

# ***Medium-Chain Acyl-CoA Dehydrogenase (MCAD) splicing mutations identified in newborns with an abnormal MS/MS profile***

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# MCAD deficiency:

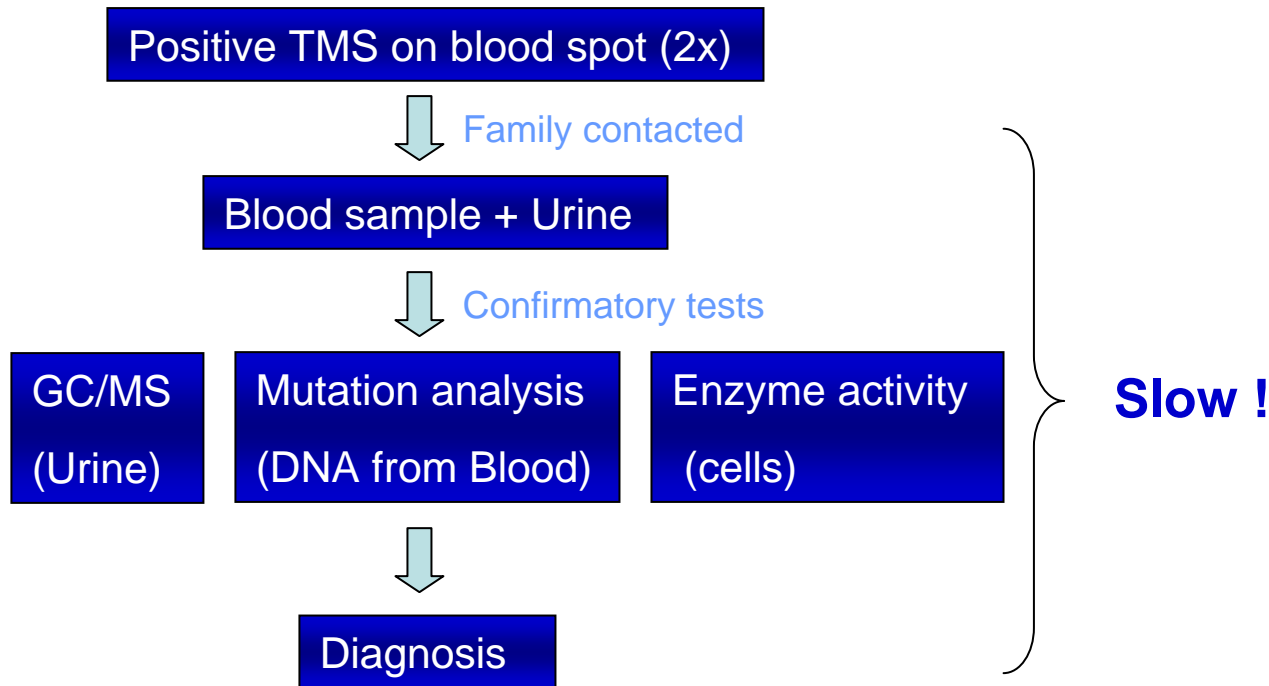
- 1. Medium-Chain AcyI-CoA Dehydrogenase (MCAD) deficiency is a potentially fatal defect in the mitochondrial fatty acid oxidation**
- 2. Disease manifestation can be reduced/avoided by fasting and diet - Good outcome if patient is diagnosed before clinical presentation**
- 3. Can be detected by Tandem MS analysis for acylcarnitines in blood spots**

**Therefore:** Included in many Tandem MS based newborn screening programs worldwide – Routine screening in UK since March 2007  
More than 600.000 newborns screened pr. year.

**We perform mutation analysis for many programs worldwide**

# Why mutation analysis?

## Traditional diagnostic procedure:



Collection of new samples

Borderline cases

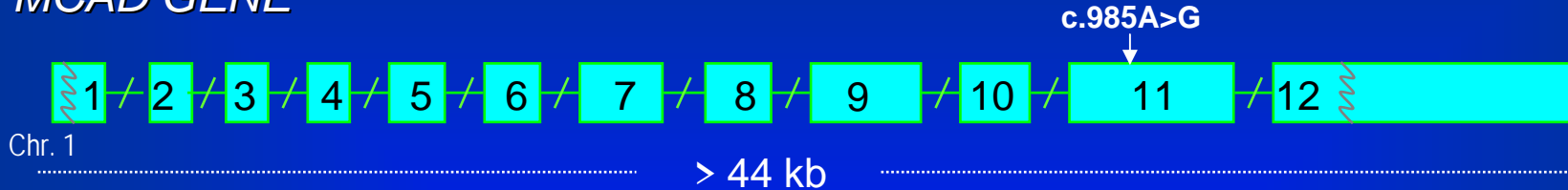
Overlapping excretion patterns

DNA analysis on original blood spot is fast !!

It is crucial to distinguish between normal variations and deleterious variations. Consequently, knowledge about the functional consequences of detected mutations is fundamental for correct diagnosis

# MCAD deficiency – Mutation spectrum

## MCAD GENE



## PROTEIN

LEADER

ACYL-COA DEHYDROGENASE

**Prevalent mutation:** c.985A>G / K304E - Carrier frequency 1/40-1/100

**Clinically presenting patients:**

Northern European descent:

80% Homozygous 18% Heterozygous 2% No c.985A>G

Denmark:

74% Homozygous 16% Heterozygous 10% No c.985A>G

**Newborn screening:**

UK:

55% Homozygous 21% Heterozygous 24% No c.985A>G

Denmark

42% Homozygous 35% Heterozygous 23% No c.985A>G

>100 different mutations known

	<b>UK:</b> (>1 mill newborns screened)	<b>Total:</b>
<b>Newborns with other genotypes:</b>	<b>69</b>	<b>&gt;350</b>
<b>Different mutations:</b>	<b>35</b>	<b>&gt;100</b>
<b>Splicing mutations:</b>	<b>10</b>	<b>29</b>
<b>Splicesite</b>	<b>7</b>	<b>15</b>
<b>Silent</b>	<b>2</b>	<b>3</b>
<b>Missense</b>	<b>1</b>	<b>3</b>

**Splicing mutations make up a significant proportion of total**

## How to evaluate:

1. **Absent in 100 chromosomes (Ethnicity? Rare variants?)**
2. **Bioinformatics (Splice site strength - ESE/ESS prediction)**
3. **Patient cells (Confirms defect – Linked or causative?)**
4. **Minigenes / *In vitro* RNA techniques (Independent of patient genetic background - Artificial effects)**
5. **Database of evaluated mutations**

## Two examples:

### 1. Splice site mutation (IVS10-6T>G)

Most prevalent splice mutation in UK

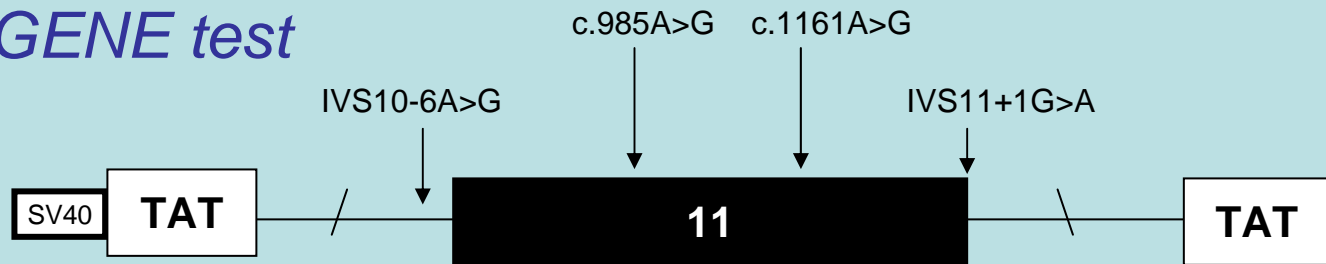
8 newborns originating from Pakistan/India

Phenotype????

