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# UNIVERSITÀ DEGLI STUDI DI TORINO

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# Two families with novel missense mutations in *COL4A1*: when diagnosis can be missed.

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**Running title:** *COL4A1* mutations

## ABSTRACT

Mutations in *COL4A1*, encoding one of the six collagen type IV proteins, cover a wide spectrum of autosomal dominant overlapping phenotypes including porencephaly, small-vessel disease and hemorrhagic stroke, leukoencephalopathy, hereditary angiopathy with nephropathy, aneurysms and muscle cramps (HANAC) syndrome, and Walker-Warburg syndrome. Over 50 mutations are known, mainly being missense changes. Intra- and inter-familial variability has been reported.

We studied two Italian families in which the proband had a clinical diagnosis of *COL4A1*-related disorder. We found two novel mutations (c.1249 G>C; p.Gly417Arg and c.2662G>C; p.Gly888Arg). Both involved highly conserved amino acids and were predicted as being deleterious by bioinformatics tools. The c.1249 G>C (p.Gly417Arg) segregated in four subjects with variable neurological phenotypes, namely leukoencephalopathy with muscle symptoms, brain small-vessel disease and mild infantile encephalopathy. A fourth case was a carrier of the mutation without any neurological symptoms and a MRI with aspecific white matter anomalies. The c.2662G>C (p.Gly888Arg) mutation was *de novo* in the proband. After a temporary motor impairment at age 14, the subject complained of mild imbalance at age 30, during the third trimester of her twin pregnancy, when an anomaly of the left brain hemisphere was documented in one fetus. Both her male dizygotic twins presented a severe motor delay, early convulsions, and leukoencephalopathy, and were carriers of the mutation.

In summary, we confirm that high intra-familial variability of *COL4A1* mutations with very mild phenotypes, the apparent incomplete penetrance, and *de novo* changes may become a “dilemma” for clinicians and genetic counsellors.

## 1. Introduction

Collagen type IV is a multimeric protein expressed almost exclusively in the basal membrane [1]. In humans, it is encoded by six paralogous genes: *COL4A1* to *COL4A6* [2]. Each gene encodes for one of six different chains (alpha1 to alpha6), which assemble to form three heterotrimers, namely  $\alpha1\alpha1\alpha2$ ,  $\alpha3\alpha4\alpha5$  and  $\alpha5\alpha5\alpha6$ . Whereas  $\alpha1$  and  $\alpha2$  chains are ubiquitously expressed, the others are tissue- or developmental time-specific [3].

Mutations in each *COL4A* gene have been found in human diseases; *COL4A3*, *COL4A4* and *COL4A5* have been associated with Alport syndrome [4]; genomic rearrangements involving *COL4A5* and *COL4A6* (located head-to-head on chromosome X) have been found in families with diffuse leiomyomatosis (a smooth muscle tumor), and Alport syndrome [5, 6]; *COL4A6* mutations are responsible for X-linked deafness [7]. More recently, mutations in *COL4A1* have been identified in a wide range of diseases, including porencephaly [8, 9], familial and sporadic small-vessel disease and hemorrhagic stroke [10-12], leukoencephalopathy [13], hereditary angiopathy with nephropathy, aneurysms and muscle cramps (HANAC) syndrome [14], Walker-Warburg syndrome [15], and isolated ophthalmological anomalies [13, 16]. It has been proposed that this spectrum of signs and symptoms may be grouped under the name of *COL4A1*-related disorders [17], thus indicating a single disease with great variability in expressivity and age at onset. Finally, *COL4A2* has been found to be mutated in the autosomal dominant porencephaly type 2 [18].

## 2. Subjects and Methods

We studied two independent Italian families with a clinical suspicion of *COL4A1*-related disorders (Figure 1). Informed consent was obtained from all participants in the study or their legal representatives; the study was approved by the internal ethics committee of the Department of Medical

Sciences, University of Torino, Italy. All coding exons and flanking intron boundaries of *COL4A1* (NM\_001845) and *COL4A2* (NM\_001846) were amplified by PCR from blood-extracted genomic DNA (Qiagen, Mannheim, Germany), and Sanger sequenced (Life Technologies, Carlsbad, CA, USA). Mutation pathogenicity was evaluated using bioinformatics tools (Figure 2).

