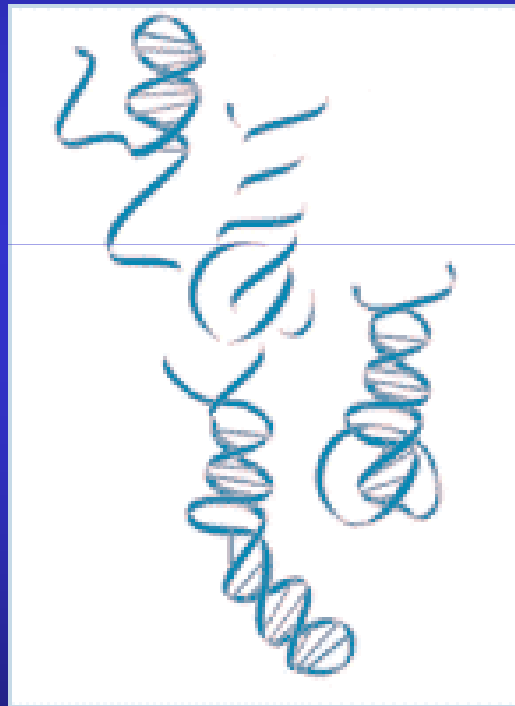


# Gli APTAMERI

# APTAMERI

Acidi nucleici a singolo filamento caratterizzati da una specifica **struttura tridimensionale** che si lega direttamente alla proteina target.



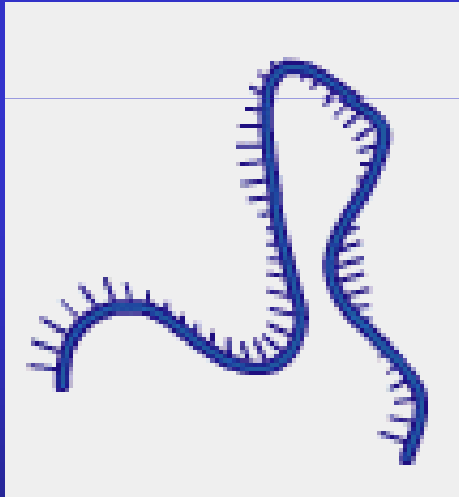
**Interazione Acido Nucleico/Proteina**

# Aptameri

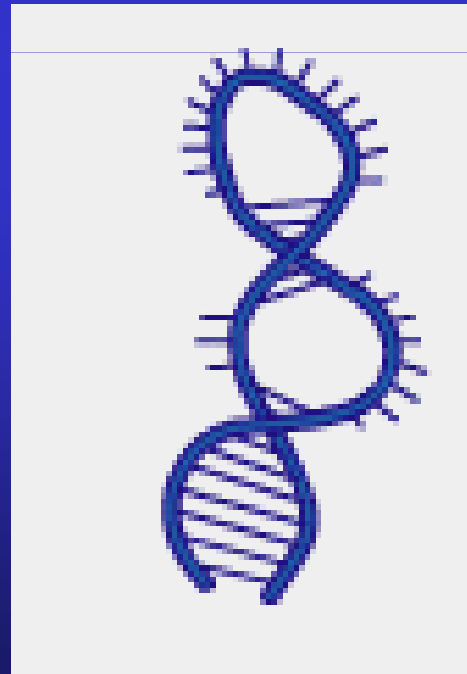
Dimensioni: 30-70 nucleotidi



Molecola Lineare



Folding

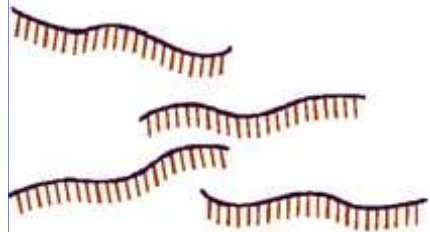


Struttura  
tridimensionale  
stabile

RNA oder ssDNA  
(<100nt)

