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# Skewed X-chromosome inactivation drives the proportion of *DNAAF6*-defective airway motile cilia and variable expressivity in primary ciliary dyskinesia

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## Background

Cilia and flagella are evolutionarily conserved organelles whose motility relies on the outer and inner dynein arm complexes (ODAs/IDAs). Defects in ODAs/IDAs result in primary ciliary dyskinesia (PCD), a rare disease characterized by recurrent airway infections, male infertility and *situs inversus* (in 50% of cases). To date, about 50 genes have been implicated in PCD, usually following an autosomal-recessive pattern.

*DNAAF6* is involved in the assembly of the dynein arm complexes. *DNAAF6* pathogenic variations were considered to cause an X-linked recessive PCD, with carrier mothers supposed to be asymptomatic.

**Purpose** : Our objective was to assess the possible clinical and ciliary consequences of allelic specificity of X-chromosome inactivation (XCI) in 6 mothers of PCD patients carrying *DNAAF6* pathogenic variations.

## Airway phenotypes of mothers heterozygous for a *DNAAF6* pathogenic molecular defect

Mother ID number Age range (yr) at the time of study	Genotype	Respiratory-symptom onset (age)	Primary ciliary dyskinesia-related investigations			Airway disease	
			nNO <sup>1</sup> (μL/min)	HSVA (% motile/immotile ciliated tufts) (CBF)	TEM Abnormal cilia (%)	Upper	Lower
M1 DCP1339 45-50	c.[3.27-Mb del],[=] p.[0],[=]	-	ND	ND	ND	None	None
M2 8822GM0145 35-40	c.[118-kb del],[=] p.[0],[=]	-	ND	ND	ND	None	None
M3 16GM00624 45-50	c.[3.73-Mb del],[=] p.[0],[=]	Adult (38 yr)	Not evaluated	Motile: 83% (8.1 Hz) Immotile: 17%	Not assessable (no ciliated cells)	Intermittent nasal obstruction	Some bronchitis episodes from 38 years old
M4 DCP1748 35-40	c.[1.93-Mb del],[=] p.[0],[=]	Childhood (10 yr)	159	Motile: 77% (4.5 Hz) Immotile: 23%	Absence of both dynein arms (3%)	Sinusitis during childhood	Wet cough during childhood; none during adulthood
M5 DCP1848 40-45	c.[511C>T],[=] p.[Gln171*],[=]	Childhood (5 yr)	248	Motile: 50% (15.5 Hz) Immotile: 50%	Absence of both dynein arms (80%)	Chronic rhinosinusitis; otitis only during childhood Partial sinus opacification	Some bronchitis episodes; one episode of pneumonia (at 39 yr)
M6 DCP1994 45-50	c.[397G>T],[=] p.[Asp133Tyr],[=]	Childhood (6 yr)	Normal	Motile* (9.5 Hz) Immotile*	Absence of both dynein arms (45%)	Serous otitis; chronic rhinosinusitis with surgery (at 6 & 20 yr); nasal polyposis Total sinus opacification Purulent secretions	Several episodes of pneumonia, chronic bronchitis, exacerbations with intermittent <i>P. aeruginosa</i> Bronchiectasis involving the left inferior lobe

nNO: nasal nitric oxide; HSVA: high-speed videomicroscopy analysis; CBF: ciliary-beat frequency; TEM: transmission electron microscopy; ND: not done.

\*ND in PCD patients <77 μL/min (2).