We genotyped local healthy subjects by PCR (Exon 21: 5'-ttgcaggcctccctgtacc and 5'-gcccagggggcccaggggaac; Exon 33: 5'-tgatccattcaagttcccagtgt and 5'-ccaagtggcatcgagaaatagac), restriction digestion analysis and agarose gel electrophoresis. A total of 550 chromosomes were analyzed for the c.1249G>C mutation (exon 21) and 522 for c.2662G>A mutation (exon 33). Digestions were performed with *TaqI* for exon 21 and *SmaI* for exon 33. For exon 21, the digestion yields a 156 bp fragment for the wild-type allele and two fragments of 134 bp and 22 bp in the mutated allele. For exon 33, the digestion yields two fragments of 468 bp and 113 bp in the wild-type allele and a fragment of 581 bp in the mutated allele.

### **3. Results**

#### *3.1 Clinical data*

##### *3.1.1 Family LEU-1-TO*

The proband, a 50 yr-old woman with a maternal family history of stroke, had a first cerebral hemorrhage at age 45, which was mainly endoventricular (Table 1, Figure 1A). Cerebral angiography demonstrated a small aneurysm of the anterior communicating artery. She subsequently suffered from a retinal hemorrhage. A retinography demonstrated marked tortuosity of retinal vessels (Figure 1B). At age 47, she presented an acute left hemiparesis after repeated sneezing. MRI showed periventricular bleeding and a diffuse signal abnormality of the supratentorial white matter (Figure 1C-D).

Proband mother displayed mild perinatal hemiparesis and had a history of epileptic seizures since the age of 3 yrs., incompletely controlled by antiepileptic therapy. She had, however, never experienced stroke. Clinical history was therefore compatible with an idiopathic form of perinatal encephalopathy. Proband sister (III-4) complained of muscle cramps and a mild impairment of the right leg since adolescence. General and metabolic work-up resulted as being normal with Creatine Phospho Kinase (CPK) at the upper level of the normal range. MRI showed diffuse leukoencephalopathy (Figure 1G) and retinography demonstrated a mild retinal vessel tortuosity (Figure 1F).

MRI also showed a diffuse leukoencephalopathy in the proband and in the mother (III-1, II-2), and a small porencephalic periventricular lesion in II-2.

Proband brother (III-3) was completely asymptomatic at age 30 and had an unremarkable clinical history except for two episodes of spontaneous pneumothorax in his youth. MRI showed just a few small non-specific T2 hyperintensities of the supratentorial white matter (Figure 1E). CPK was normal. Brain angio MR and ophthalmological examination were not performed.

### *3.1.2 Family LEU-2-TO*

The proband was a 34 yr-old woman, the eldest of three siblings, born from non-consanguineous neurologically-healthy parents. She had a history of congenital glaucomas and cataracts (Table 1, Figure 1A).

At age 14, the patient presented a sudden and reversible motor impairment, which was not further investigated. At age 30, during the third trimester of her twin pregnancy, she started complaining mild imbalance and gait disorder. At that time, an anomaly of the left brain hemisphere was documented in one fetus by ultrasounds. The patient underwent an elective caesarian section, the decision of which was taken after 1 month of clinical worsening, represented by repeated episodes of vomiting with

weight loss and stepwise gait unbalance. Her blood pressure and routine blood examinations were normal. Soon after the delivery (~ 1 hour), she presented right hemiparesis with dystonic posture of the upper arm and early hypertonus of both the right arm and the right leg. The CT scan showed a left parietal hemorrhage and a diffuse hypointensity of supra- and infra-tentorial white matter. The MRI confirmed a severe leukoencephalopathy and displayed multiple supra and infra-tentorial micro-hemorrhages, together with lacunar infarcts (Figure 1H-I). Cerebral angiography revealed diffuse anomalies of the carotid axis; specifically, focal areas of vessel narrowing interspersed with pseudoaneurysmatic dilatations of both cavernous segments, and the major findings were two small (less than 1 mm) left carotid-ophthalmic and left carotid siphon aneurysms. The posterior vascular axis was relatively free from anomalies.