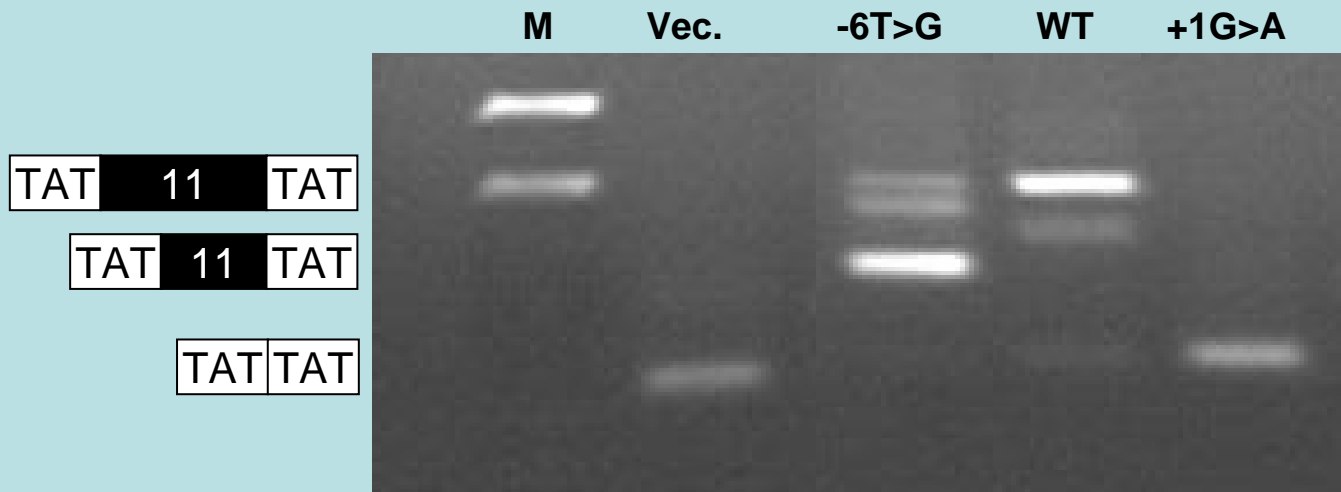
### 2. Silent mutation in exon 2 (c.87A>G)

# Exon 11 missplicing – Caused by IVS10-6T>G

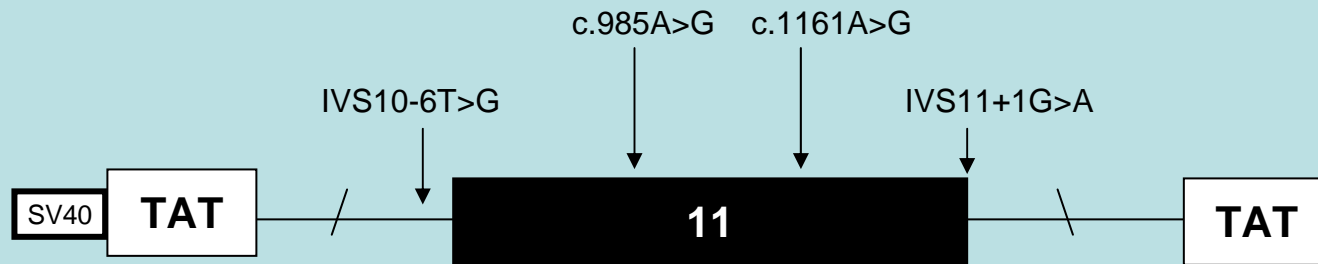
## MINIGENE test



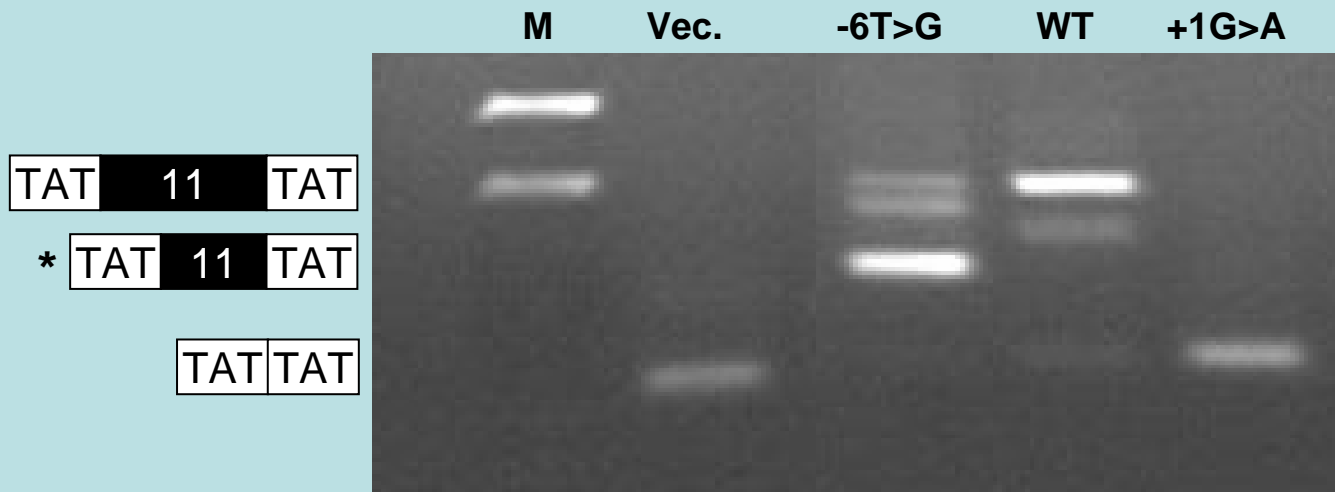
	Score		Score	
<b>WT</b>	: ttttaattct <u>ag</u> CAC	87	<b>WT</b> : TCAG/gtaagg...	86
<b>-6A&gt;G</b>	: tttta <u>ag</u> tct <u>ag</u> CAC	83 83	<b>+1G&gt;A</b> : TCAG/ <u>a</u> taagg...	--



# Exon 11 missplicing – Caused by IVS10-6T>G



	Score		Score	
WT	:t <b>tt</b> aattct <b>ag</b> CAC	87	WT:TCAG/gtaagg...	86
-6A>G	:t <b>tt</b> a <b>ag</b> tct <b>ag</b> CAC	83 83	+1G>A:TCAG/ <b>a</b> taagg...	--
*Pseudo site	:ctattgcaaagGCA	77		



**Splice site mutation (IVS10-6T>G):**

**Prediction: New pseudosite and old acceptor equal score**

**Expect: Some normal splicing and some with 5 bp ins (new pseudo splice site) .**

5bp ins -> FS -> premature stop codon -> NMD

**Instead: New site with low score activated – No use of new site**

**No significance here as both scenarios results in loss of function**

**BUT: Other genes**

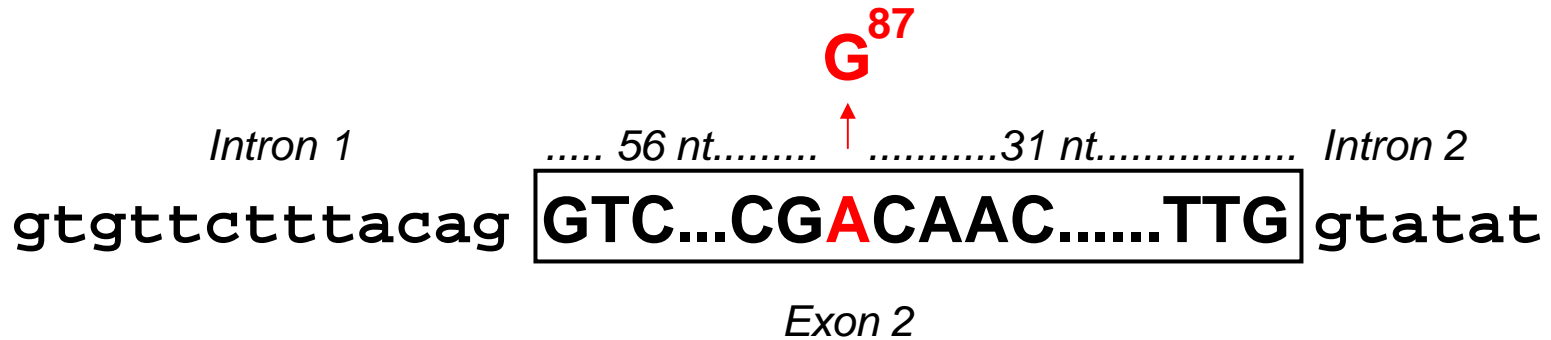
# A c.87A>G synonymous mutation in MCAD Exon 2 in two unrelated newborns

1. Two unrelated newborns with a positive tandem MS screening
2. Sequence analysis of the entire MCAD gene showed:  
Heterozygosity for c.985A>G (Prevalent – K304E)  
Heterozygosity for c.87A>G (CGA>CGG (Arg<sup>4</sup> > Arg<sup>4</sup>) (Silent))
3. Analysis of parental DNA showed that the mutations are located in separate alleles
4. c.87A>G is not present in controls (>100 chromosomes).



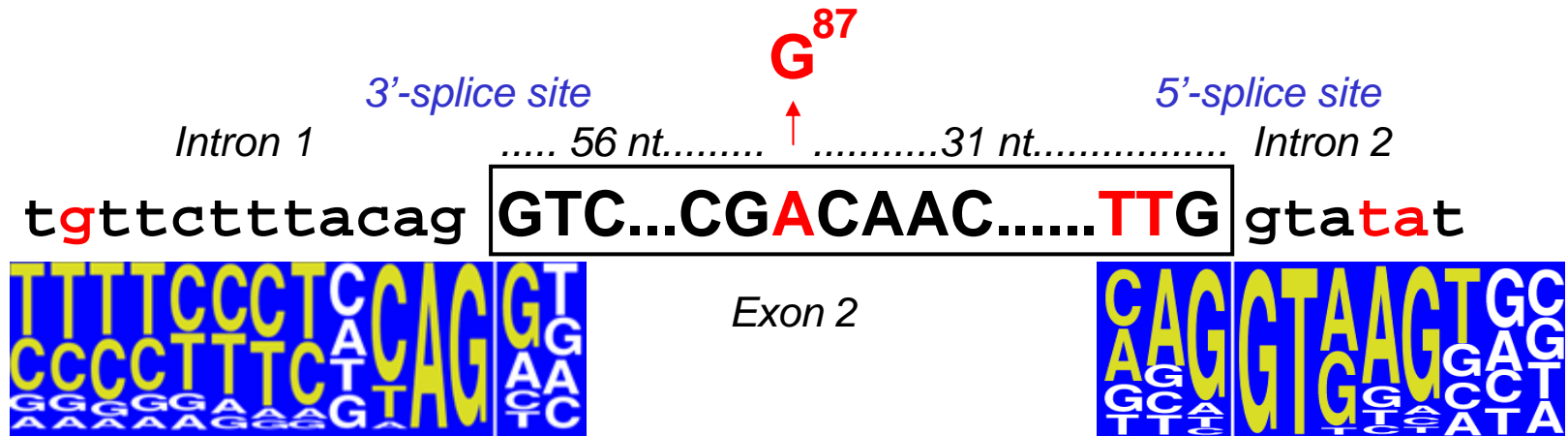
**Is the c.87A>G mutation deleterious despite being a silent mutation? Splicing??**

# Does the c.87A>G mutation affect splicing?



1. c.87A>**G** is located in the middle of exon 2 – It does not affect splice sites directly, nor does it create a pseudo-splice site.