defined  
three-dimensional  
structures

aptamer-target  
complex

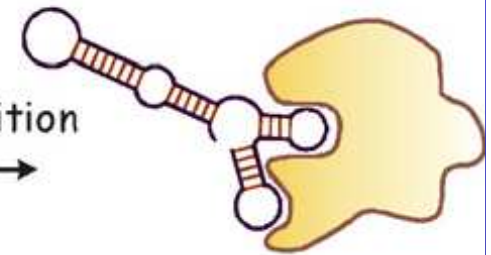


folding



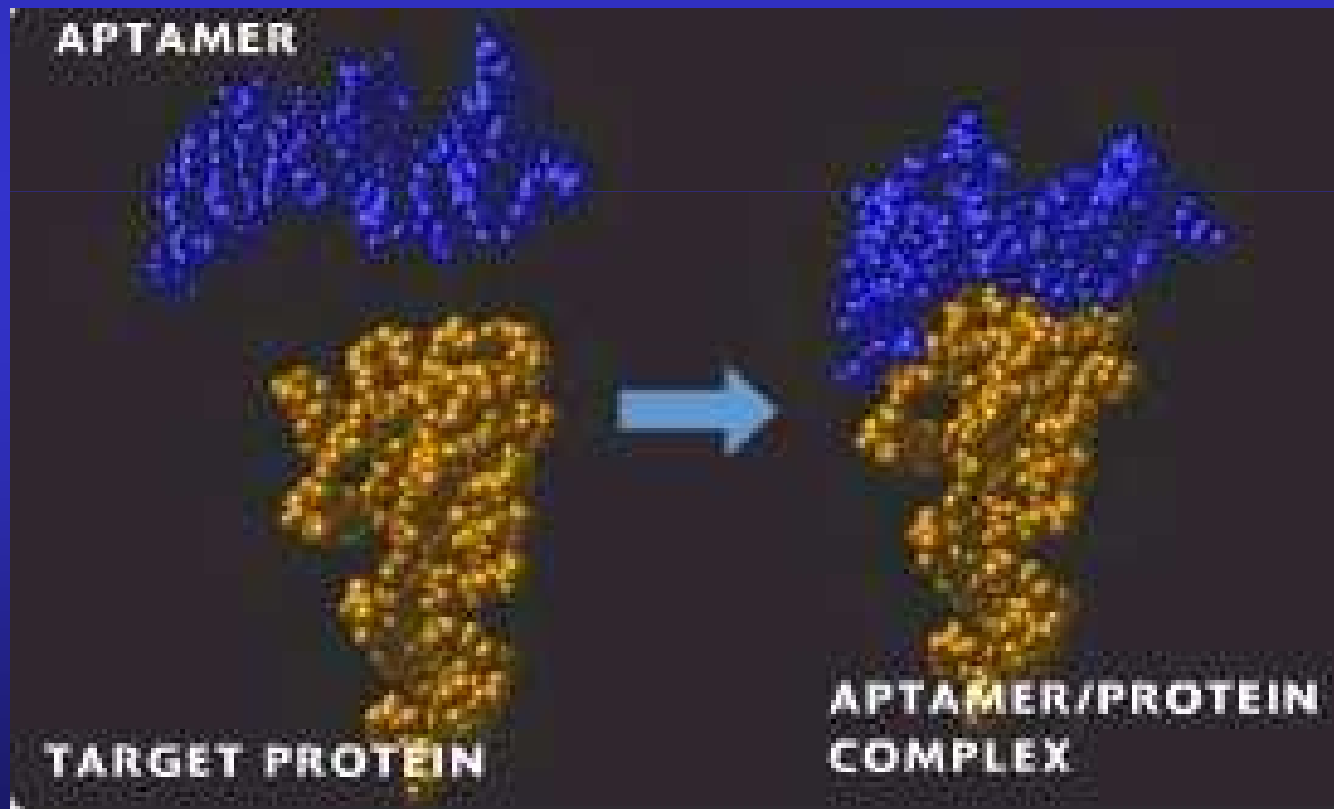
molecular recognition

binding



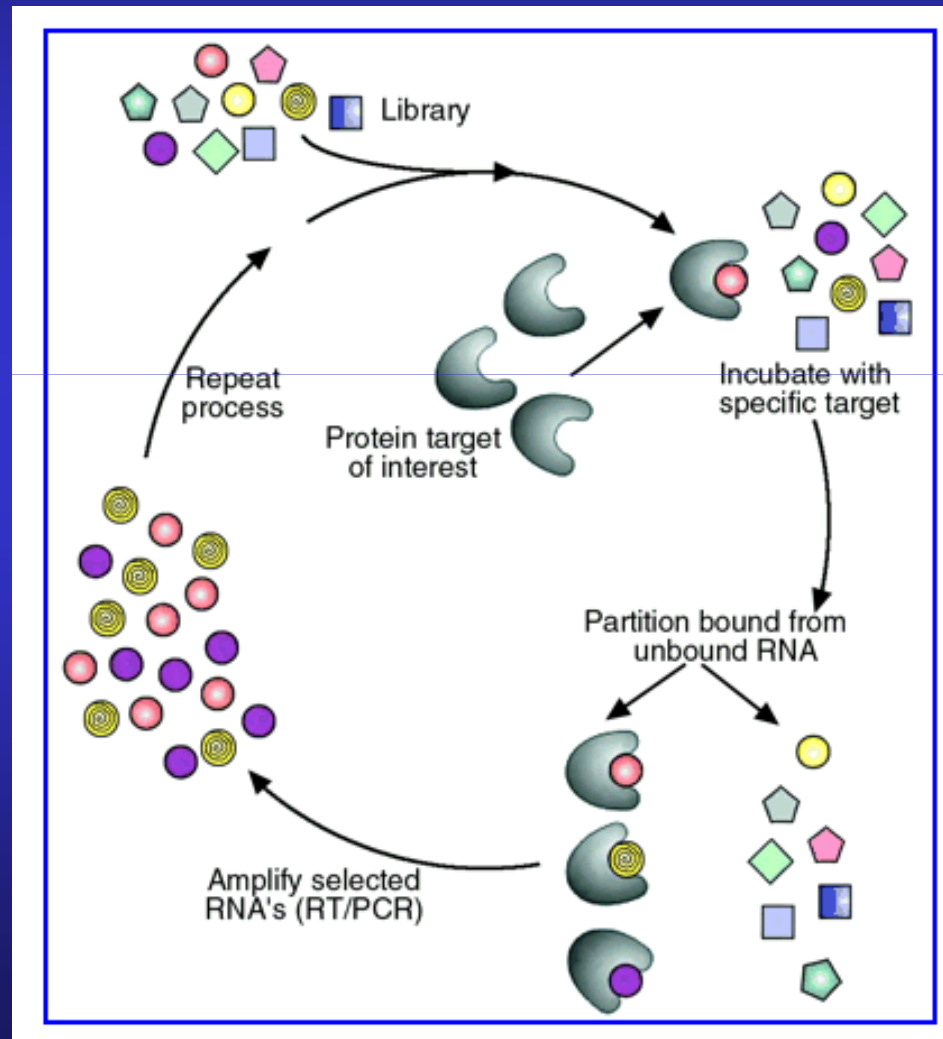
# Anatomia degli Aptameri

Gli aptameri sono molecole selezionate per legarsi in modo specifico ad una predefinita *proteina target*



# Selezione in vitro degli Aptameri:

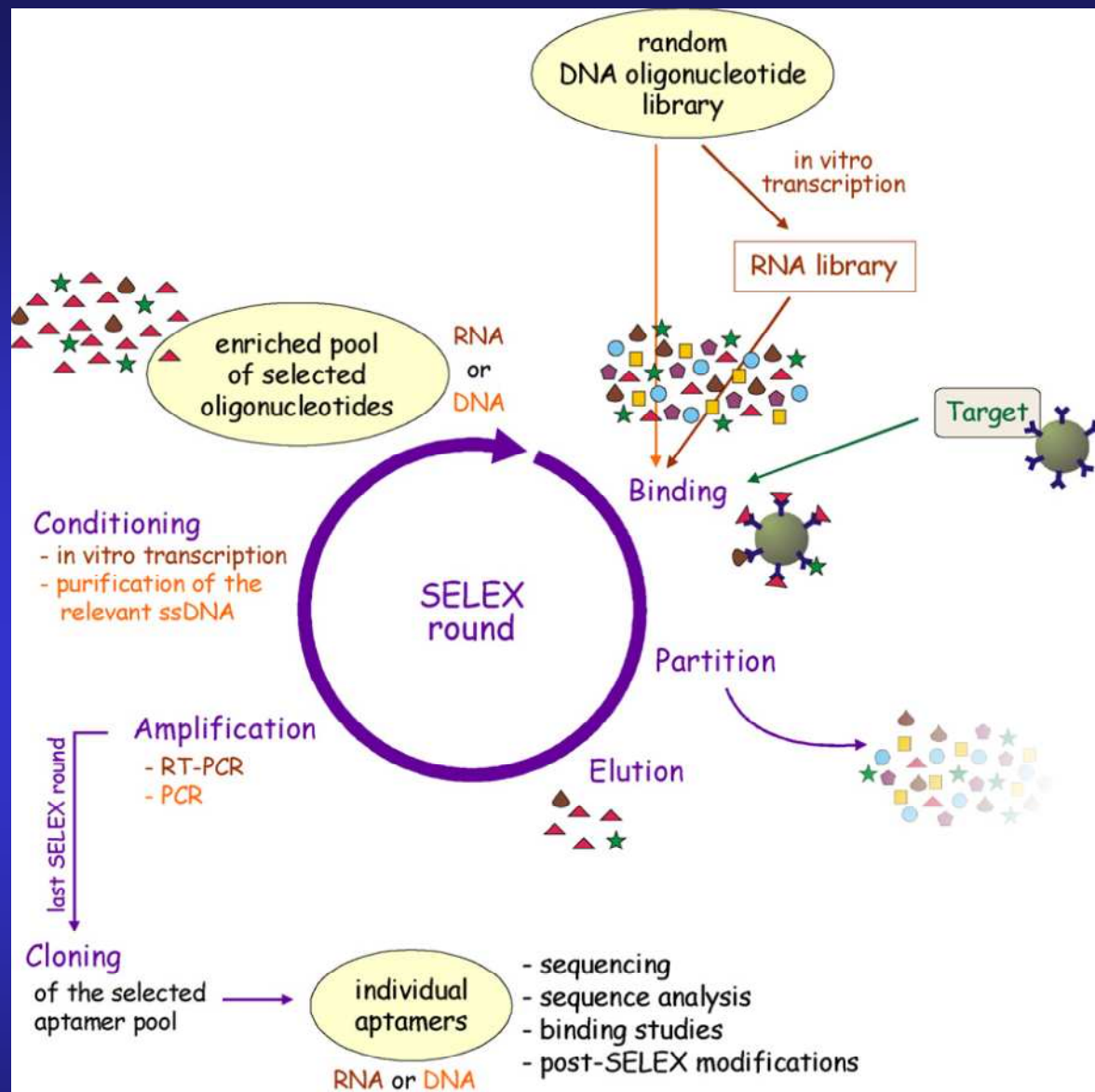
**SELEX** (systematic evolution of ligands by exponent enrichment)



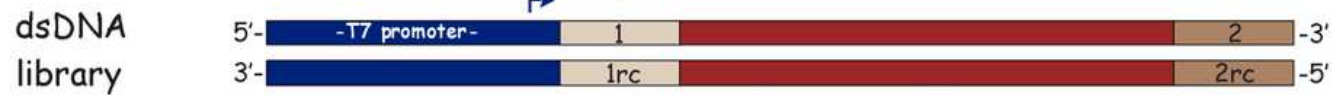
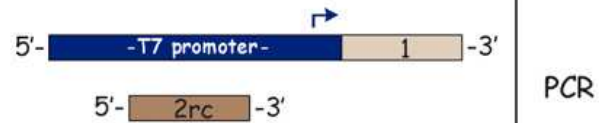
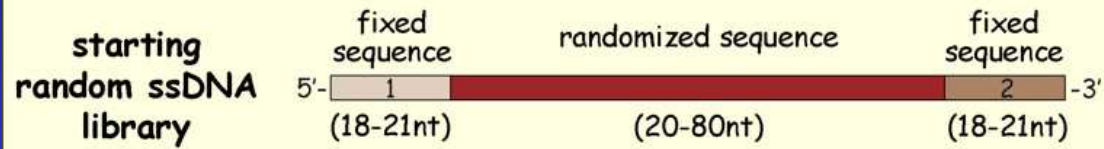
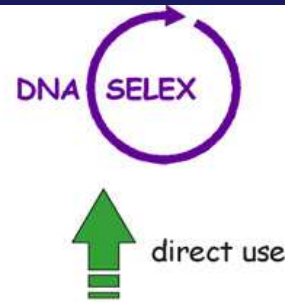
# Selezione in vitro degli Aptameri:

**SELEX** (systematic evolution of ligands by exponent enrichment)

1. Sintesi chimica di  $10^{14}$  RNA o DNA (Libreria)
2. Incubazione con la proteina target: cromatografia per affinità
3. Rimozione degli oligo *non legati* mediante buffer di lavaggio
4. Rimozione degli oligo *legati* alla proteina target con una soluzione contenente la proteina target
5. Retrotrascrizione e PCR (RNA) o solo PCR (DNA) degli oligo che si sono legati
6. Trascrizione in vitro (RNA) o solo denaturazione (DNA) per separare i filamenti
7. Inizio di un nuovo ciclo fino a 5-10 cicli

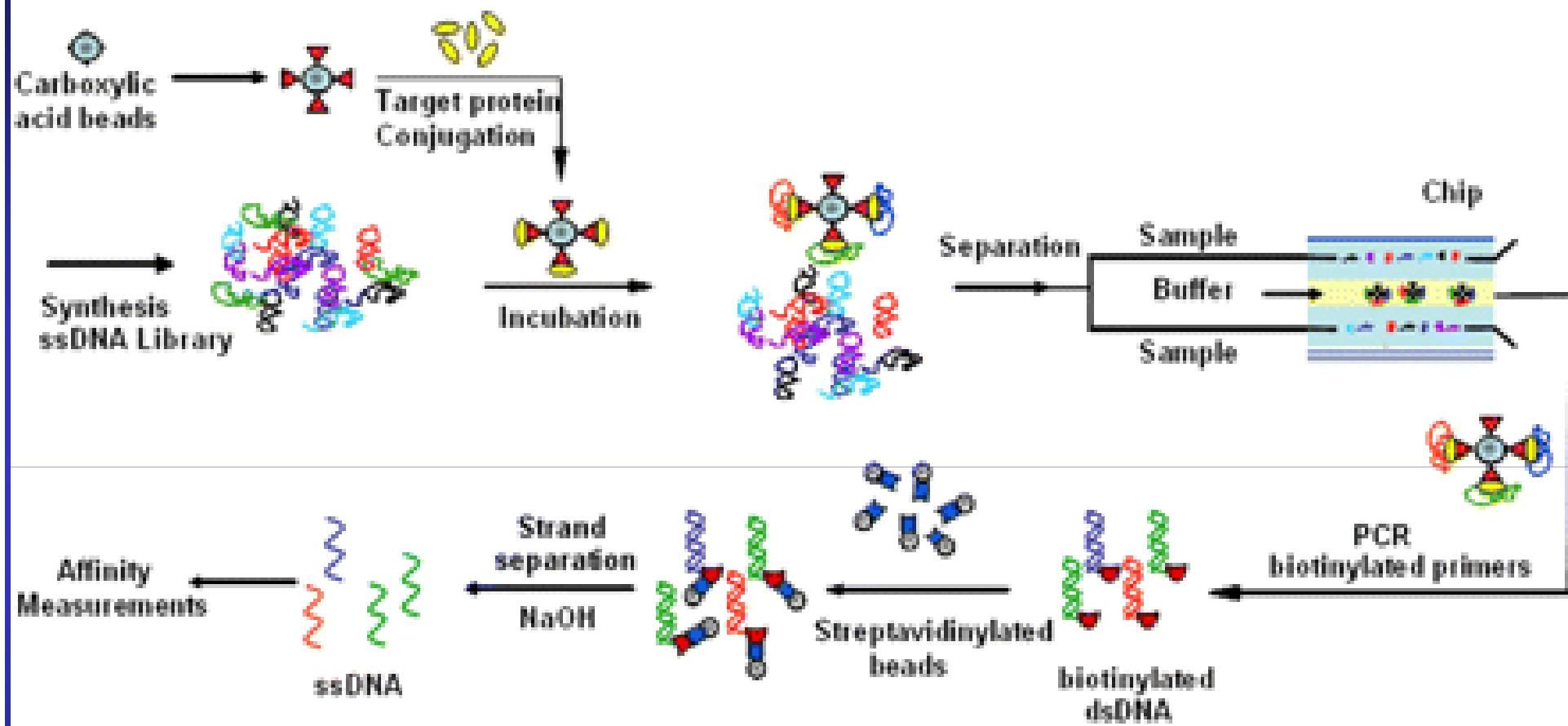






in vitro transcription  
by T7 RNA polymerase





# Applicazioni degli Aptameri:

**1. Ricerca**

**2. Diagnostica**

**3. Terapia**

# Applicazioni degli Aptameri:

## ALTERNATIVA AGLI ANTICORPI

- elevate specificità e affinità unite a ridotte dimensioni
- sintesi chimica (vs sintesi in animali o colture cellulari)
- facilmente modificabili: marcatura con radioattivo, code fluorescenti e biotinilate...

## **APPLICAZIONI IN VIVO:**

**→ nessuna tossicità dimostrata (facilmente eliminabili da sangue e reni)**

**→ non immunogenici**

**→ tessuto-specifici**

Table 3  
Examples of applied aptamers

Target	Aptamer/assay	Field of application	Research or product state	Reference
VEGF	RNA aptamer, chemically modified	Therapy, wet age related macula degeneration	Product: Macugen <sup>®</sup> (Pfizer Inc.)	Maberley (2005) and Chapman and Beckey (2006)
Thrombin	DNA aptamer (thrombin inhibitor ARC-138)	Therapy, anticoagulant	Product development, Phase 1 studies in August 2004 (Archemix Corp.)	Nimjee et al. (2005a)
Factor IXa	RNA aptamer (factor IXa inhibitor) and its antidote REG1	Therapy, anticoagulant	Product development, Phase 1 studies completed in 2006, (Regado Biosciences Inc.; Archemix Corp.)	Nimjee et al. (2005a) and Dyke et al. (2006)

## **TERAPIA:**

**Condizioni patologiche acute e spazialmente confinate**

**→ Trombosi: aptameri contro trombina, FVIIa, FIXa**

**→ Cancro: aptameri contro proteine segnale (es. Crescita, differenziazione, trasformazione cellulare...)**

**→ Patologie virali: identificazione e inibizione di proteine virali**

# Gli APTAMERI: un'applicazione

Rusconi CP, Scardino E, Layzer J, Pitoc GA,  
Ortel TL, Monroe D, Sullenger BA

RNA aptamers as reversible  
antagonists of coagulation  
factor IXa

Nature 2002; 419: 90-94

([www.nature.com](http://www.nature.com))



