental triggers, starting from the very comprehensive study by Kirkman and colleagues (2009). Our attention was drawn to one particular figure of their study (Fig. 3A in the original article and reshown here as Fig. 2A). Their Kaplan-Meier curve showed that those patients who had the heaviest smoking history (defined as 'cumulative smoking' of pack-years) had the oldest age of onset, whereas, light smoking slightly accelerated the rate of visual loss compared to non-smokers. This paradox, that heavy cumulative smoking looked apparently protective, was not explained by the authors and the issue was commented on by Nancy J. Newman (2009) in her dedicated editorial. In the light of new evidence, as exemplified by the three brothers above, we reconsidered the published curve of heavy smokers as representing a separate group of affected LHON patients that can be defined with three propositions:

(i) by definition all those who lose vision from LHON have an mtDNA mutation as the primary hit;
(ii) visual loss requires a second 'hit' that can be either genetic (nuclear modifier) or environmental (such as smoking) or a combination of the two;
(iii) the population of pure genetic cases (LHON type I) can be distinguished from environmentally triggered cases (LHON type II) in several regards including age of onset, clinical features (tempo and severity) and exposure history.

As noted above, the three brothers exemplified the type I and II categories of LHON affected (Case 1 type I; Cases 2 and 3 type II). Obviously, a subset of type I LHON may coincidentally smoke, as was for the Brazilian Case 1, but for reasons to follow, we think that types I in general are predetermined to become affected because of genetic nuclear modifiers.

Thus, we tested this hypothesis in our large cohort ($n = 260$) of LHON affected ($n = 134$) and unaffected mutation carriers ($n = 126$) from 75 Italian pedigrees by re-interviewing the patients with the Kirkman criteria for purposes of comparison. Despite the differences between our cohort of 260 Italian individuals carrying one of the LHON primary mutations and the cohort collected by Kirkman in their study (Table 1), we obtained a Kaplan-Meier curve (Fig. 2B) remarkably similar to that of Kirkman and colleagues (2009). We found, as they did, that the heavy smokers had the latest onset (Fig. 2A and B). We then differentiated LHON affected types I and II by considering separately those likely to be pure genetic cases (no smoking/drinking) from those exposed to common environmental triggers (smoking/drinking) and then computed the age of onset for each group. The results are shown in Fig. 3A–C. The pure genetic cases (type I) formed a classical binomial distribution peaking at ~age 15 (Fig. 3A). Those with an environmental trigger had a broader and later age of onset of visual loss peaking at ~age 28. This latter group probably contained both type I and type II LHON affected. Stratifying by gender, we obtained similar results except that there were fewer affected females, with shifted age of onset for both types I and II, the latter cases over 40 possibly reflecting menopause as a further disease trigger (Fig. 3B). In fact, females are at lower risk, probably due to the protective effect of oestrogens during the fertile period (Giordano *et al*., 2011). On the other hand, females are also vulnerable to environmental factors such as smoking. Although the discriminating threshold of age <30 versus >30 is arbitrary, one can see from Fig. 3A and C that the two groups of patients largely divide at age 30 with most of the non-smokers falling to those <30 and only nine non-smokers being >30 (Fig. 3C). Interestingly, four of these nine individuals had significant exposure to other environmental toxins (not shown). Considering all smokers in Fig. 3C, there was a tight, almost linear (P-value $= 1.4 \times 10^{-10}$, $r^2 = 0.63$) paradoxical correlation between age of onset of visual loss and pack-per-years. We believe there is an inverse correlation of genetic modifying factors and cumulative smoke exposure in this population, reflecting increasing risk with prolonged exposure, but decreasing influence of genetic modifying factors. Hence, we conclude that high cumulative smoking is not protective but represents a higher proportion of type II cases, whereas individuals becoming affected at <30 years of age most probably represent a combination of genetics and tobacco smoking, but with the predominant weight of genetic modifying factors determining type I cases.

Overall, we reason that the pure type II cases (no genetic modifiers) would probably have remained as unaffected carriers, if they had not smoked. In other words, type II would not be 'visible' as affected if not for their heavy, long-lasting smoke exposure. This should be kept in mind when advising LHON carriers not to smoke. It may seem arbitrary to parse the two groups by age (<30 versus >30). However, this came from comparing the distribution of the curves and noting that in non-smokers, there is a cluster of younger patients and that in smokers the cluster is of older patients. Of course, based on our distribution of cases shown in Fig. 4C, as previously remarked, there are