# Does the c.87A>G mutation affect splicing?



1. c.87A>G is located in the middle of exon 2 – It does not affect splice sites directly, nor does it create a pseudosplice site.
2. Exon 2 5' splice site is weak, with a score of only 1.76 (Threshold for functional sites is 6.67).

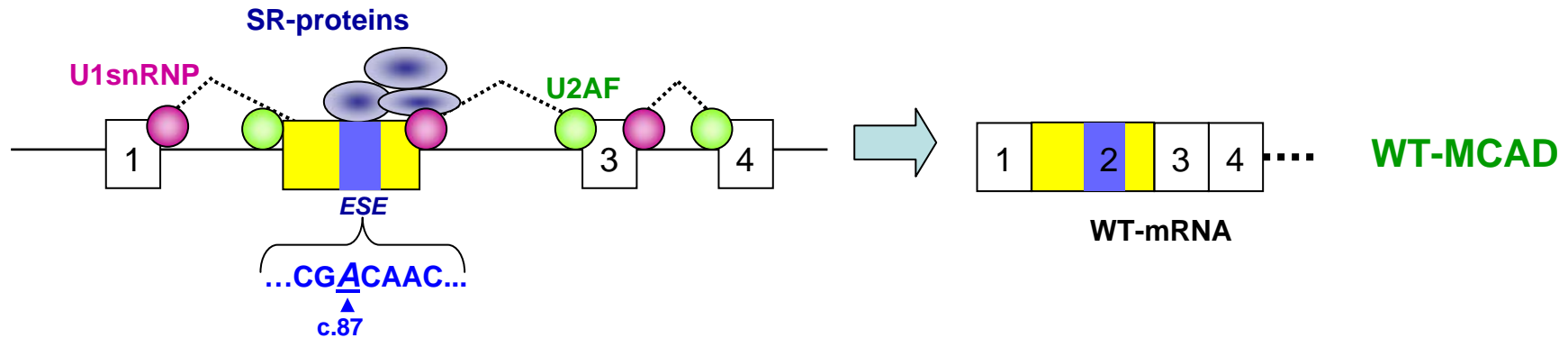
Exon 2 has a very weak 5' splice site, which is hard to recognize for splicing factors such as U1snRNP



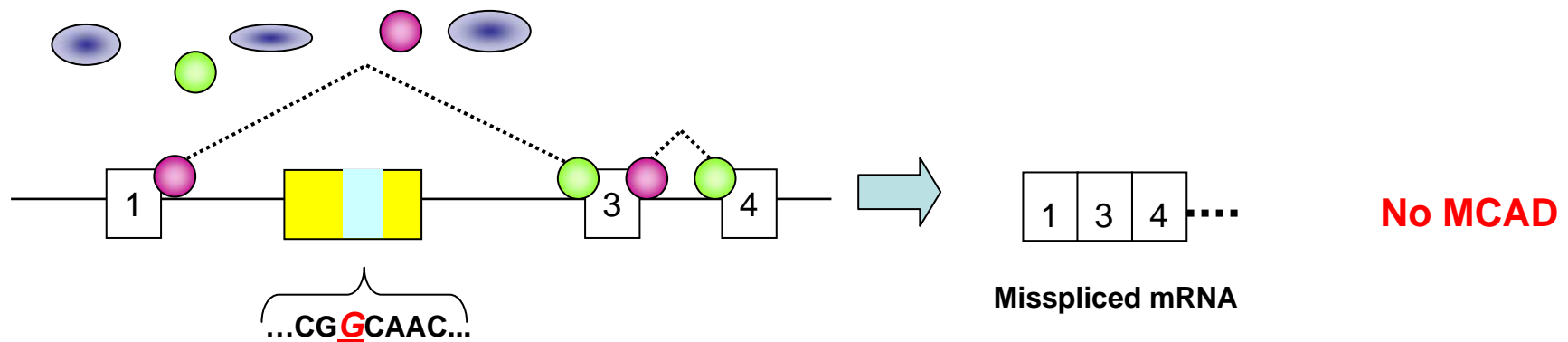
Does the c.87A>G mutation disrupt a splicing regulatory element which is necessary for recognition of the weak splice site ?

# Hypothesis 1: Exon Splicing Enhancer (ESE) inactivation

*Splicing of exon 2 with “c.87A” :*



*Splicing of exon 2 with “c.87A>G” :*





# In silico analysis of MCAD exon 2: c.87A>G and c.85C>T

## RESCUE ESE and FASS ESS analysis:

WT	(85C-87A)	GCCAAT <u>CG</u> ACAACGT	ACAACG ESE motif
("Control") 85C>T	(85T-87A)	GCCAAT <u>TG</u> ACAACGT	ACAACG ESE motif
87C>G	(85C-87G)	GCCAAT <u>CG</u> <u>G</u> CAACGT	No ESE motif

No inhibitory motifs predicted by FASS-ESS prediction



This may suggest that the c.87A>G mutation disrupts an ESE, and that it does not create an ESS.

# In silico analysis of MCAD exon 2: c.87A>G and c.85C>T

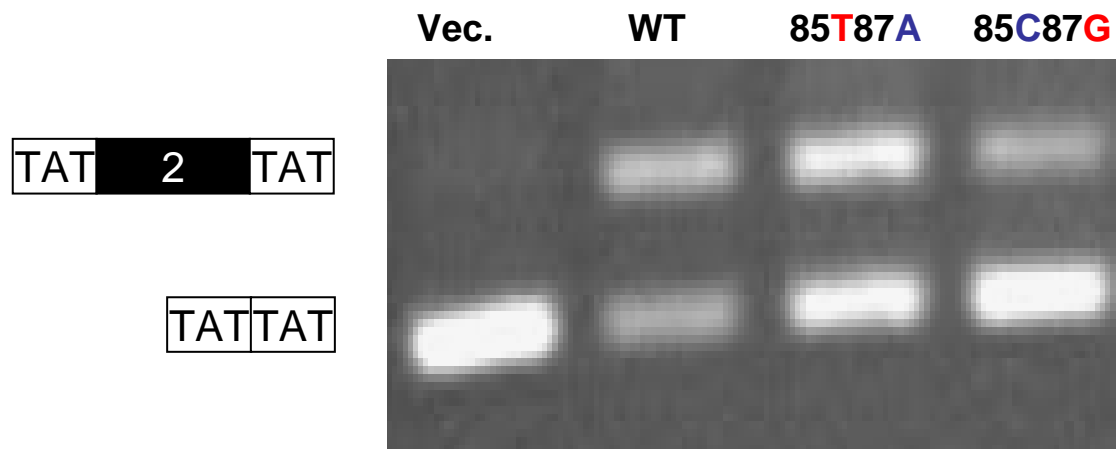
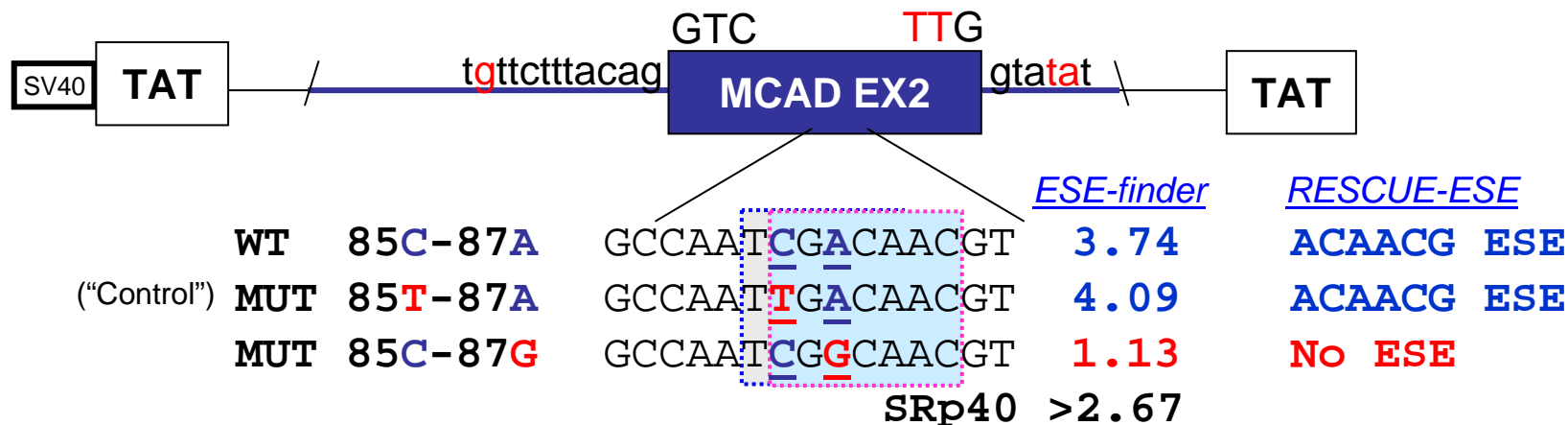
## ESE finder analysis:

		<i>ESE-finder</i>	
WT	(85C-87A)	GCCAATCGACAACGT	3.74
("Control") 85C>T	(85T-87A)	GCCAATTGACAACGT	4.09
87A>G	(85C-87G)	GCCAATCGGCAACGT	1.13
		SRp40	>2.67



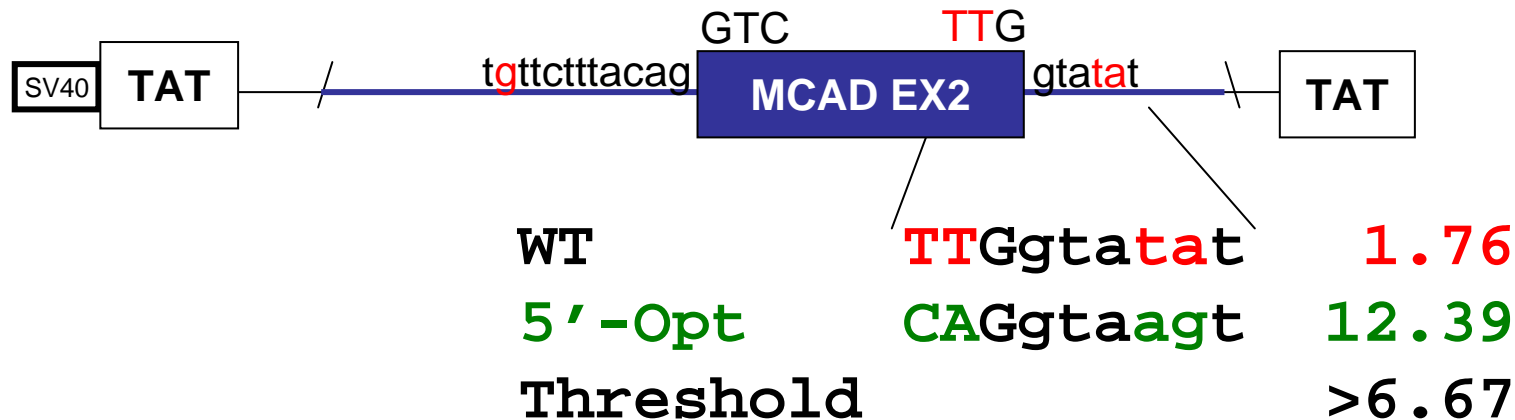
This may suggest that the c.87A>G mutation disrupts an SRp40 binding ESE

# Minigene studies of MCAD exon 2: c.87A>G and c.85C>T

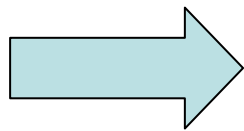
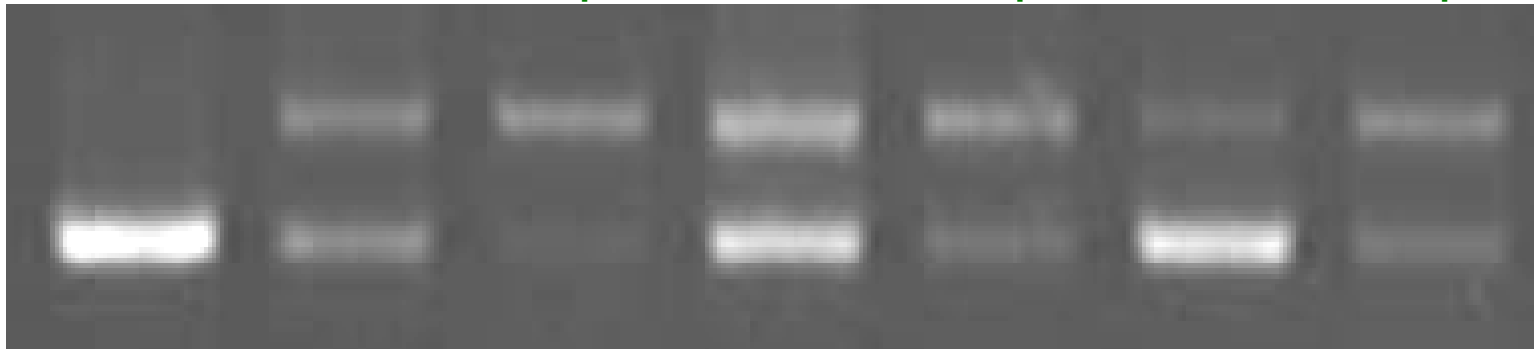


➔ This shows that wild type exon 2 is difficult to splice. It confirms that c.87A>G causes missplicing and is consistent with disruption of an SRp40 binding ESE

# Optimization of 5'-splice site of MCAD exon 2:



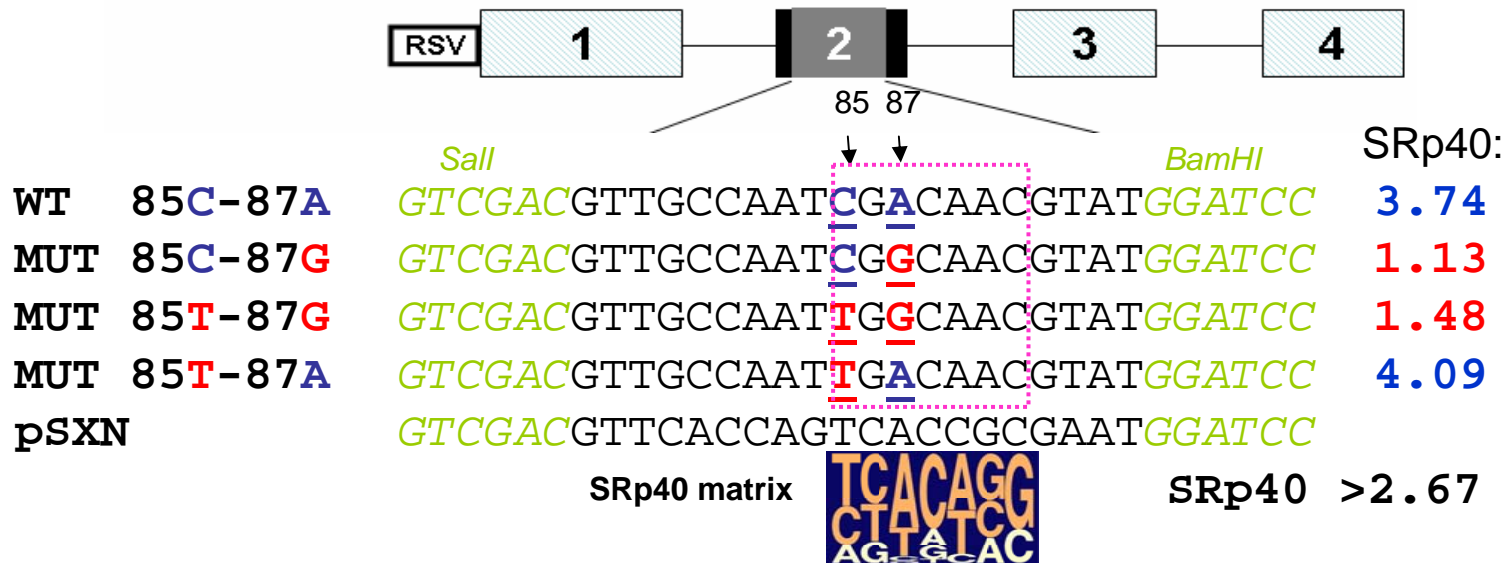
Vec.                      WT                      85T87A                      85C87G  
    5'-opt                      5'-opt                      5'-opt