# COAGULAZIONE DEL SANGUE

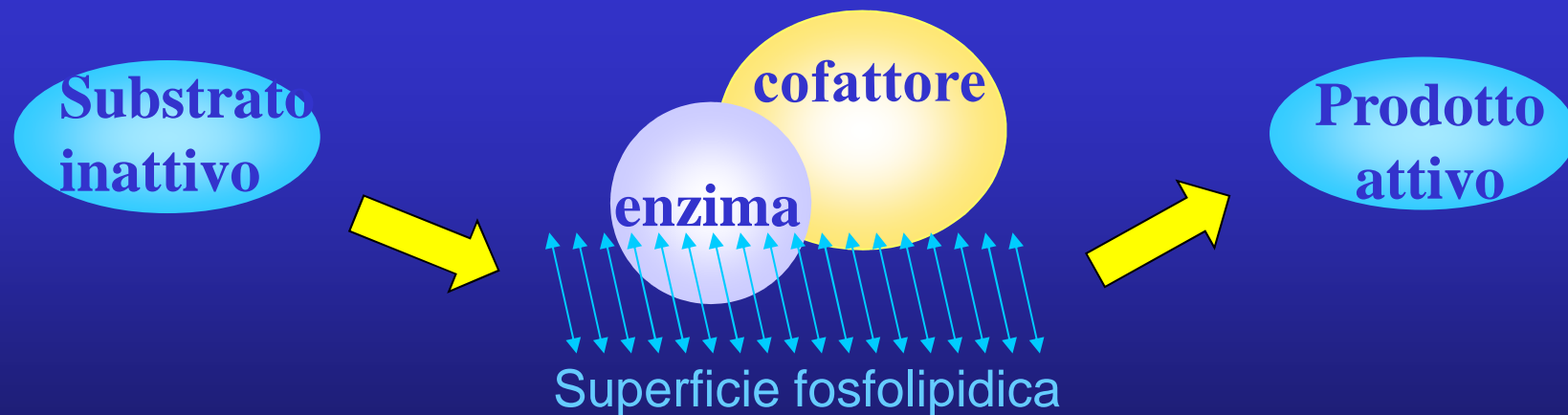
Danno vascolare



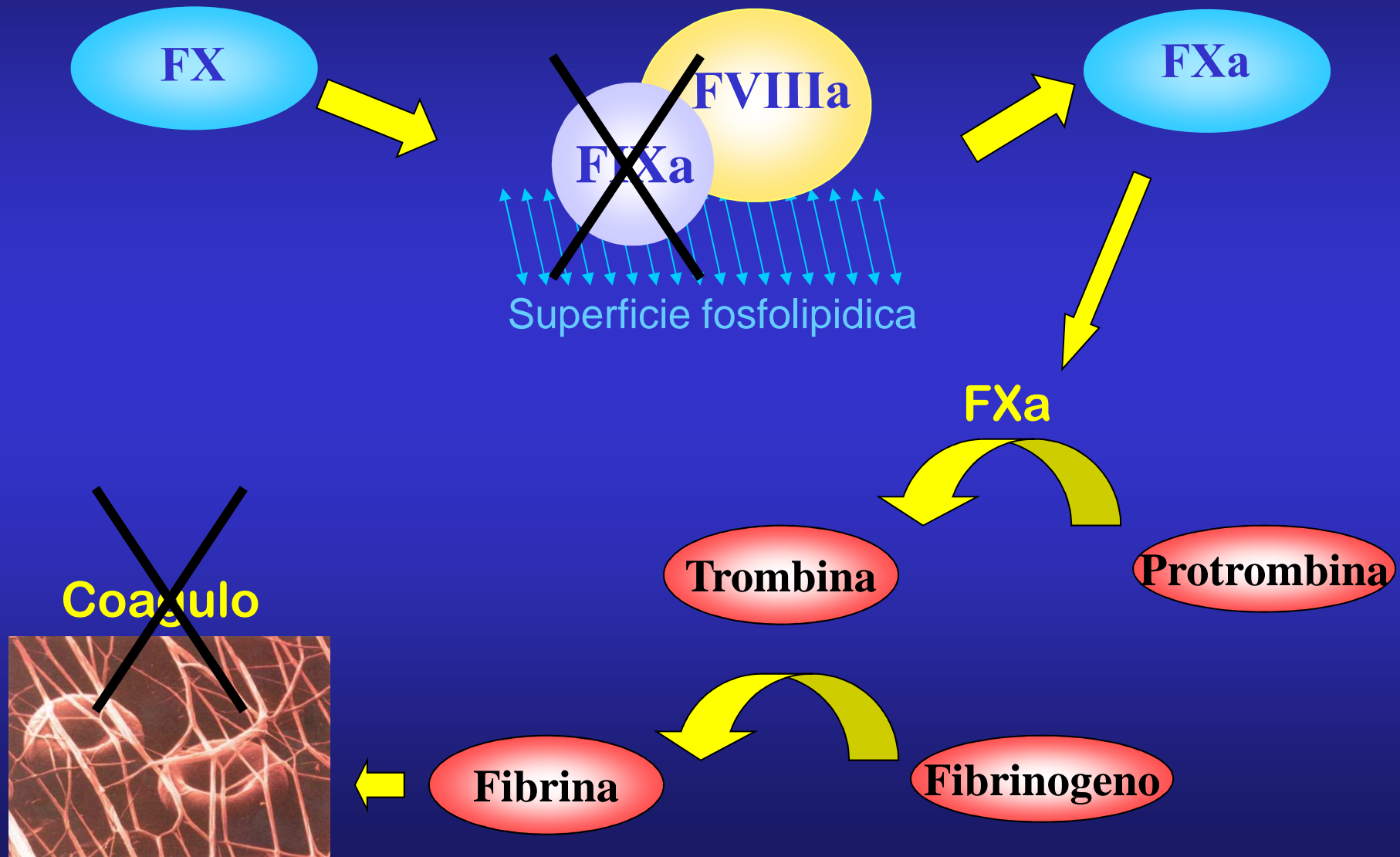
Attivazione a cascata di fattori e cofattori plasmatici



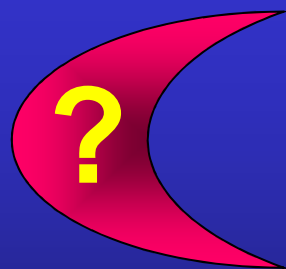
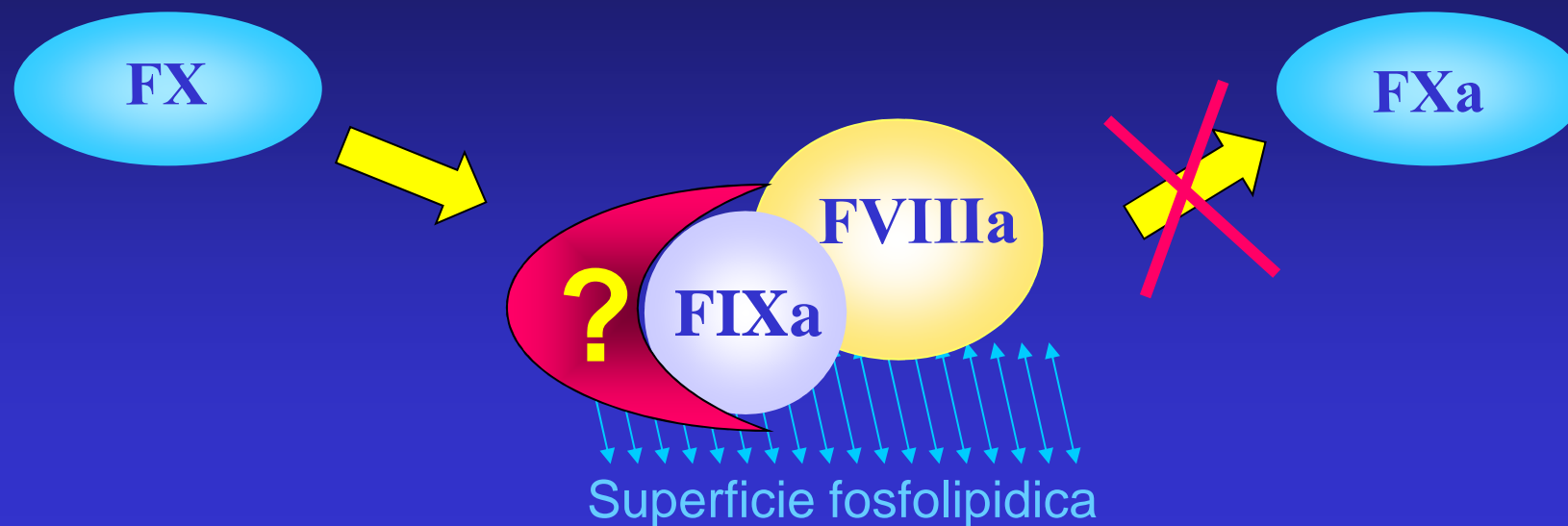
Complessi macromolecolari