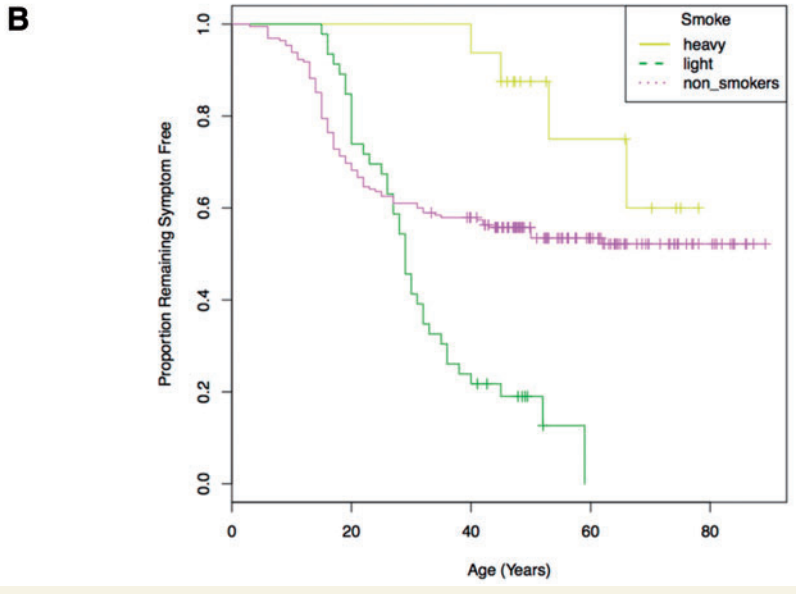


Figure 2 Kaplan-Meier curves for age of onset in independent LHON cohorts. (**A**) Kaplan-Meier curve from Kirkman *et al.* (2009). Remarkably, the LHON heavy smokers (defined as 'cumulative smoking' of pack-years) present with delayed age of onset at ~40 years or later, whereas the LHON non-smokers and light smokers present the steepest decline across the age of 20. (**B**) Kaplan-Meier curve in the Italian cohort. The same analysis as in Kirkman *et al.* (2009) applied to an Italian cohort of LHON patients and unaffected carriers faithfully reproduced the same observation that 'LHON heavy smokers' become affected at age 40 or later as opposed to LHON non-smokers and light smokers who become affected across the age of 20.

probably mixtures of types I and II in the 20–35 age range. Dimitriadis and colleagues (2014) recently proposed a similar distinction in a large LHON cohort (251 affected and 277 unaffected LHON mutation carriers, apparently from the UK and Germany) by using the cut-off age of 50 to define 'late-onset cases'. They found 20 such cases failing to observe any significant difference between late onset- and typical LHON patients with regard to daily tobacco and weekly alcohol consumption before disease onset (Dimitriadis *et al*., 2014). It is unclear if this cohort is the same or partially overlaps with that of Kirkman and colleagues (2009), but it would be very interesting to analyse their data in a parallel fashion as we presented in this letter.

In conclusion, there are two broad subgroups of LHON affected types with very different ages of onsets, differences in tempo and clinical features and extent of histopathology or OCT parameters, well illustrated by the examples of the three Brazilian brothers. These two types of LHON affected constitute a major confounding factor hampering the recognition of genetic modifiers. We envision future studies, aimed at identifying LHON genetic modifiers,