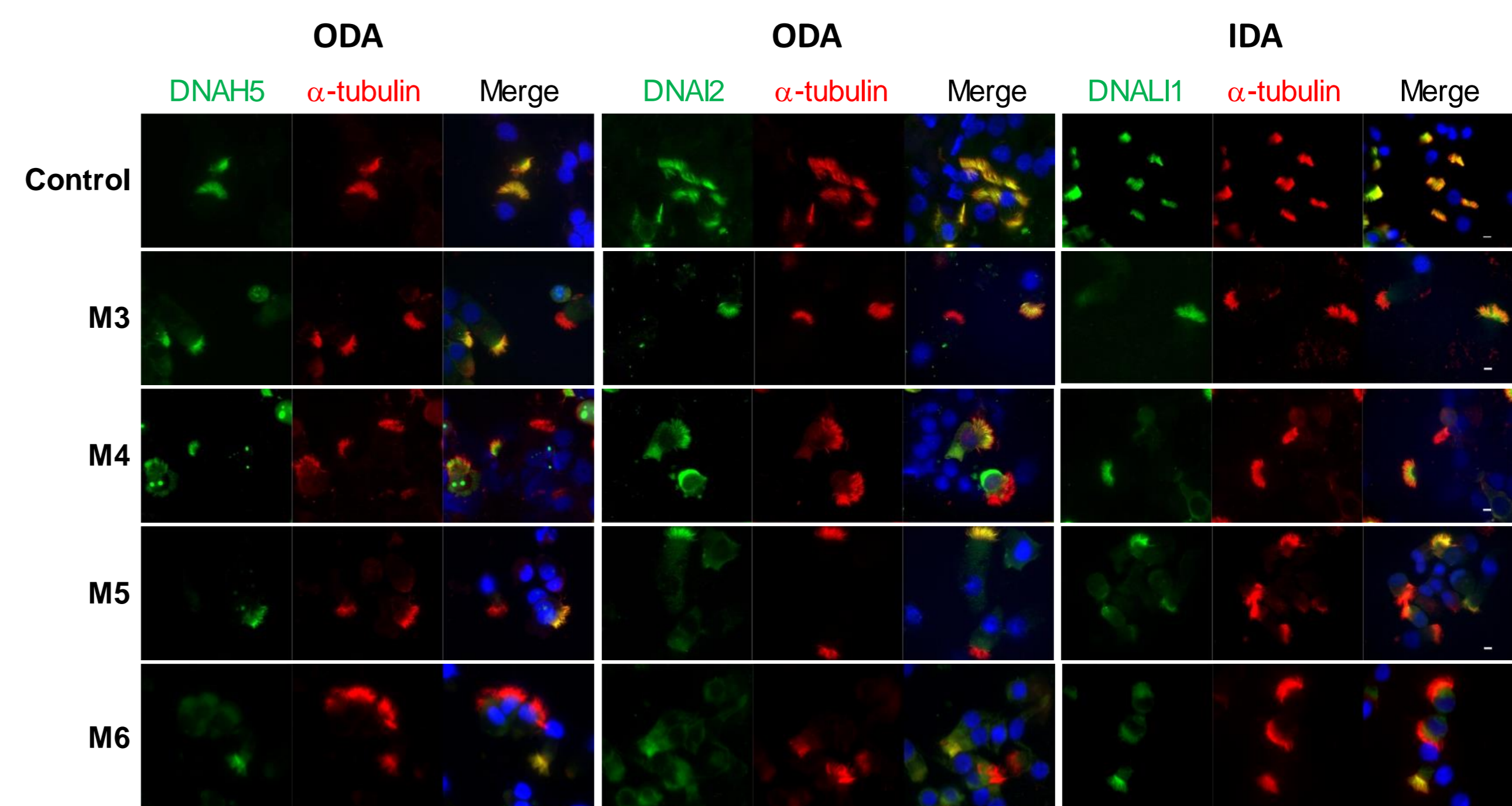
\*Examined visually but % of immotile ciliated cells not evaluated

□ No symptoms □ Mild phenotype □ Moderate phenotype □ Severe phenotype

- Clinical phenotypes differed, ranging from no respiratory symptoms (mothers M1 and M2) to variable disease severities (M3, M4, M5, M6)
- M6's greater symptom severity is close to typical PCD
- None of the six mothers had *situs inversus*