# Complesso di attivazione del FX



# Complesso di attivazione del FX



Aptamero selezionato

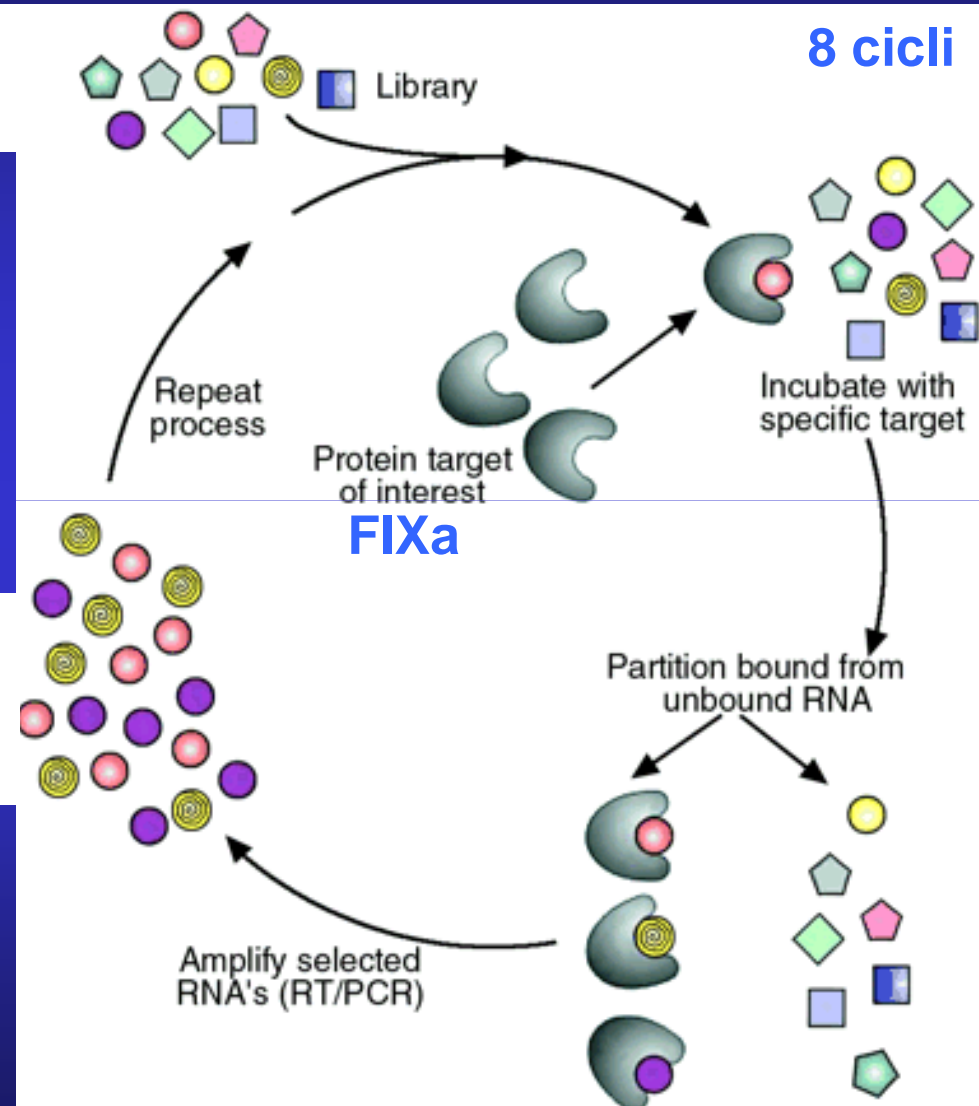
1. Elevata AFFINITA' con il FIXa

2. SPECIFICITA' per il FIXa

# Selezione degli Aptameri SELEX

Libreria:  $10^{14}$   
oligonucleotidi (RNA)

Retrotrascrizione degli  
RNA selezionati e  
sequenziamento

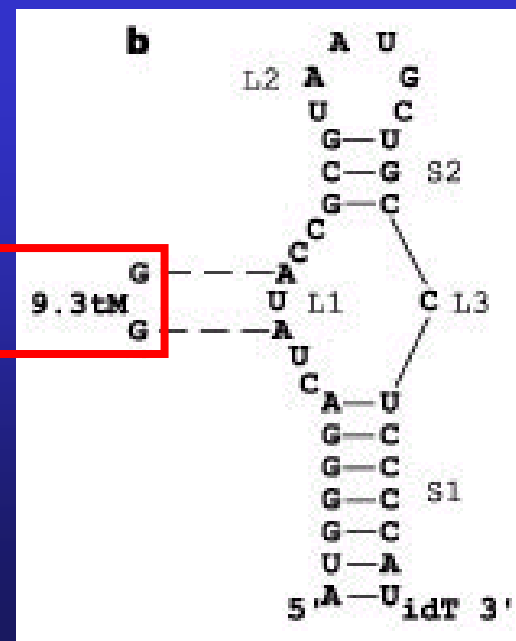


# 1. AFFINITA' con il FIXa

Aptamer	S1	L1	S2	L2	S2	L3	S1	
9-3	5' <u>GGGAUGGGGA</u>	CUAUACC	GCG	UAAUGC	UGC	C	UCCCCAUUCC	GGAACGCU 3'
9-20	5' <u>GGGGA</u>	CUAUACCG	GCA	AUUG	UGC	A	UCCCC	UGGACCUAACAAUA 3'
9-19	5' <u>GGaUGGGGA</u>	CCAUUA	ACGA	CUAC	UCGU	GAA	UCCCCACC	AUCAGCGCACAA 3'
9-4	5' <u>GGGaUGGGC</u>	ACUAUAC	GCA	UCU	UGC	U	GCCUGCCC	GCGAGUCAAUUG 3'
9-12	5' <u>GGGaUGGG</u>	CGAUA	UAC	ACAUG	GUG	AU	CCCACCC	ACAUGAAACCACAG 3'
9-17	5' <u>GAGGgaUGGGGA</u>	CCAUAC	GCA	CAU	UGC	UGAA	UCCCCUC	AAUAGCACCUC 3'
9-25	5' <u>GGGAUGGGGA</u>	CCAUUA	ACUC	UAAC	GGU	GAA	UCCCgCAUCUC	GACAAUA 3'
9-26	5' <u>GGGaUGGG</u>	UGAUA	ACCA	CUC	UGU	GAA	CCCeUCCC	GACUUGCUUGCA 3'
9-11	5' <u>GGGaUGGGGA</u>	CUAUA	UUUGG	AAU	CUGGA	C	UCCCCCU	GCCUGCCCCAGA 3'
9-2	5' <u>GGGAUGGG</u>	CUAUUA	CAC	GCUG	GUG	AU	CCCADCUC	AAUUGAAACACA 3'
9-7	5' <u>GGAUUGGG</u>	CGAUA	ACCA	ACA	UGU	GAU	CCCANUC	AUCAUACCCUACAA 3'
9-28	5' <u>GGGAUGGGCG</u>	CCAUAC	GCA	CAU	UGC	UGCAU	CGCCUCCCC	GUAAGAAC 3'
9-16	5' <u>GAGGGaUGGG</u>	CCAUAC	GUUG	ACGA	CUGC	A	CCCGaCCCUU	CAGCCCAGSUC 3'
9-18	5' <u>GGGaUGGG</u>	CCAUUA	ACCA	CUU	UGU	GAA	CCCACCC	AGCUCCUGUGAUUG 3'
9-14	5' <u>GGGAUGGGGA</u>	CUAUA	CGU	GAAUG	ACU	GCA	UCCaCUUCCC	CGCCAUGG 3'
9-27	5' <u>GGGaUGGG</u>	UAAUA	ACU	GUA	UGG	UGAA	CCCACCC	AAACUCCCCAUGGCUA 3'