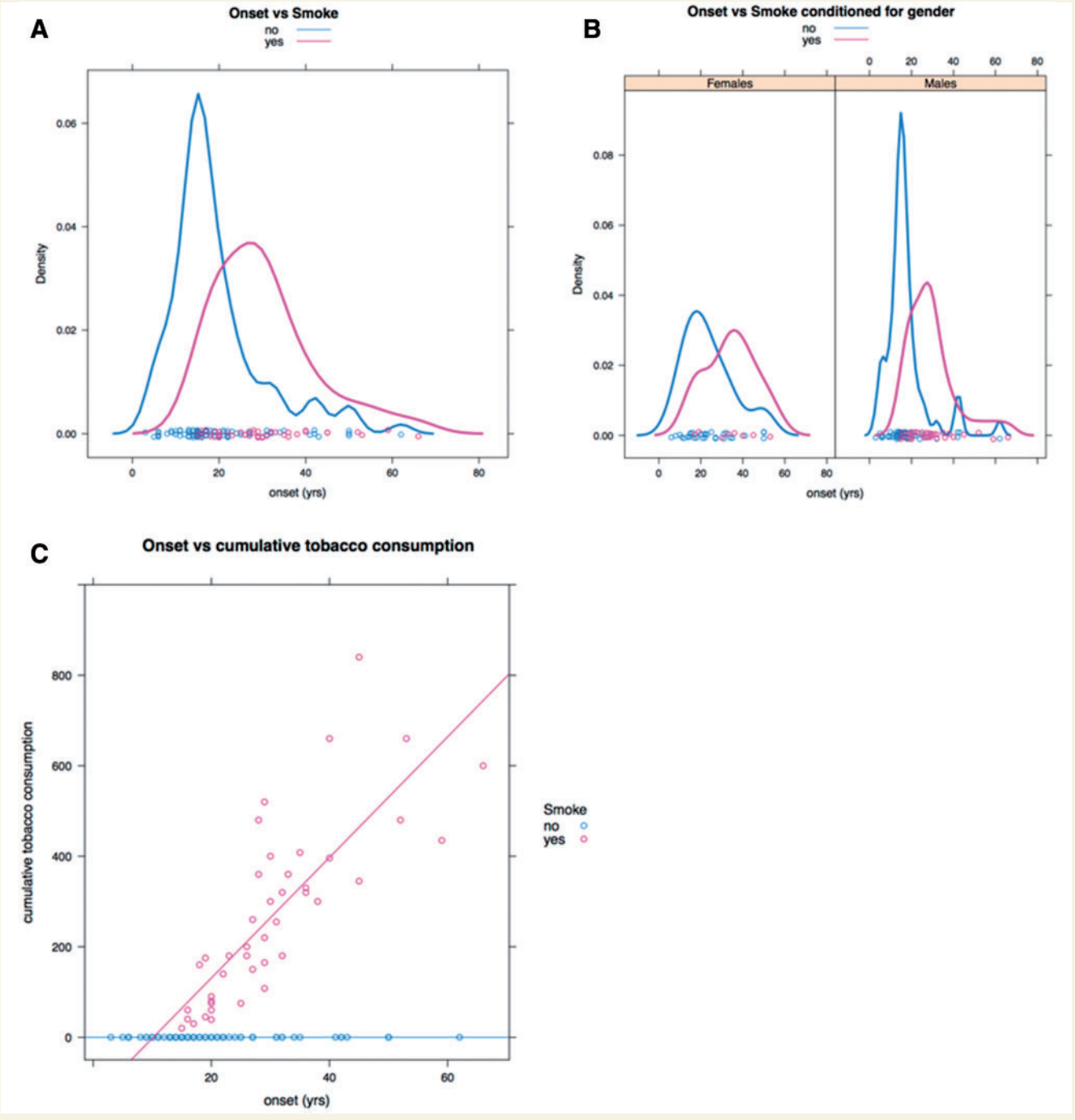


Figure 3 Distribution of age at onset for non-smokers versus smokers in the Italian LHON cohort. (**A**) Age at onset versus tobacco smoke. A tight bell-shaped distribution, peaking at about 15 years of age, characterized the age at onset of the LHON non-smokers (pure genetic cases, type I), whereas the group of LHON smokers (type I and type II) had a wider distribution, peaking at about age 28. (**B**) Age at onset versus tobacco smoke conditioned by gender. Stratifying the previous data by gender, females show a shift to later age at onset in both groups, the LHON non-smokers and smokers. (**C**) Age at onset versus cumulative tobacco smoke. The age at onset of the LHON non-smokers (pure genetic cases, type I) largely separate from that of LHON smokers (types I and II) at age 30 years. Thus, LHON affected with age at onset <30 are mostly non-smokers mixed with some light-smokers, qualifying the type I patients for which the prevalent disease trigger is the genetic background characterized by still unknown modifying variants.

that concentrate on type I patients whose loss of vision was at a younger age and therefore mostly genetic. Patients with type II LHON may have a previously unrecognized potential for visual recovery. Genetic factors other than the primary mtDNA mutations might still play a role in type II LHON patients, particularly in how they cope with toxic exposure. It should be remembered that tobacco smoking exerts many different effects on mitochondrial function,

Table 1 The Kirkman and Italian cohorts

Cohort	Kirkman *et al.* (2009)	This letter
Proportion of affected males	74.5%	69%
Mean age at onset of visual loss	27.9 years (SD = 14.9)	22.9 years (SD = 12.4)
Visual failure occurred before the age of 10 years	*n* = 15 (7.7%)	*n* = 9 (6.8%)
Visual failure occurred after the age of 50 years	*n* = 18 (9.2%)	*n* = (3.8%)
Proportions of smokers in the affected group	~66%	32%
Proportions of smokers in the carriers group	~50%	16%

SD = standard deviation.

including an increase in reactive oxygen species (Rua Ede *et al*., 2014) and inhibition of mitochondrial respiration (Miró *et al*., 1999). To unwrap the mechanism of tobacco toxicity in LHON we studied affected and carrier individuals from our cohorts and their cells (Giordano *et al*., 2015). This extended set of results show that tobacco smoke and its derivatives lower mtDNA copy number, affect oxidative phosphorylation and reactive oxygen species detoxification pathways, possibly interfering with mitochondrial biogenesis. Thus, tobacco smoke ultimately can hamper the compensatory response needed to keep unaffected mutation carriers from converting.

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