Both male twins of the patient presented severe motor delay and early convulsions. MRI confirmed severe encephalopathy in both children (Figure 1J). At age 6 years, twin A presented with severe psychomotor delay with tetraparesis, absence of speech and severe intellectual disability; twin B had epileptic encephalopathy, which was partially non-respondent to therapy. Twin B can walk but has never acquired complete language skills.

### *3.2 Genetic findings*

Sequencing of *COL4A1* revealed the c.1249 G>C mutation in exon 21 (p.Gly417Arg) in family LEU-1-TO, and the c.2662G>A mutation in exon 33 (p.Gly888Arg) in family LEU-2-TO (Figure 1A).

Both mutations were novel, and not reported as polymorphisms in the 1000 Genomes ([www.1000genomes.com](http://www.1000genomes.com)), dbSNP138 ([www.ncbi.nlm.nih.gov/projects/snp](http://www.ncbi.nlm.nih.gov/projects/snp)) and HapMap ([www.hapmap.com](http://www.hapmap.com)) databases. To exclude population stratification artifacts, we screened, by

restriction enzyme assays, a cohort of local controls (more than 500 chromosomes) and the two variants were not identified.

Both mutations involved alterations of highly conserved glycine residues within a Gly-X-Y repeat of the triple helical rod domain, and were predicted to be likely pathogenic by different bioinformatics tools (see Fig. 2).

#### 4. Discussion

*COL4A1*-related disorders cover a spectrum of autosomal dominant overlapping phenotypes including porencephaly type 1 (OMIM #175780), brain small-vessel disease with hemorrhage (OMIM #607595) and HANAC (OMIM #611773). Intra- and inter-familial variability are common findings in all *COL4A1*- related disorders (<http://www.ncbi.nlm.nih.gov/books/NBK7046/>).

We found two novel mutations (c.1249G>C; p.Gly417Arg and c.2662G>A; p.Gly888Arg) confirming the phenotype variability and an apparent incomplete penetrance of these diseases. Their pathogenicity is strongly supported by bioinformatics predictions including the physical-chemical properties of the amino acid changed, the evolutionary conservation, and the lack of known SNPs at the mutant site.

Indeed, 42 of the 46 pathogenic missense mutations described in *COL4A1* affect a glycine within Gly-X-Y repeats, suggesting that this amino acid change is not tolerated. Moreover, Gly-to-Arg mutations have been shown to be pathogenic in the *C.elegans Col4a1* orthologue [19, 20] and in human *COL4A5* [21]. Finally, the mutation in family LEU-2-TO is *de novo*, a strong indicator of pathogenicity [22].

A total of 58 mutations have been described in *COL4A1*, the majority of which affect the triple-helical central rod domain (88%; Figure 2). HANAC is almost exclusively associated with mutations located in the initial third of the rod domain (exons 9-25), with a cluster of mutations in exons 24-25. A genotype-phenotype correlation is absent for mutations that affect the remaining part of the rod domain

(exons 26-48) [2]. The c.1249G>C (p.Gly417Arg) mutation was found to be associated with three different clinical presentations [leukoencephalopathy with muscle symptoms (III-4), brain small-vessel disease (III-1), and mild infantile encephalopathy (II-2)], with the absence of a neurologic phenotype in one subject.

In the LEU-2-TO family, c.2662G>A (p.Gly888Arg) resulted in a severe antenatal intracerebral hemorrhage leading to porencephaly in two cases, a rare finding reported in literature without a strict correlation with mutation type or location [23-25].

Both probands in the two families present intracerebral aneurysms involving the carotid axis, a distinctive feature of HANAC phenotype, but also present at various degrees in other *COL4A1*-related phenotypes. In family LEU-1-TO, aneurysms are combined with a dysplastic change of the artery as previously reported [26].

Both genetic and/or environmental modifiers could be responsible for this intra-familial variability. Among these, mechanical traumas may have a role in triggering an acute hemorrhage in agreement with other reports, as was the case for III-1 in family LEU-1-TO (repeated sneezing) [27, 28]. In proband II-3 (LEU-2-TO), we speculate that physical stress and unknown factors present in late pregnancy caused one or more episode of subacute stroke. These lead to clinical worsening in the third trimester followed by a more severe hemorrhage and a right hemiparesis soon after the caesarian section.