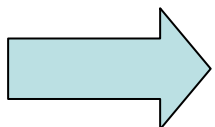
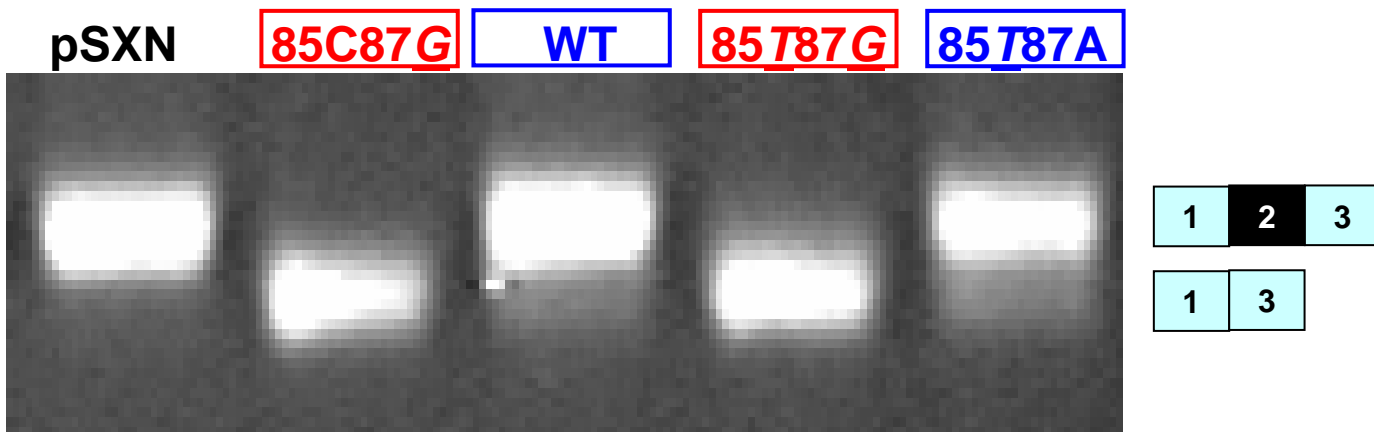
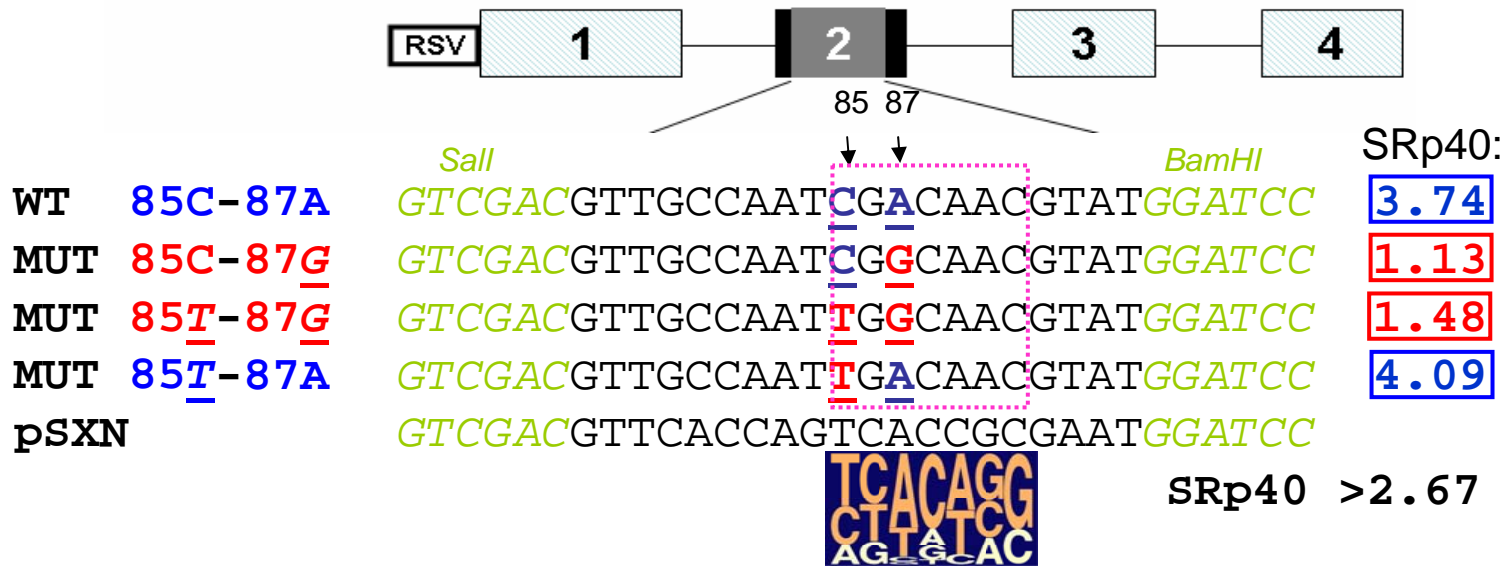
This shows that exon 2 is difficult to splice, mainly due to the weak 5'-splice site. It suggests that the putative ESE is needed for recognition of this weak 5' splice site.

# pSXN Minigene studies of the putative ESE

1. Testing of target sequence in a heterologous context like the pSXN reporter can confirm if it can function as an **ESE**
2. Distinguish between **ESE** disruption and **ESS** creation by the c.87A>G mutation
3. Possible to explore the consensus for an **ESE**



# pSXN Minigene studies of the putative ESE



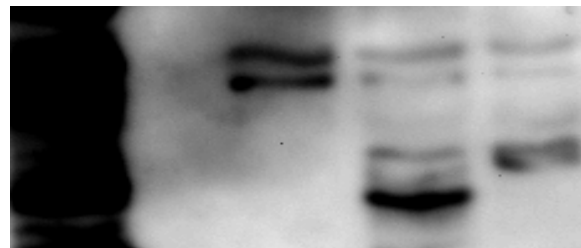
This confirms that the c.87A>G mutation disrupts an ESE, with a consensus sequence like the SRp40 binding motif

# RNA-affinity purification

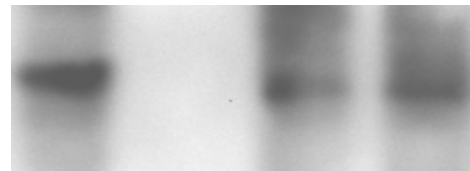


Western blot analysis of affinity purified proteins:

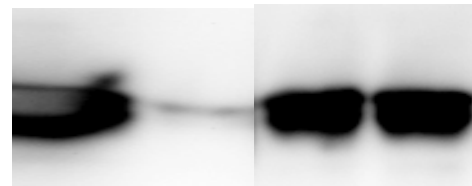
NE --- 0 WT 87G



← SRp40

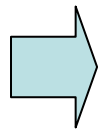


← 9G8



← hnRNPH

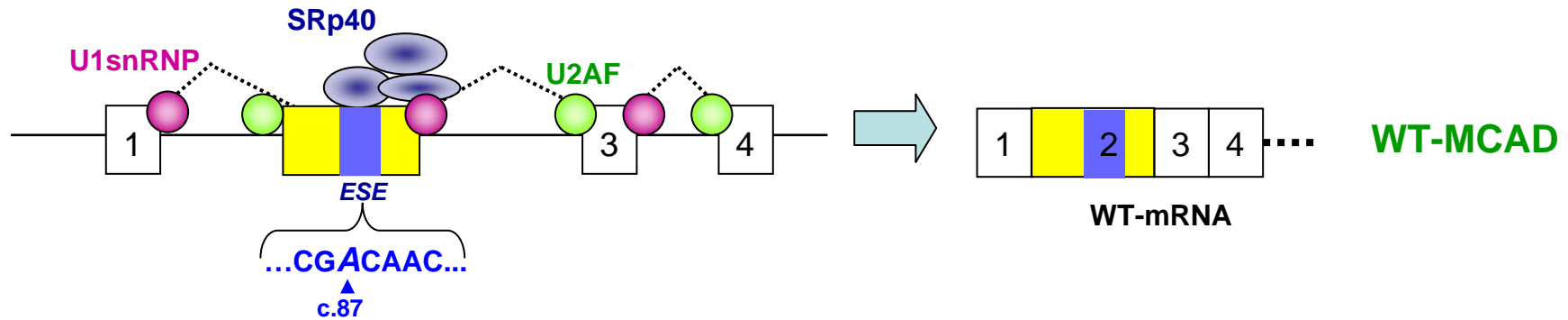
NE 0 WT 87G



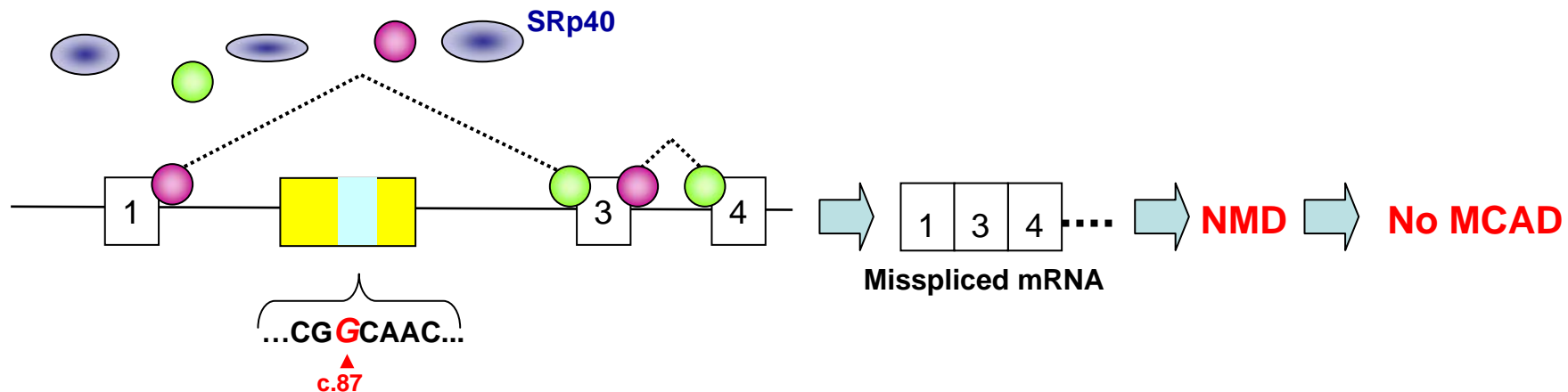
The c.87A>G mutation disrupts binding of SRp40

# c.87A>G causes ESE inactivation by disruption of SRp40 binding

Splicing of exon 2 with “c.87A” :



Splicing of exon 2 with “c.87A>G” :



# Confirmation of the splicing defect in patient cells

Cells from 2 controls, the patient and her father (Both heterozygous for c.87A>G) were cultured with and without cyclohexamide (Blocks NMD).