## Impact of heterozygous *DNAAF6* mutations on dynein arm-specific proteins in airway epithelial cells (AECs)

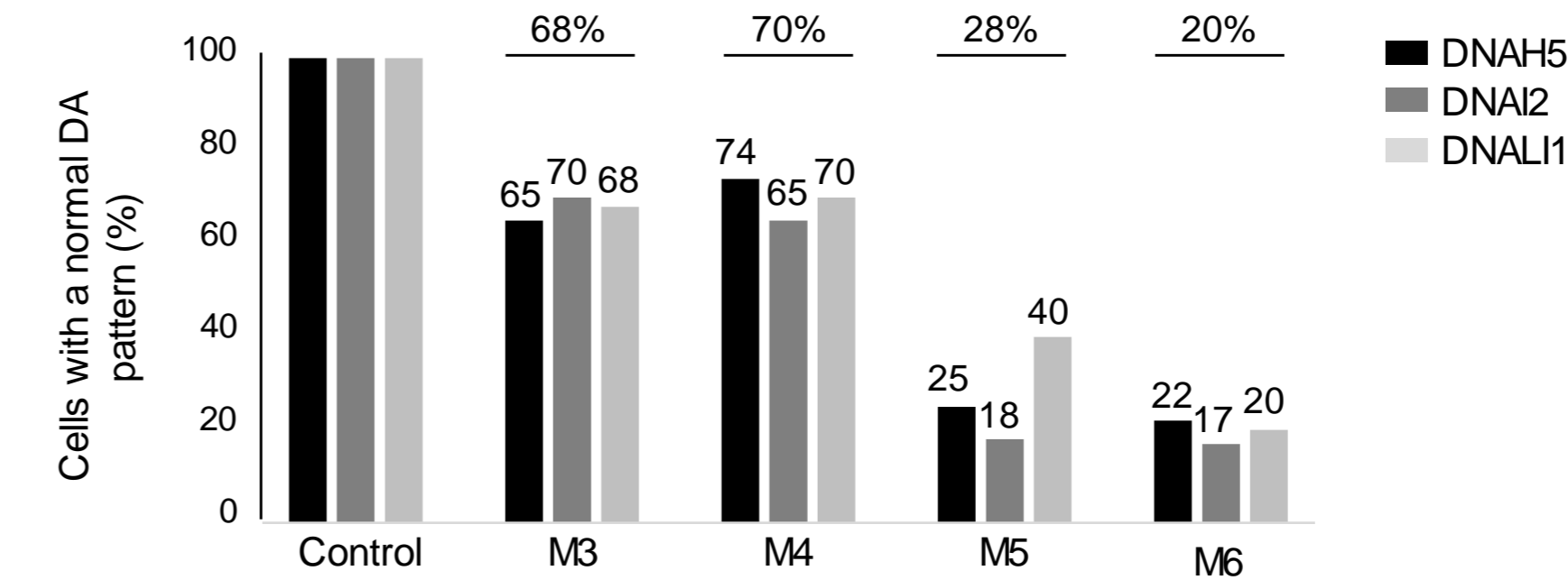
### Functional mosaicism in airway multiciliated cells