We suggest a diagnosis of *COL4A1*-related disorders should be suspected in cases with compatible clinics/neuroradiology, even in the absence of a familial history. For instance, a *de novo* mutation can be present (e.g., c.2662G>A), or the variable expression with age of onset and/or the lack of detailed clinical examination may mimic incomplete penetrance (e.g., c.1249G>C) [29]. These disorders represent a challenge for genetic counselling: there is a 50% risk of transmitting the genetic defect to a

child, but establishing the phenotype in a newborn is impossible due to the variability in disease expression.

#### *4.1 Conclusions*

We describe two novel *COL4A1* missense mutations and confirm the high intra-familial variability of *COL4A1*-related disorders with very mild phenotypes and apparent incomplete penetrance that, together with *de novo* events, may represent a “dilemma” for clinicians and genetic counsellors.

#### **ACKNOWLEDGMENTS**

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#### **DISCLOSURE OF CONFLICTS OF INTEREST**

The authors declare there are no conflicts of interest to disclose.

## FIGURE LEGEND

**Figure 1. Genealogic trees, mutation analysis, MRI scans and retinography.** (A) LEU-1-TO and LEU-2-TO family trees. Lower panels: sequence electropherograms showing *COL4A1* mutations. (B) Fluorescein angiography showing typical retinal arteriolar tortuosity in subject LEU-1-TO:III-1 (arrows). (C) axial fluid-attenuated inversion recovery (FLAIR) image shows a diffuse periventricular leukoencephalopathy; (D) axial T2 gradient echo shows multiple micro-bleeds (arrows). (E) axial T2 gradient echo in LEU-1-TO:III-3 demonstrates small hyperintensity in subcortical white matter (arrows). (F) Fluorescein angiography shows mild retinal vessel tortuosity (arrow) in LEU-1-TO:III-4. (G) axial fluid-attenuated inversion recovery (FLAIR) image shows a diffuse periventricular leukoencephalopathy in LEU-1-TO:III-4. (H) Sagittal FLAIR image shows a diffuse periventricular leukoencephalopathy with some lacunar infarcts in LEU-2-TO:II-3 (arrow); (I) axial T2 gradient echo demonstrates multiple micro-hemorrhages (arrows); (J) axial T2 turbo-spin echo shows an insular encephalomalacia with peripheral low signal intensity due to an old parenchymal hemorrhage (arrow).

**Figure 2. *COL4A1* mutations.** (A) and (B) The p.Gly417Arg and p.Gly888Arg changes are shown. Both mutations affected residues highly conserved in vertebrates as shown by the “Multiz Alignment of 100 Vertebrates” track of the UCSC genome browser ([www.genome.ucsc.edu](http://www.genome.ucsc.edu)). The mutation pathogenicity was assessed using eight different software. (C) The *COL4A1* protein structure is showed with the amino-terminal 7S domain, the central collagen triple helical domain, and the carboxy-terminal NC1 domain. Mutations in *COL4A1* are in red when associated with porencephaly. Black lines indicate the two novel mutations reported in this paper (A and B).