## Aptamero 9.3t

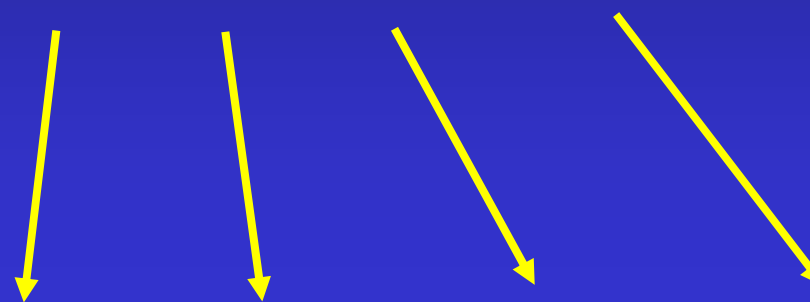
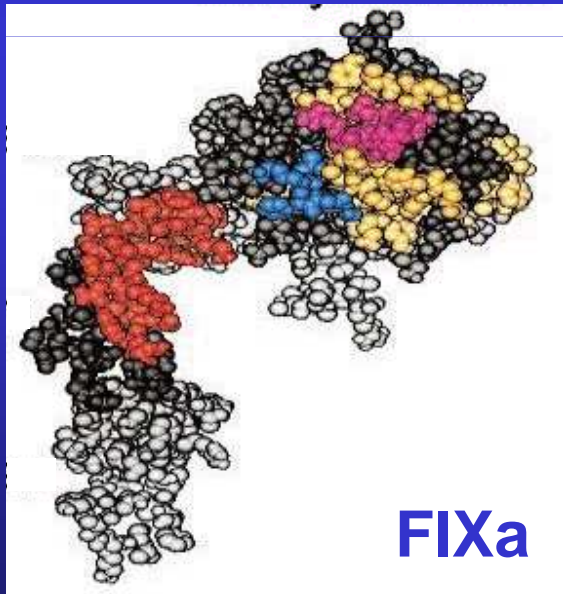
9.3 tM: controllo negativo (aptamero inattivo)



