AlphaMissense versus laboratory-based pathogenicity prediction of 13 novel missense *CPA1* variants from pancreatitis cases

We have read with great interest the study by Wang *et al*[1] in which the authors evaluated the utility of the AlphaMissense prediction programme[2] (https://alphamissense.hegelab.org) in the classification of missense *CPA1* variants with respect to pathogenicity in chronic pancreatitis. While the AI-driven prediction performed relatively well, the authors highlighted potential shortcomings that can limit its value in clinical practice. Defining the pathogenic potential of *CPA1* variants detected in pancreatitis cases can be challenging because the mechanistic basis of disease risk is unrelated to loss of CPA1

function and seems to be determined by mutation-induced misfolding and the ensuing endoplasmic reticulum (ER) stress.[3–5] Recently, we used transiently transfected HEK 293T cells to measure the secretion efficiency and induction of BiP mRNA expression, a marker of ER stress, for 50 missense *CPA1* variants from pancreatitis cases and healthy controls.[6] We found that the best predictor of pathogenicity was loss of secretion (<10% of wild type) irrespective of BiP levels. This data set can serve as a reference for the assignment of clinical significance of novel *CPA1* variants. In the present study, we set out to examine what fraction of novel *CPA1* variants detected in real-world genetic testing can be classified as pathogenic and whether AlphaMissense can replace laboratory-based functional analysis in variant prediction.

We collected 13 novel *CPA1* missense variants that were recently identified in

patients with pancreatitis from several international centres (online supplemental table S1). A query of gnomAD V.4.1.0 revealed that nine variants had ultra-low allele frequency (≤0.008%) and four variants were absent. The variants have not been described in association with pancreatitis so far. Variant p.R386C was reported in two cases of pancreatic cancer.[7 8] We used our previously published protocol[6 9] to measure proenzyme secretion and BiP mRNA levels (online supplemental table S2), and we plotted BiP levels as a function of secretion (figure 1). We included wild-type *CPA1* and the p.N256K pathogenic variant as reference constructs and expressed secretion as % wild type and BiP levels as % p.N256K. As observed previously,[6] we found an inverse relationship between proenzyme secretion and BiP mRNA expression; that is, lower secretion levels were associated with high BiP values. Surprisingly, using the previously
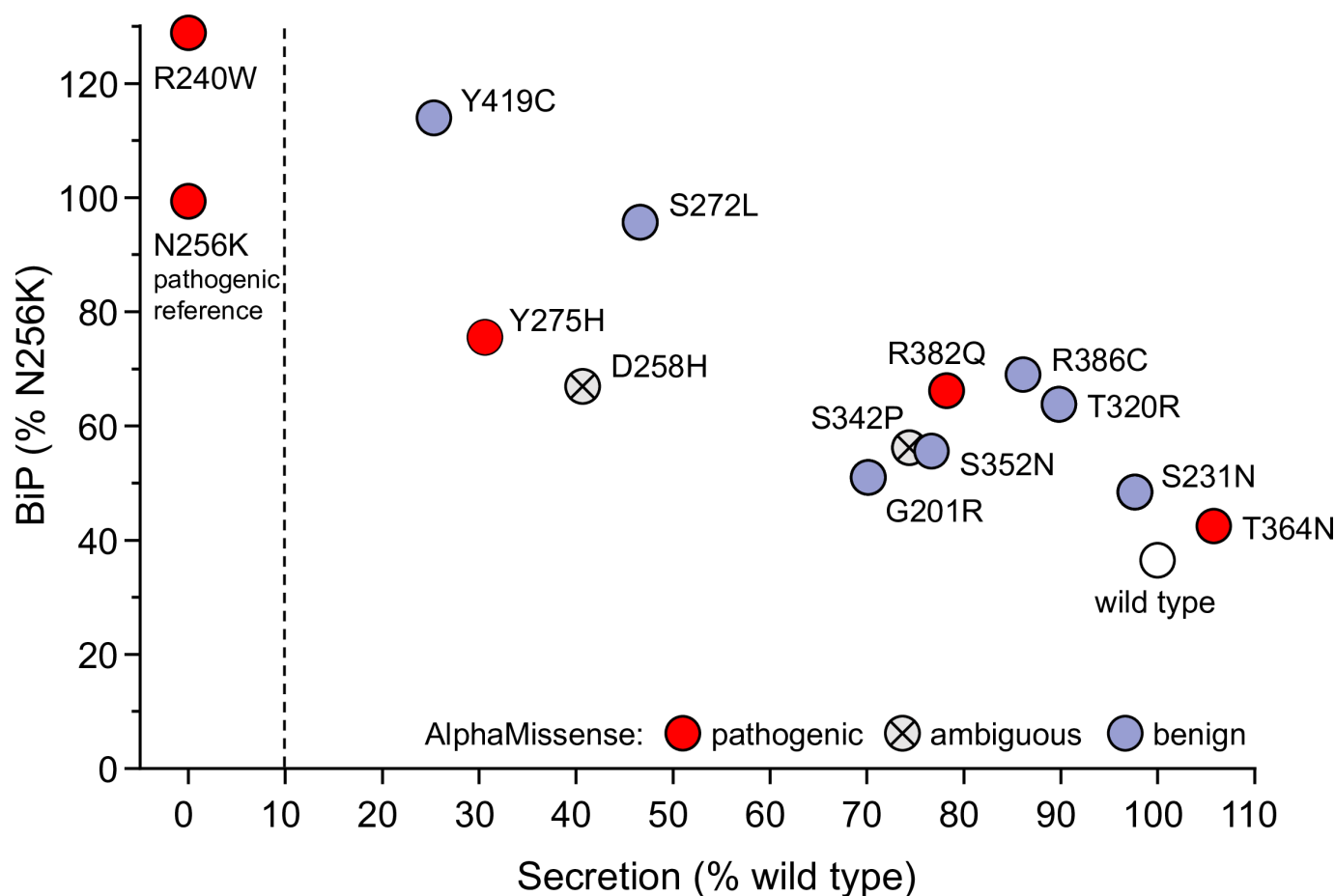