EXON 2 skipping



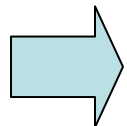
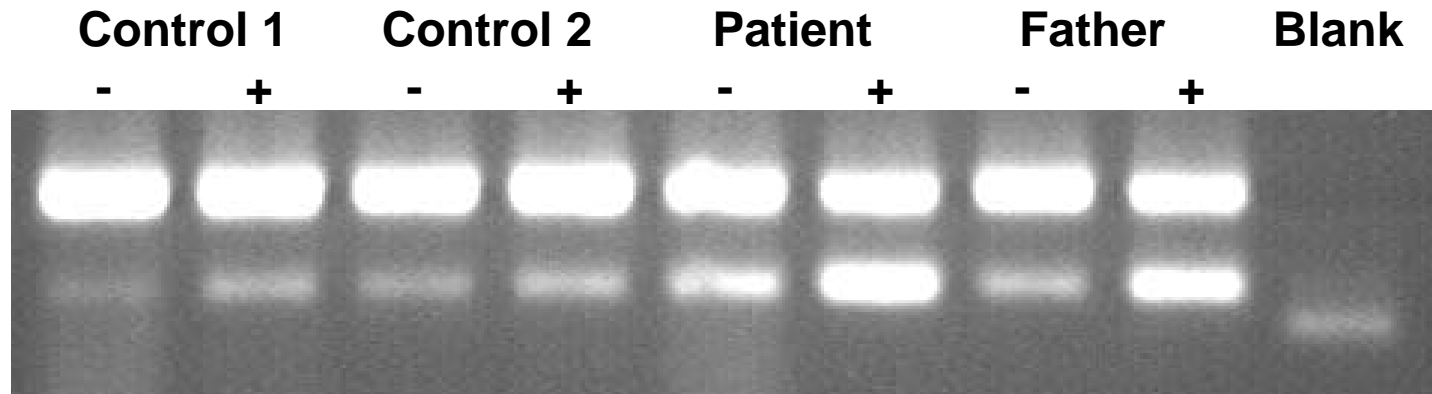
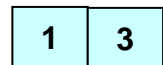
Shifted reading frame



PTC (TAA stop) at c.230-32 in exon 4



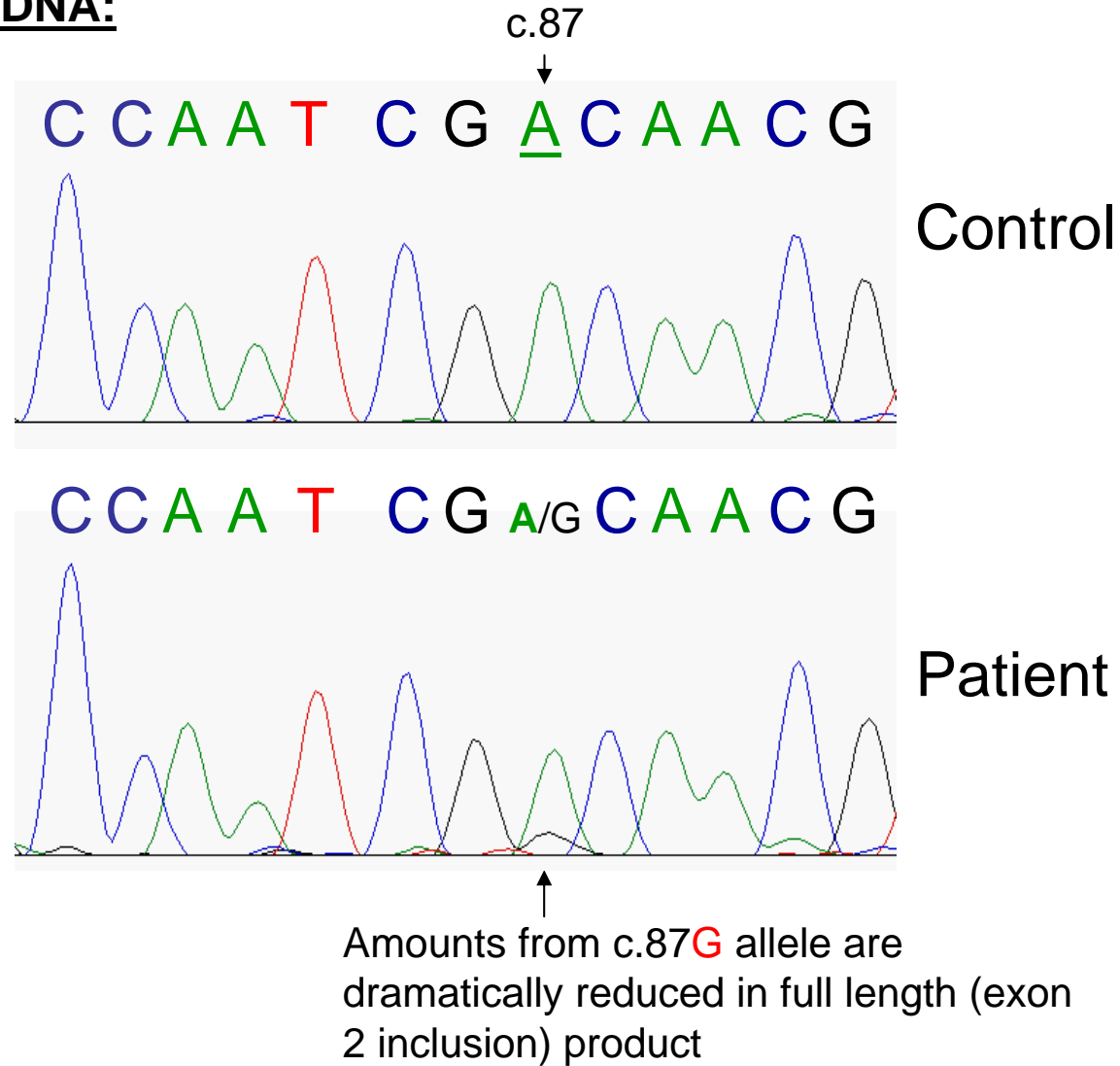
NMD (Degradation)



This confirms that the c.87A>G mutation causes exon 2 skipping and NMD in patient cells.

# Confirmation of the splicing defect in patient cells

## Sequence analysis of patient cDNA:



# Confirmation of the splicing defect in patient cells

## Sequence analysis of patient cDNA:

EXON 2 skipping



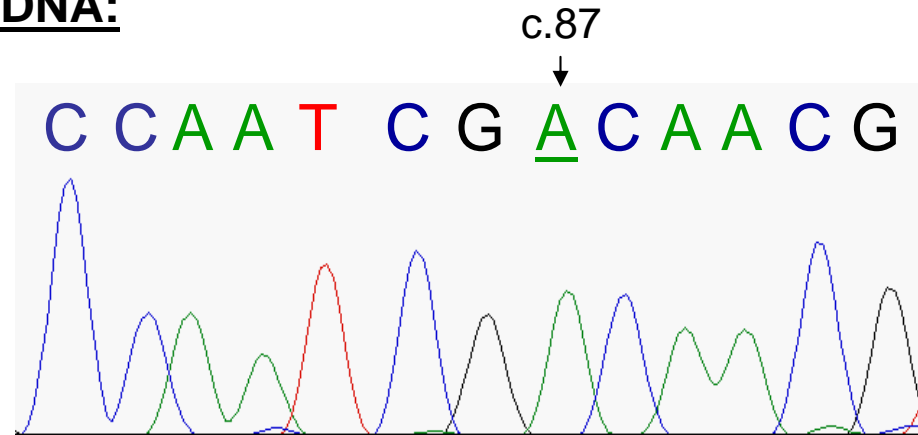
Shifted reading frame



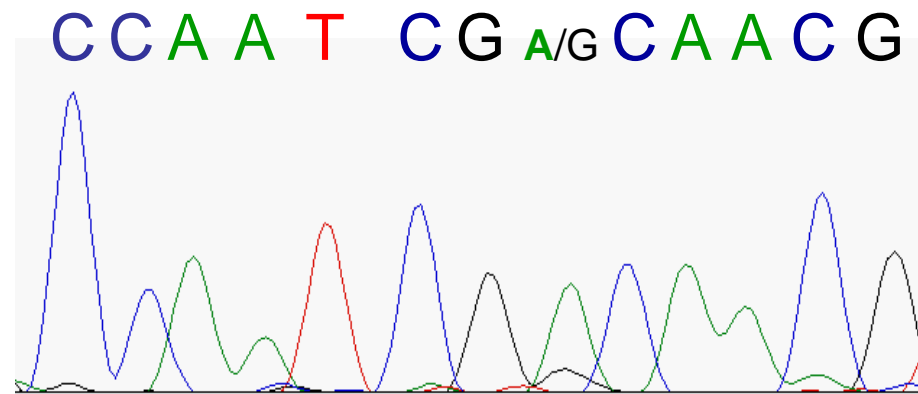
PTC (TAA stop) at  
c.230-32 in exon 4



NMD (Degradation)