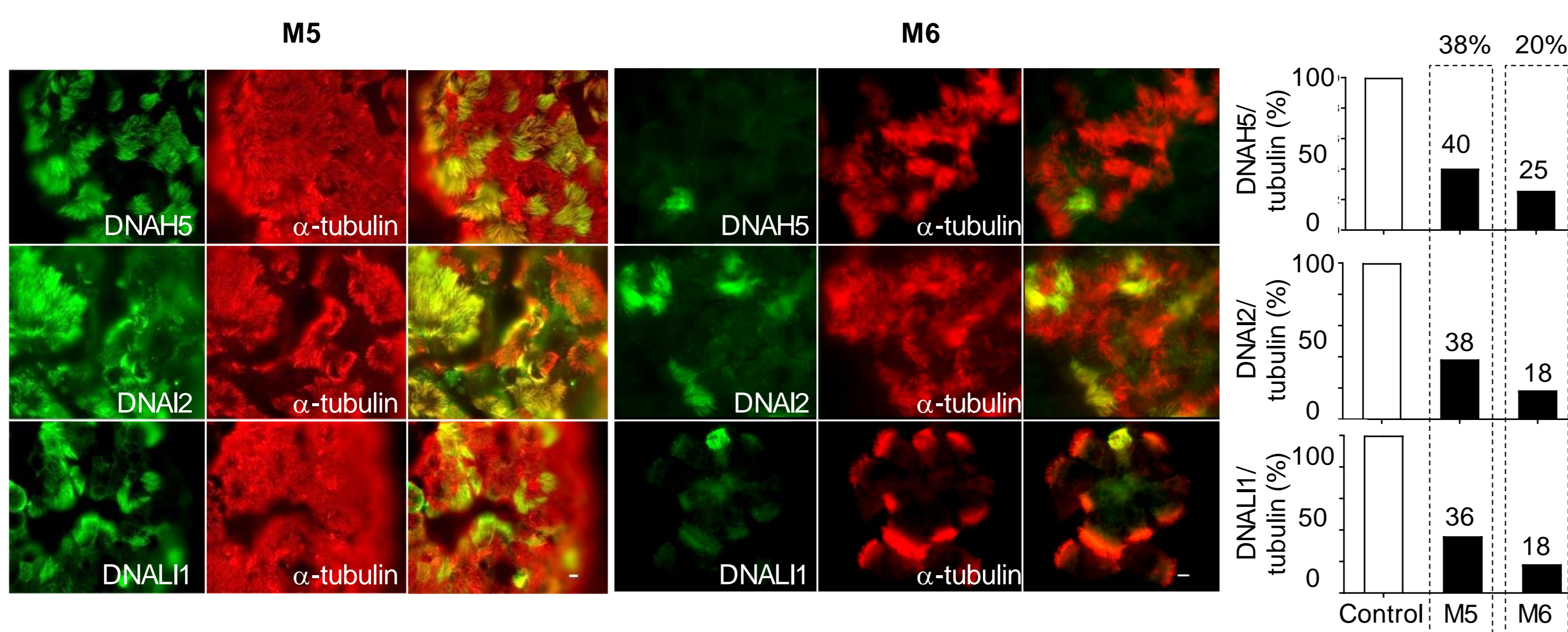
→ Normal ciliated cells expressing the normal *DNAAF6* allele show dynein arm components along the axoneme (DNAH5 and DNAI2 in ODA; DNAL1 in IDA)

→ Abnormal cells expressing the mutated *DNAAF6* allele lack the ODA and IDA markers