## 2. SPECIFICITA' per il FIXa

**Aptamero  
9.3t**

5000 volte più  
specifico



**FVIIa**

**FXa**

**FXIa**

**APC**

**Serin-proteasi**

# Inibizione IN VITRO dell'attività del FIXa

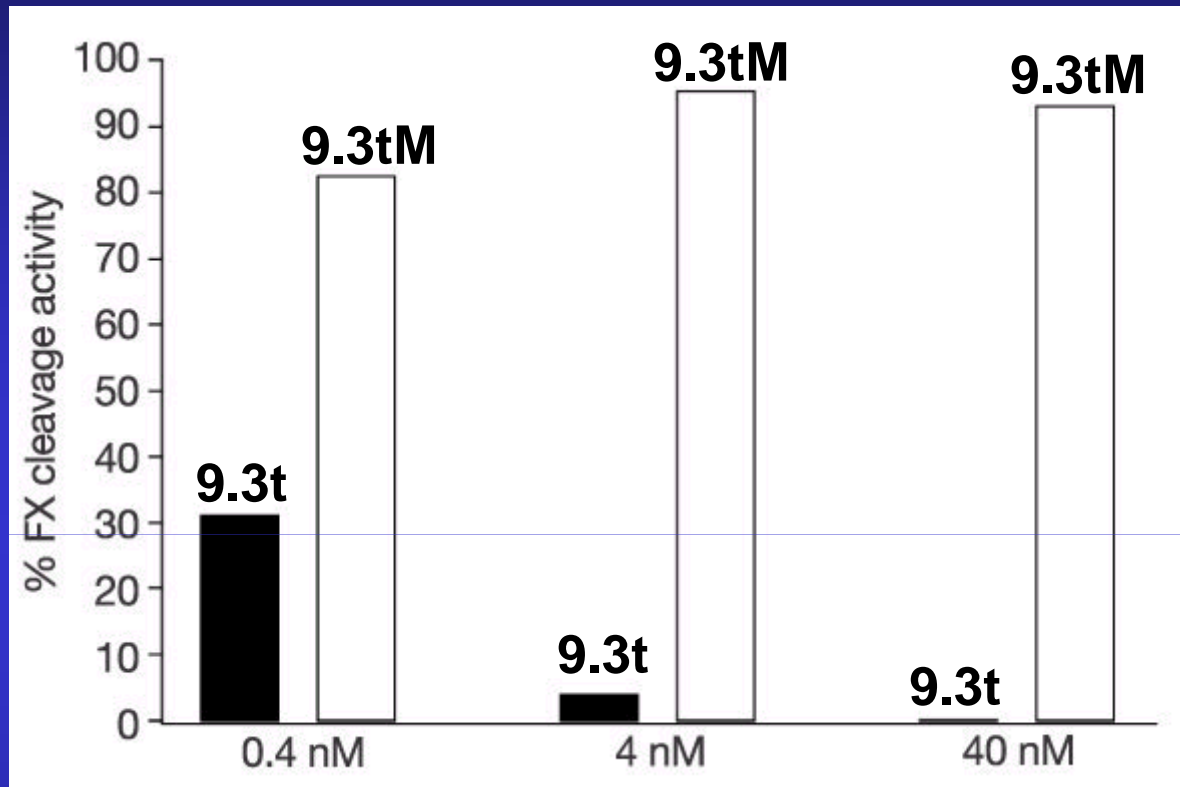
## Assemblamento del complesso



## Introduzione dell'aptamero 9.3t



# Inibizione IN VITRO dell'attività del FIXa



9.3t: aptamero selezionato

9.3tM: controllo negativo

**L'aptamero blocca l'attività del FIXa  
in vitro**

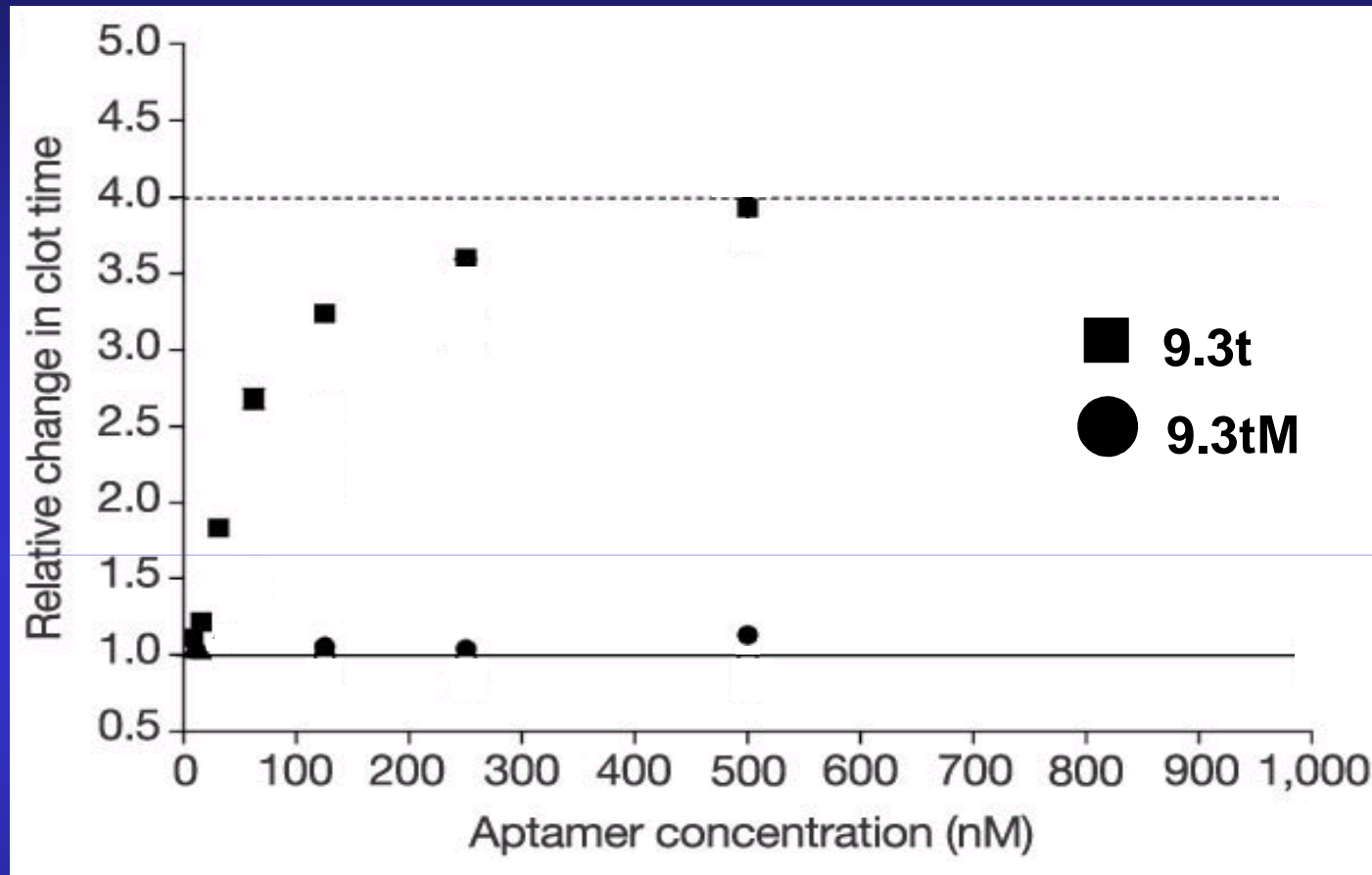


# Inibizione in plasma umano dell'attività del FIXa

## Procedimento:

1. Aggiunta di diverse concentrazioni di aptamero (9.3t) e controllo negativo (9.3tM) a plasma umano
2. Misurazione del tempo di coagulazione del plasma

# Inibizione IN VIVO dell'attività del FIXa

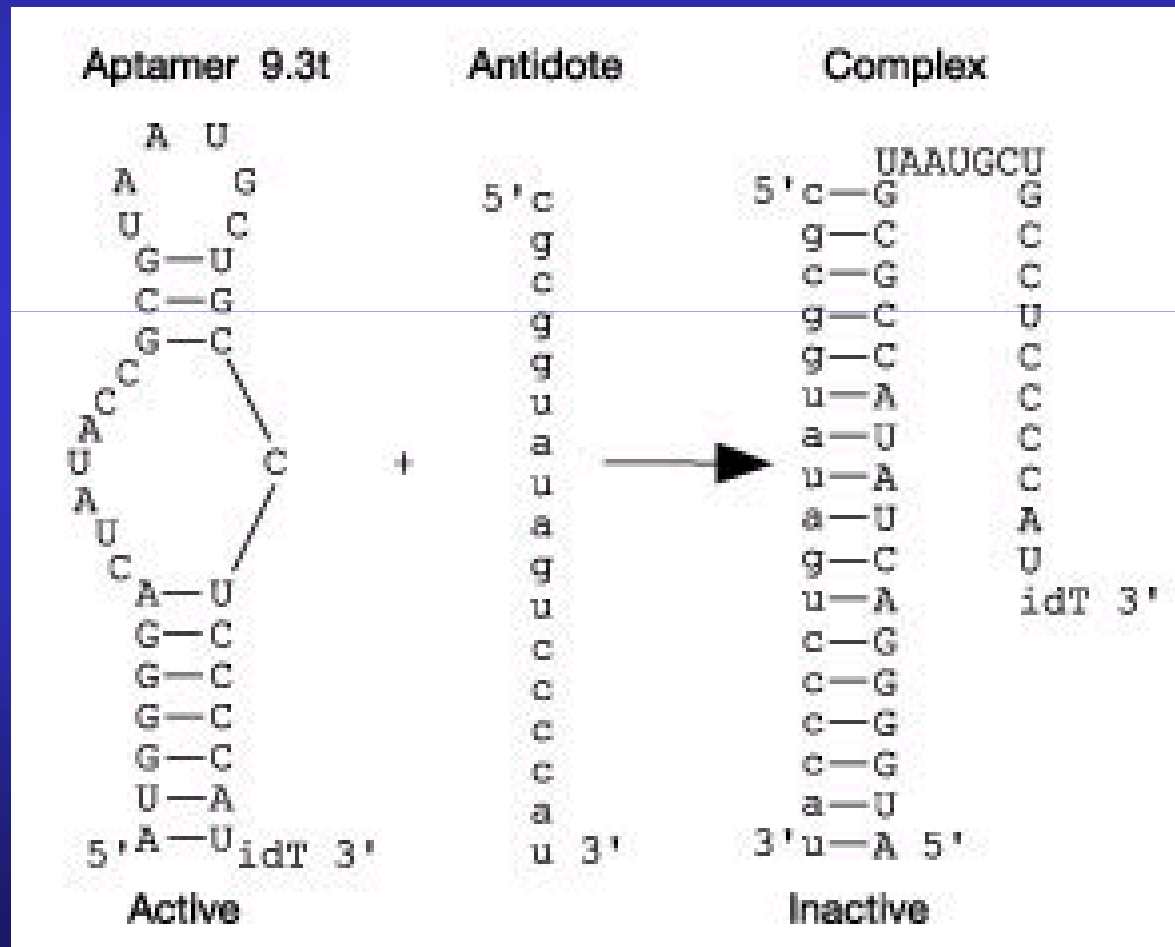


**9.3t prolunga il tempo di coagulazione in modo dose-dipendente**

**L'aptamero inibisce l'attività del FIXa  
in vivo**

# Reversibilità dell'azione dell'aptamero: ANTIDOTO

Antidoto = oligo complementare all'aptamero, in grado di alterare la sua conformazione