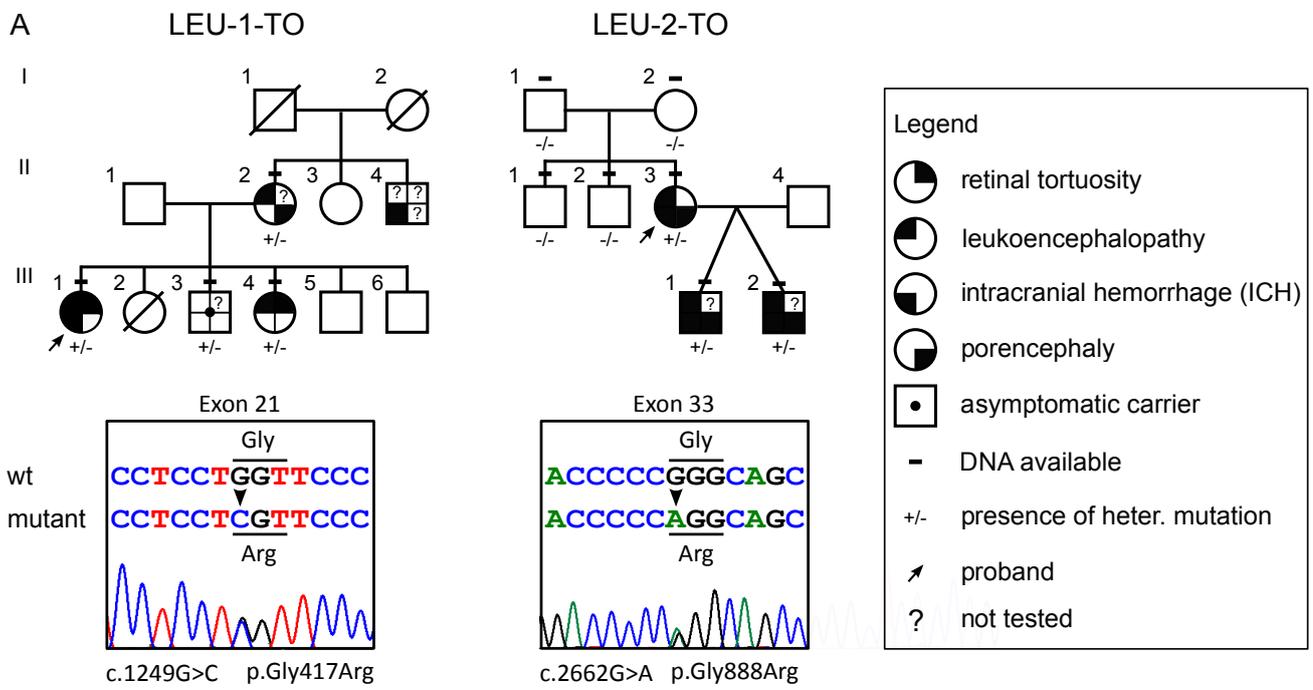
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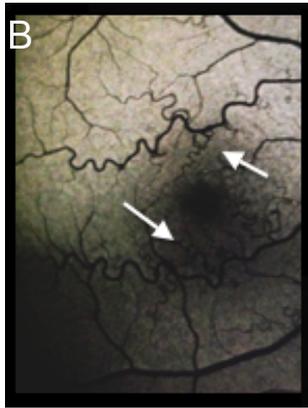
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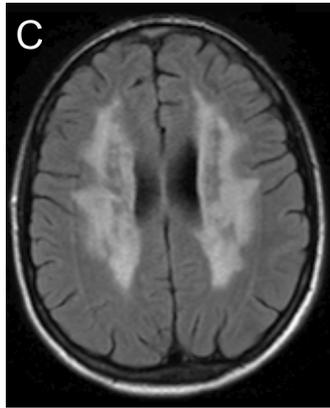
Figure 1



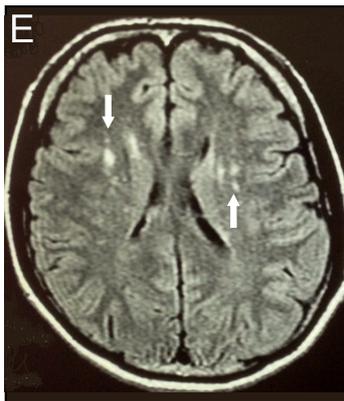
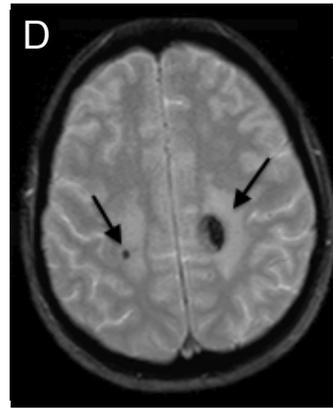
LEU-1-TO: III-1



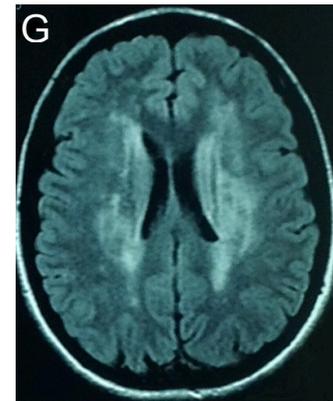
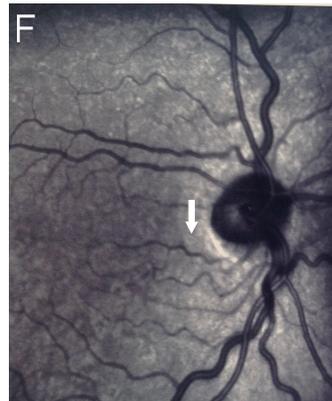
LEU-1-TO: III-3