### Proportion of normal AECs

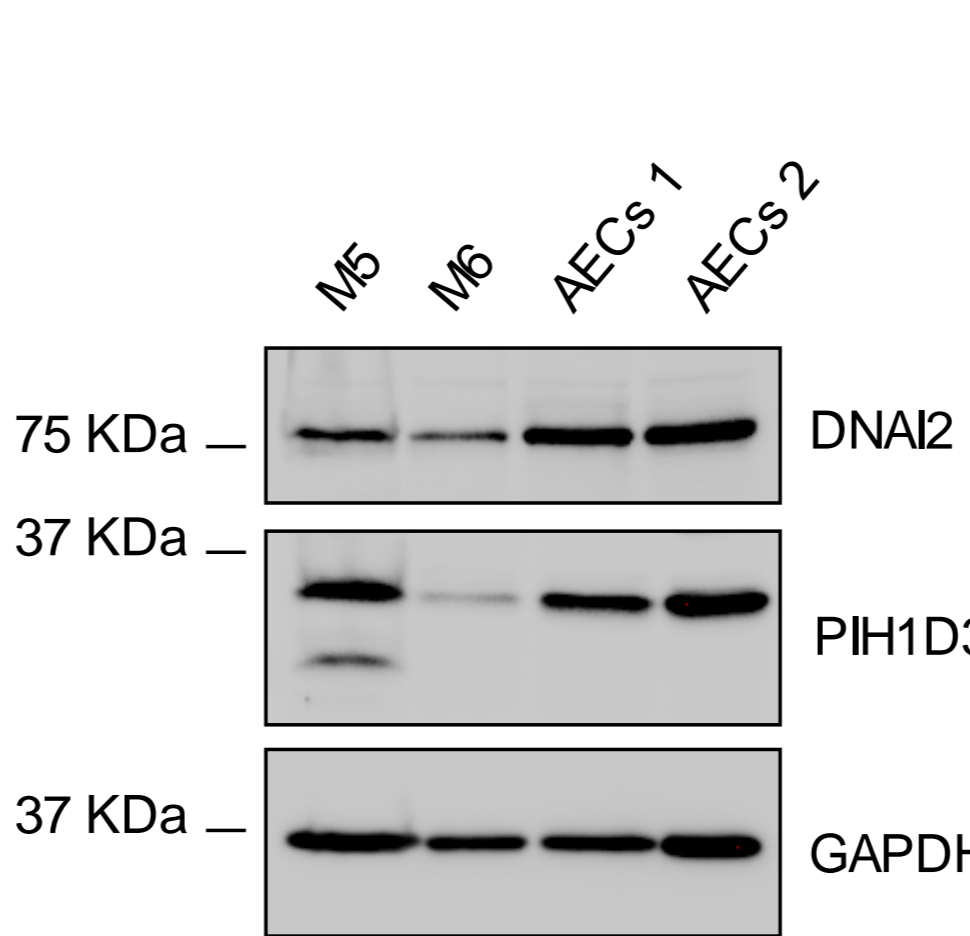


## Deep phenotyping of air-liquid interface (ALI)-cultured AECs from mothers M5 and M6

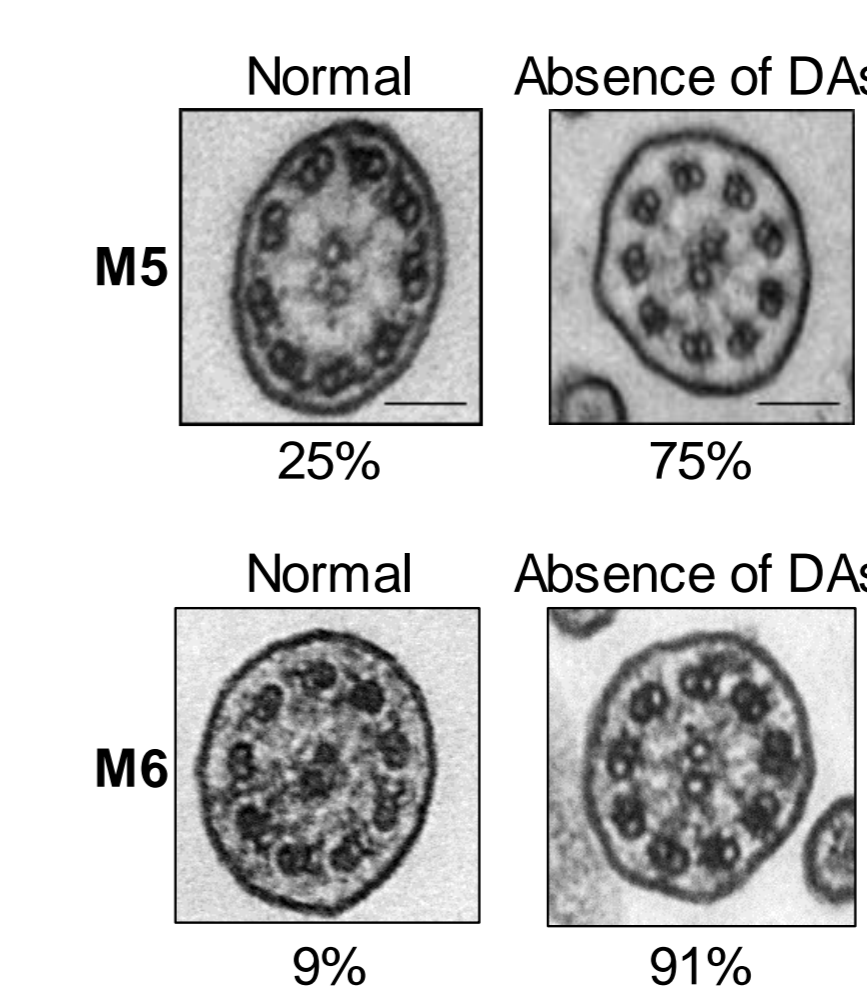


- Immunolabelling showed patches of AECs expressing either the normal or the mutated *DNAAF6* allele (DNAH5, DNAI2 and DNAL1: markers of dynein arms in ciliated cells)