Control



Patient

↑  
Amounts from c.87G allele are  
dramatically reduced in full length (exon  
2 inclusion) product

# Confirmation of the splicing defect in patient cells

## Sequence analysis of patient cDNA:

EXON 2 skipping



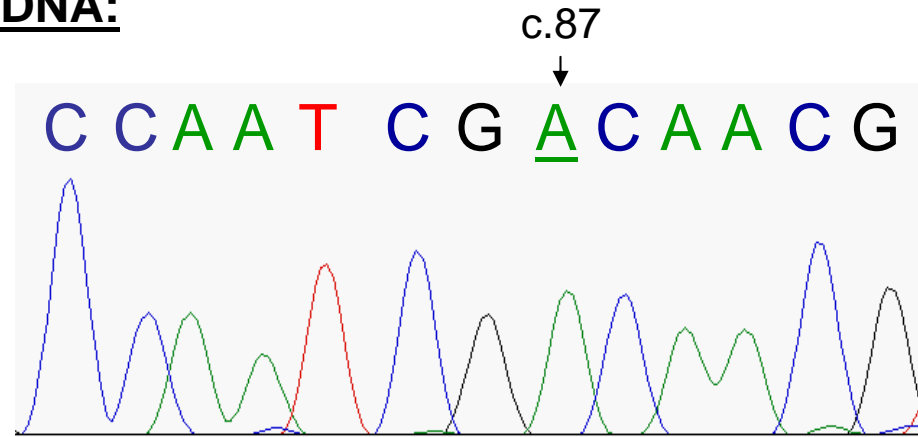
Shifted reading frame



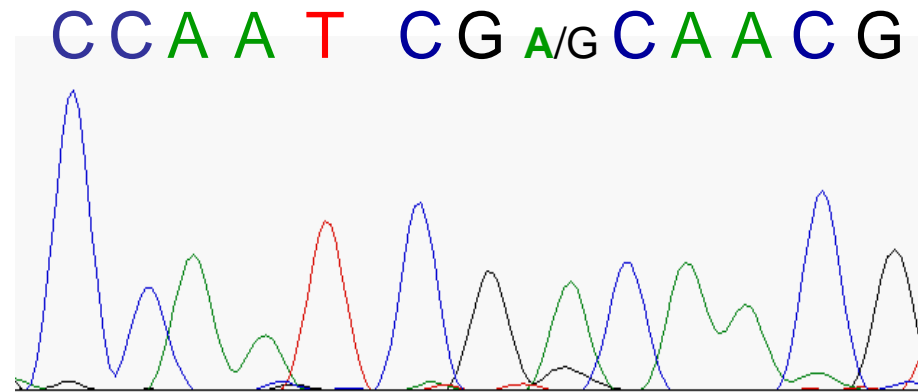
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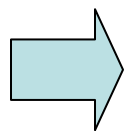
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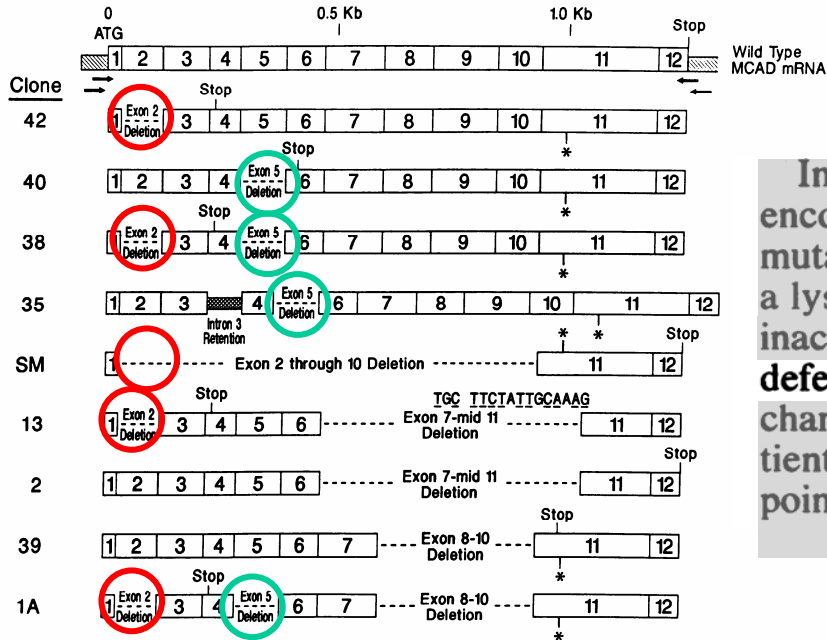


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# Unexplained mis-splicing in MCAD patients homozygous for c.985A>G - A mystery for more than 15 years

Medical Sciences: Kelly *et al.*

*Proc. Natl. Acad. Sci. USA* 87 (1)



Kelly DP *et al.* (1990) *PNAS* 87:9236-9240:

In summary, we have shown that the gene and mRNA encoding the defective MCAD in this family contain a point mutation that results in the substitution of a glutamic acid for a lysine and most likely results in an unstable, catalytically inactive protein. In addition, the patient has an unusual defect in the splicing of the MCAD pre-mRNA. Molecular characterization of additional unrelated MCAD-deficient patients will allow us to determine the frequency of the G<sup>985</sup> point mutation.

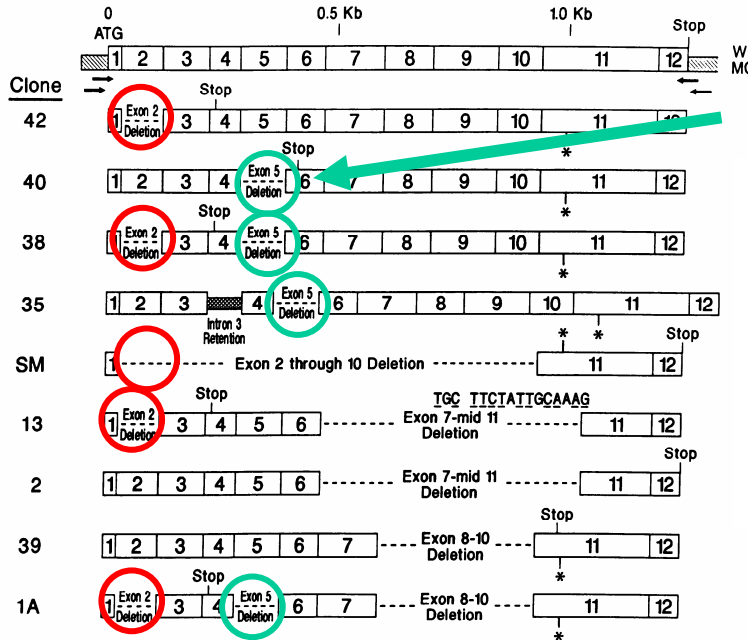
Gregersen, Andresen *et al.* (1991) *Hum Genet* 86(6):545-51:

“.. It seems that the amount of cDNA containing deletions is higher in the patient than in normal persons. .... The deletions of exons 2, 5 and 8 can only be explained by mis-splicing.....This secondary pathophysiological phenomenon might seriously contribute to the course of life-threatening attacks”.

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*Proc. Natl. Acad. Sci.*



Nielsen *et al.* (2007) *Am J. Hum Genet* 80:416-32

ESE in Exon 5 needed for correct splicing

inactive protein. In addition, the patient has an unusual defect in the splicing of the MCAD pre-mRNA. Molecular characterization of additional unrelated MCAD-deficient patients will allow us to determine the frequency of the G<sup>985</sup> point mutation.