LEU-1-TO: III-4



LEU-2-TO: II-3



LEU-2-TO: III-1

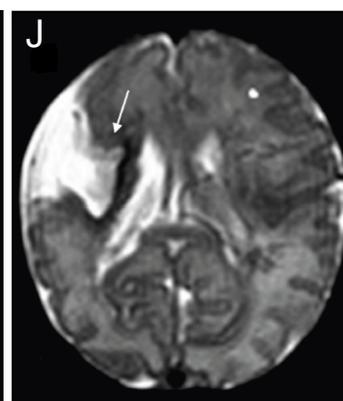
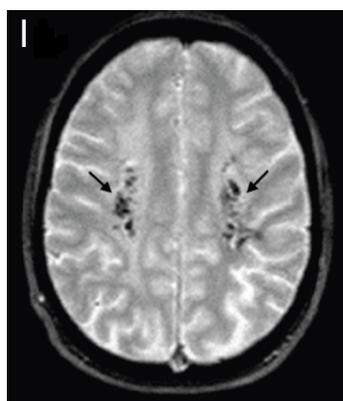
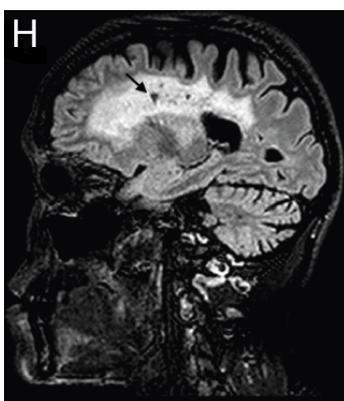


Figure 2

A LEU-1-TO FAMILY (p.Gly417Arg)

Human	GPPGSPG	Sloth	GPPGSPG
Rhesus	GPPGSPG	Opossum	GLPGPPG
Mouse	GPPGPPG	Chicken	=====
Rabbit	GLPGPPG	X-tropicalis	GPPGLPG
Cow	GPPGPPG	Zebrafish	=====
Elephant	GLPGPPG		

Software	p.Gly417Arg		p.Gly888Arg		Range of reference	Bibliography	PMID
	Description	Value output	Description	Value output			
SIFT	damaging	0.01	damaging	0.00	(≤ 0.05 pathogenic)	Ng and Henikoff (2001)	11337480
PolyPhen-2	benign	0.107	Probably damaging	1.00	(benign -damaging: 0-1)	Adzhubei IA et al. (2010)	20354512
I-Mutant 2.0	increase stability	-0.01	decrease stability	0,36	(> 0: decrease stability; < 0: increase stability)	Capriotti et al. (2005)	15980478
Mutation t@ster	disease causing	0,999	disease causing	0,999	(Probability of pathogenicity: 0-1)	Schwarz et al., (2014)	24681721
PMUT	pathogenic	0.69	pathogenic	0.70	(> 0.5 pathogenic)	Ferrer-Costa et al. (2005)	15879453
SNAP-F	non-neutral	5	non-neutral	5	(> 0 non neutral)	Bromberg and Rost (2007)	17526529
PON-P	n.a	n.a	pathogenic	1	(Probability of pathogenicity: 0-1)	Olatubosun A et al. (2012)	22505138
PHD-SNP	disease	n.a	disease	n.a	n.a	Capriotti et al. (2006)	16895930

B LEU-2-TO FAMILY (p.Gly888Arg)

Human	GTPGQPG	Sloth	GTPGQPG
Rhesus	GTPGQPG	Opossum	GTPGQPG
Mouse	GTPGQPG	Chicken	GTPGLPG
Rabbit	GTPGQPG	X-tropicalis	GMPGSIG
Cow	GTPGQPG	Zebrafish	GMA GPPG
Elephant	GTPGQPG		

C