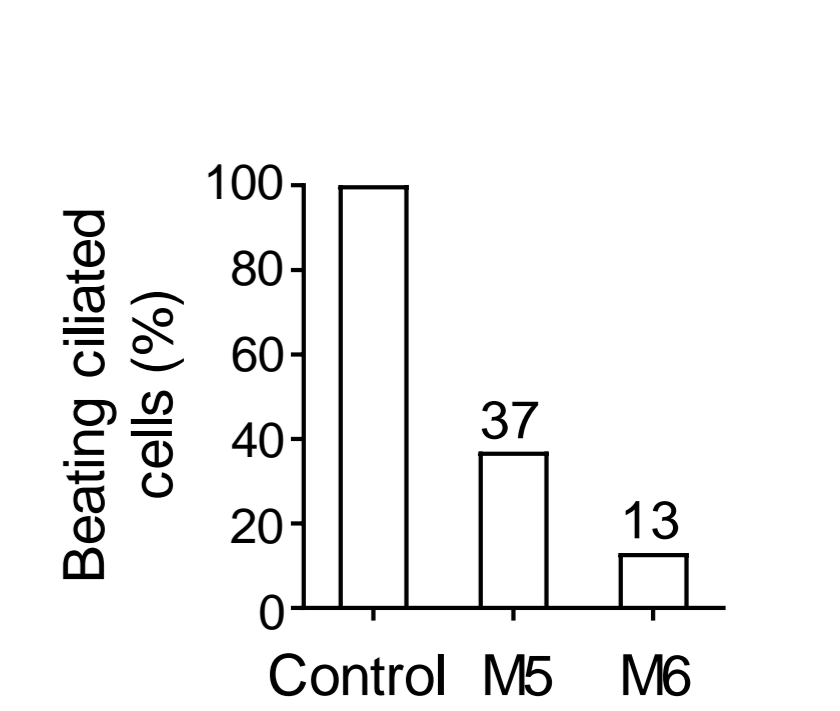
### Western blot



### Transmission electron microscopy (TEM)

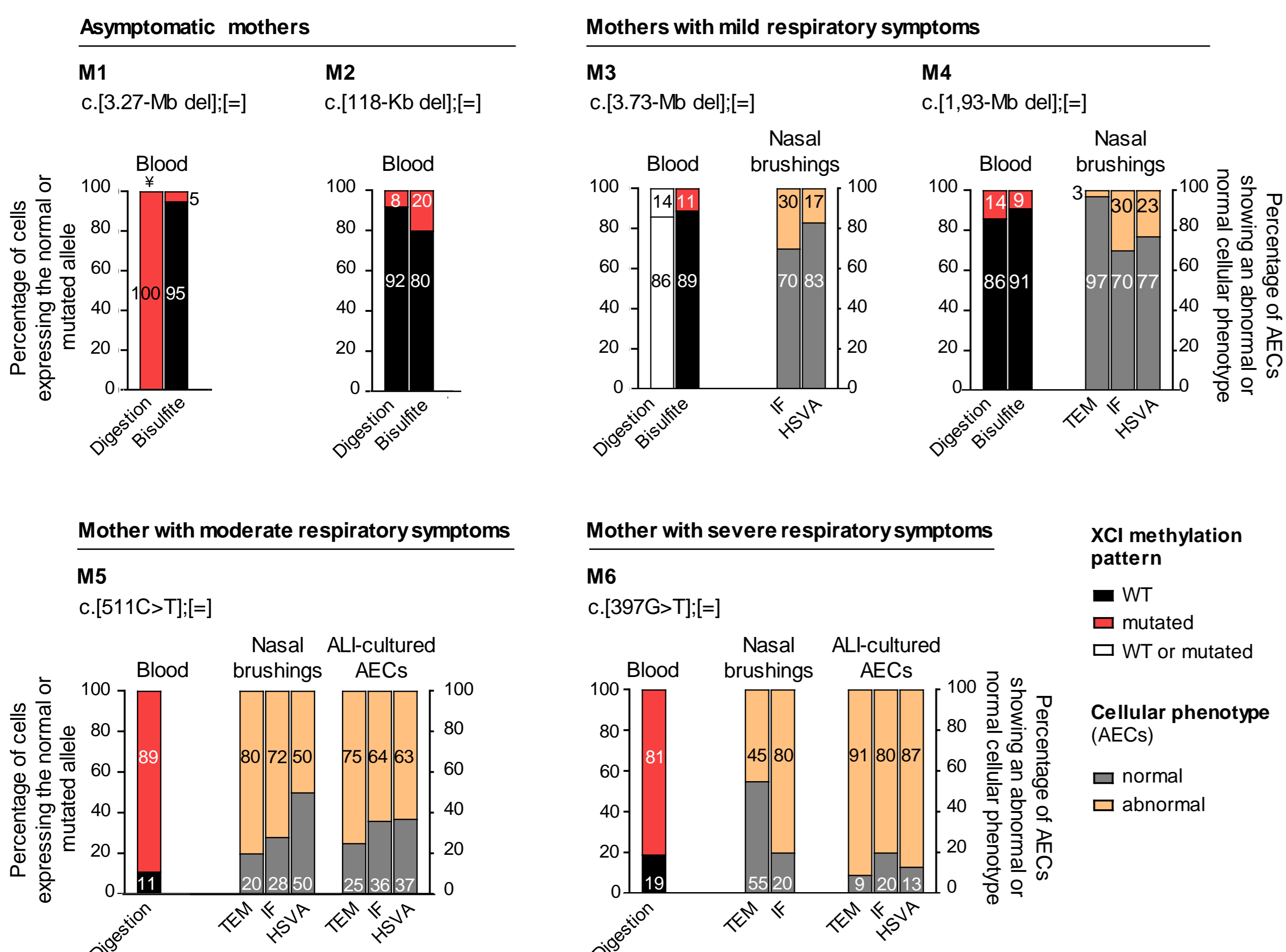


### High Speed Videomicroscopy



- Cellular mosaicism : ≈35% of normal ciliated cells in M5 (moderate phenotype) and ≈15% of normal cells in M6 (severe phenotype)

## X-chromosome inactivation (XCI) patterns vs clinical and cellular phenotypes



XCI measured in blood and/or AECs by two methods:

- 1) methylation-sensitive digestion at the *HUMARA* locus
- 2) bisulfite conversion of cytosine at the *DNAAF6* locus (only feasible for whole gene deletions)

- The digestion method revealed limitations due to the distance between *HUMARA* and *DNAAF6* and probable recombination events:

- failure to phase the XCI status with the *DNAAF6* allele in M3 (white histogram)
- inconsistent result in M1 (¥).

- The bisulfite method effectively addressed the aforementioned issues.

- XCI skewing among mothers heterozygous for mutated *DNAAF6*:
  - favouring the normal allele expression in M1, M2, M3 and M4 (asymptomatic & mild phenotypes)
  - favouring the mutated allele in M5 and M6 (moderate and severe phenotypes)

## Conclusion and perspectives

- The clinical phenotypes of the mothers are reflective of their XCI pattern.
- In the airways of symptomatic mothers, there is a coexistence of normal cells and cells with immotile cilia lacking dynein arms, with a ratio closely reflecting their XCI pattern.
- The bisulfite-conversion method, which is specific of the *DNAAF6* locus, is a more reliable than the methylation-sensitive digestion technique.
- The preferential inactivation of X chromosomes harbouring large multigenic deletions is protective in mothers: asymptomatic and mild phenotypes correspond to large multigenic deletions (M1, M2, M3 and M4). Conversely, M5 and M6 who carry point mutations, exhibited moderate and severe phenotypes respectively.
- It is crucial to provide testing for all female relatives of male PCD patients with a *DNAAF6* defect, and potentially to females consulting for mild chronic respiratory symptoms.
- In the 6 mothers, the nodal flow, which results from the beating of nodal cilia, may have been sufficient to result in normal organ lateralisation.
- Our findings also indicate that approximately 30% of normal ciliated airway cells were sufficient to prevent severe PCD, a result that paves the way for gene therapy.

These data have been published in Thomas et al., J Med Genet, 2024

The authors have no conflict of interest.