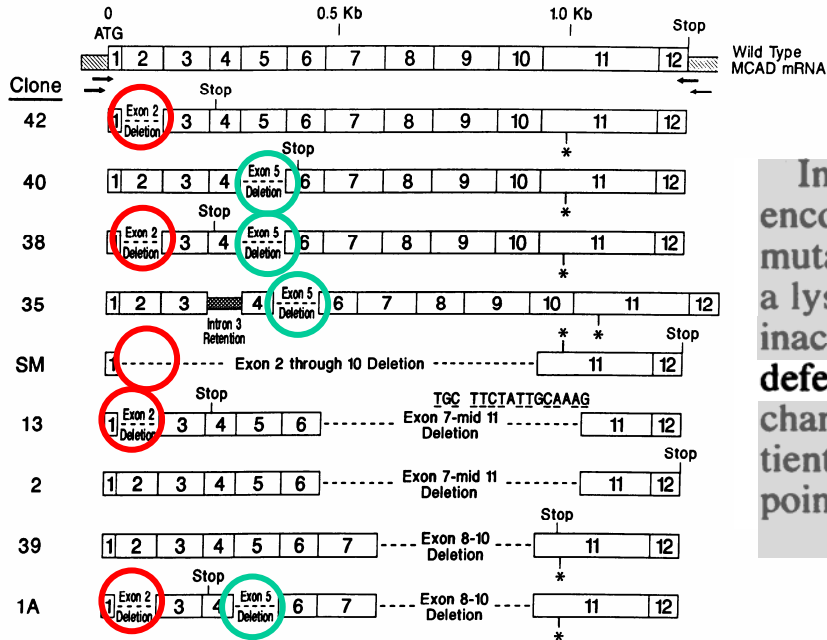
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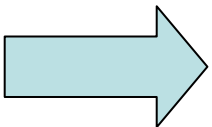


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**Exon 2 and 5 are vulnerable and dependent on ESEs – Increased skipping of exon 2 and 5 in patients with other mutations**

# Conclusions

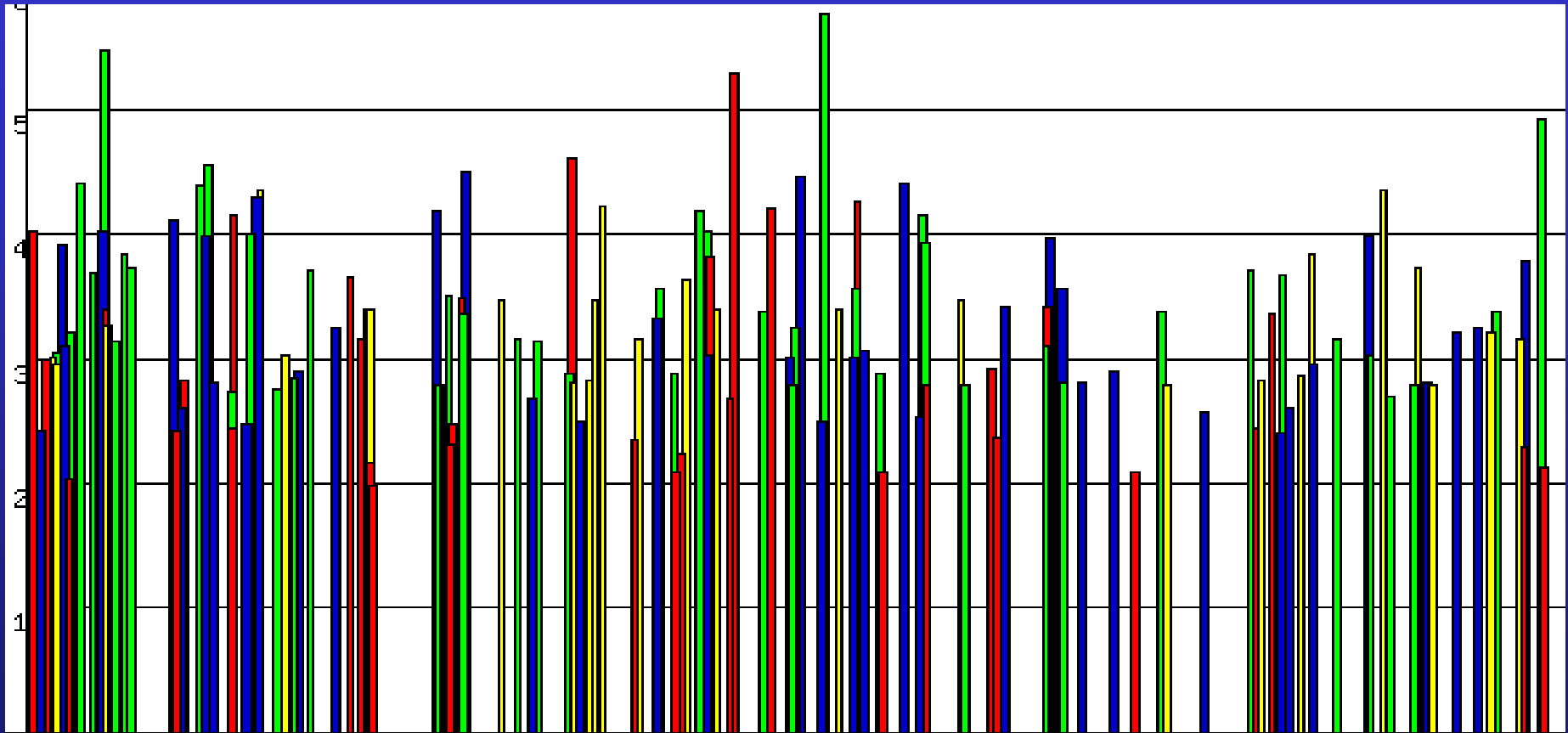
1. Splicing mutations are common  
Total 25-30% of these as many as 5-10% are silent/missense mutations
2. Prediction programs are pretty good, but functional testing and data collection vital for future
3. Effect is highly dependent on context (weak splice sites – flanking ESS/ESE etc.).
4. All genes have vulnerable exons, which may be misspliced if mutations hit exon splicing regulatory elements or compromise splice sites

# SEARCH BY ESE FINDER

1

MCAD cDNA

1269



93 mutations in exons

42 mutations either create/abolish high score motifs

24 (26%) destroys high score motifs



# Conclusions

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Total 25-30% of these as many as 5-10% are silent/missense mutations
2. Prediction programs are pretty good, but functional testing and data collection vital for future
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# ACKNOWLEDGMENTS:

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*Anne Vested Jensen*

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*Lisbeth D. Schroeder*

*Karsten Bork Nielsen*

*Pia P Madsen*

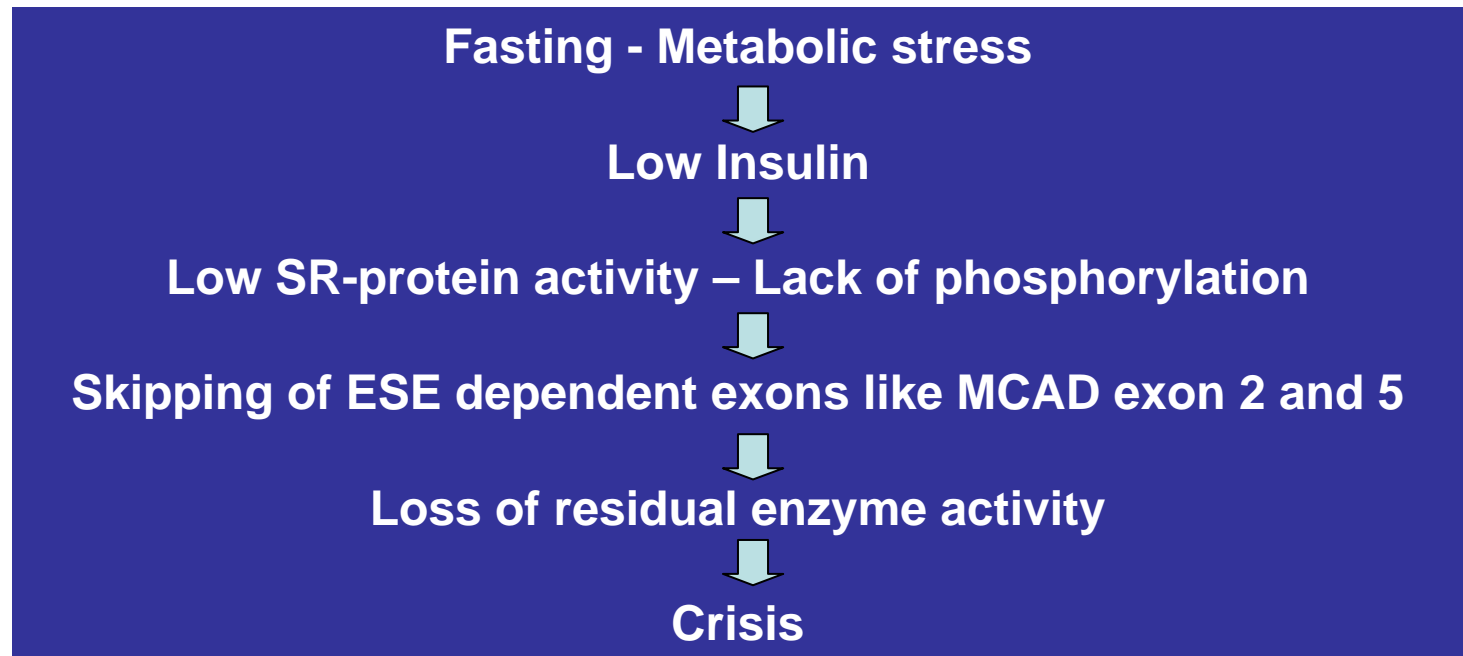
*Birthe Gahrn*

MUTATION ANALYSIS OF UK NEWBORNS PART OF THE UK  
COLLABORATIVE STUDY

# Missplicing and pathophysiology – Increased MCAD exon 2 and 5 skipping when patients are metabolically stressed?

1. SR-proteins and SRp40 activity is regulated by phosphorylation by SR-kinases
2. Insulin activates these kinases (for instance Akt-2 kinase)
3. Insulin has been shown to regulated Glucose-6-Phosphate dehydrogenase and PKCbeta through SRp40 activation (by phosphorylation).

## Hypothesis:



**So far purely speculative, but it might be a contributing factor!**