Figure 1 Effect of 13 missense *CPA1* variants on procarboxypeptidase A1 secretion and BiP mRNA expression in transiently transfected HEK 293T cells. Transfections (n=≥6) and measurements were carried out as described previously.[6 9] Conditioned media and cells were harvested after 48 hours. Secreted proenzyme levels were expressed as per cent of wild-type CPA1. BiP levels were first calculated as fold change over vector, and then expressed as per cent of the p.N256K value within the same experiment. For clarity, error bars have been omitted; SD values are listed in online supplemental table S2. AlphaMissense predictions are indicated by colour coding: red, pathogenic; grey, ambiguous; blue, benign. AlphaMissense scores are listed in online supplemental table S1. The dashed line indicates the 10% secretion cut-off value under which missense variants can be classified as pathogenic with high confidence.

established conservative secretion cutoff value of <10%, only a single variant (p.R240W) was identified as pathogenic. This variant was found in four independent paediatric recurrent acute pancreatitis cases; two of which also had a family history of the disease. We then colour-coded the symbols for the individual mutations using a simplified AlpaMissense prediction scale; where red indicates likely pathogenic variants (score >0.564), blue highlights likely benign variants (score <0.34) and grey denotes ambiguous variants (score range 0.34–0.564). Strikingly, AlphaMissense misclassified three benign variants as likely pathogenic and designated two benign variants as ambiguous. We found that three of five miscategorised variants were prone to degradation by trypsin (online supplemental table S2), confirming the reported observation that increased proteolytic susceptibility of CPA1 variants is often associated with erroneous prediction by AlphaMissense (see table 1 in Wang et al[1]).

The results indicate that laboratory-based functional analysis of *CPA1* variants is essential for accurate prediction of pathogenicity. In silico variant analysis with AlphaMissense cannot replace wet-lab studies as the rate of erroneous predictions is relatively high. AlphaMissense identifies variants with loss of catalytic function or susceptibility to proteolytic degradation as likely pathogenic, even though these properties are irrelevant to pancreatitis risk. Importantly, the majority of novel *CPA1* variants detected during real-world genetic testing of pancreatitis cases are benign and should not be considered pathogenic without experimental verification.

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