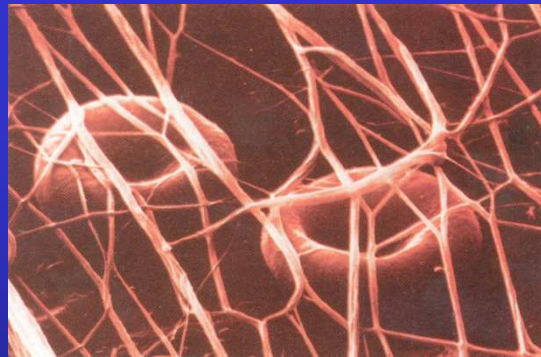


# Reversibilità dell'azione dell'aptamero: ANTIDOTO

**Plasma + aptamero = inibizione della coagulazione**

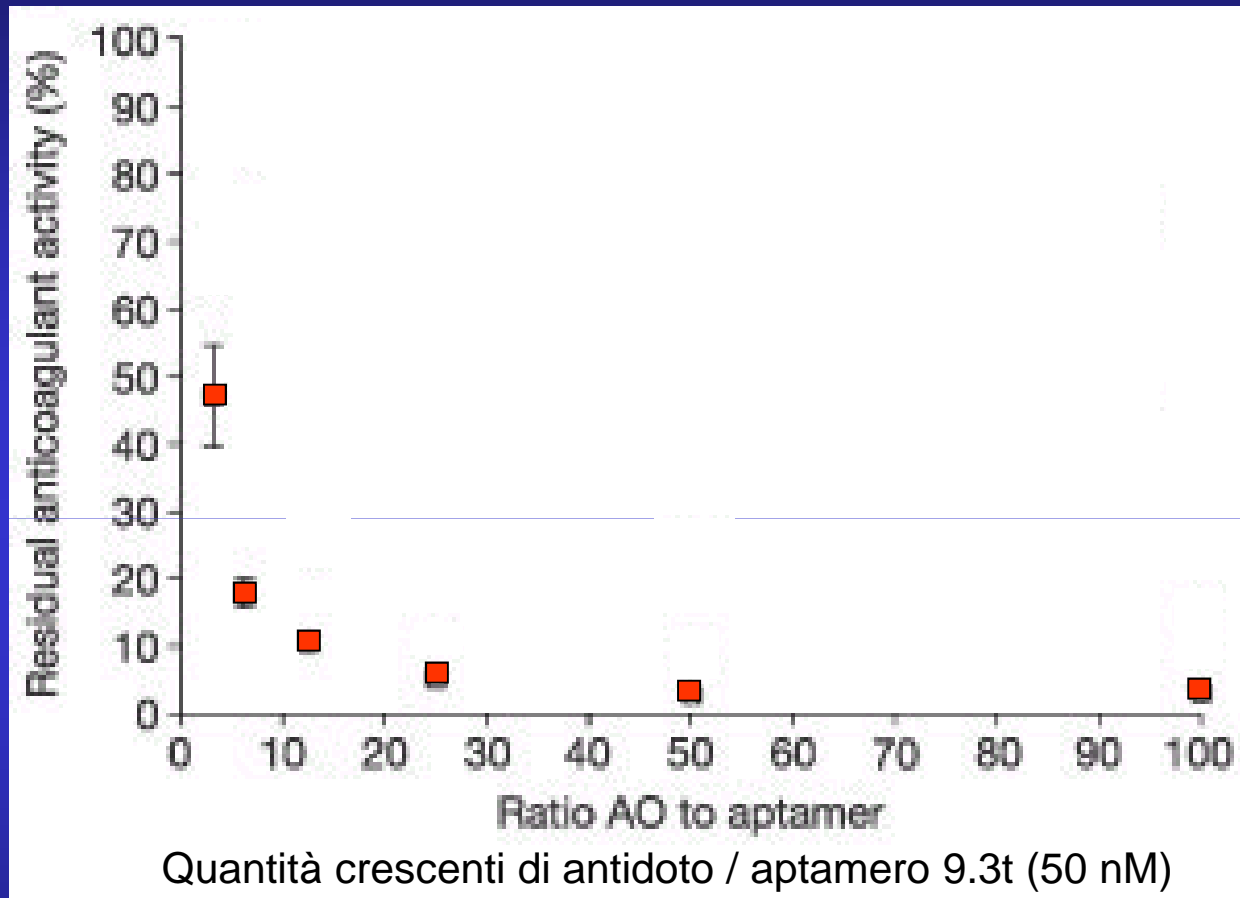


**Plasma non coagulato + antidoto = coagulazione**



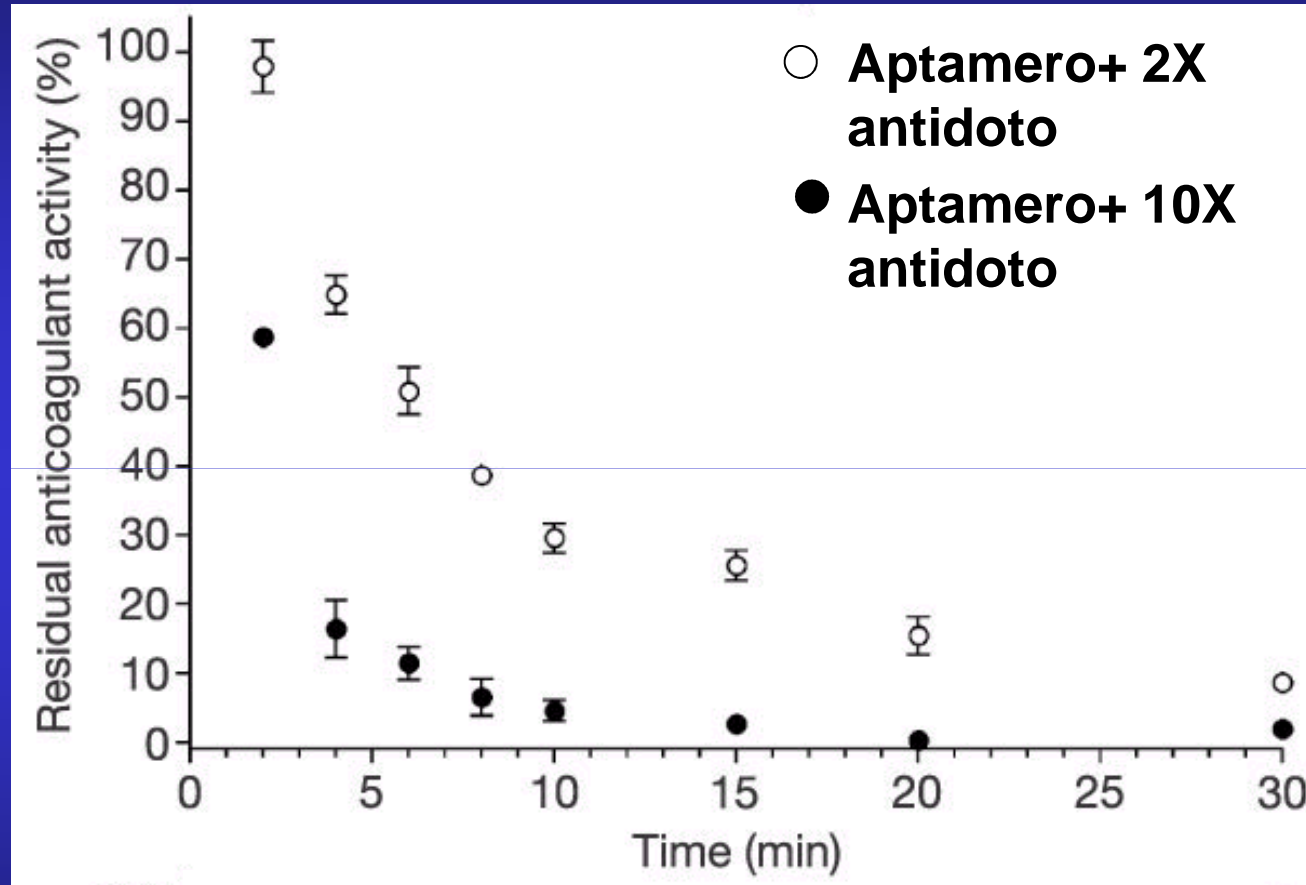
**Si misura la capacità del plasma di coagulare  
entro 10 min.**

# Reversibilità dell'azione dell'aptamero: ANTIDOTO



**L'antidoto neutralizza l'azione  
dell'aptamero**

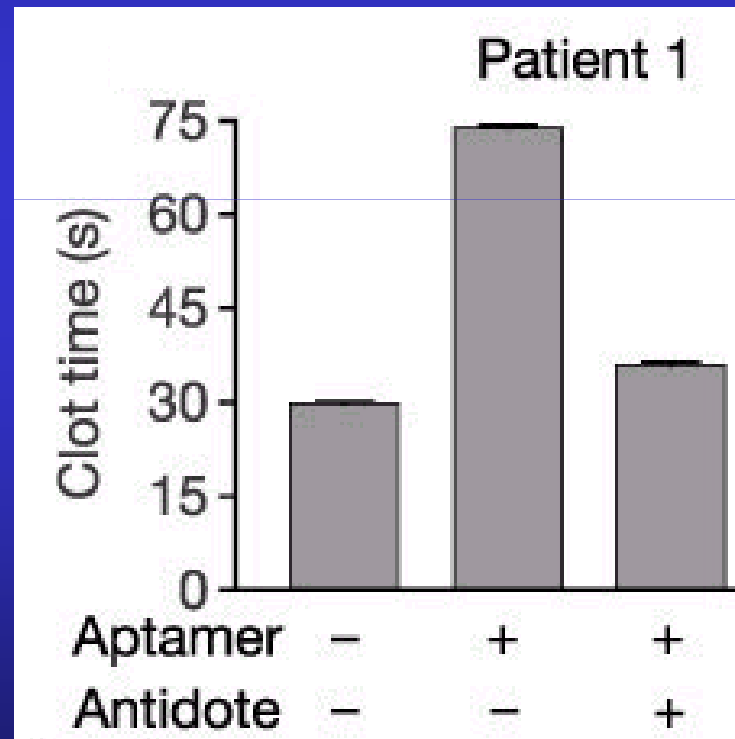
# Reversibilità dell'azione dell'aptamero: ANTIDOTO



**L'azione dell'antidoto è rapida e dose-dipendente**

# Efficiacia di aptamero e antidoto su pazienti

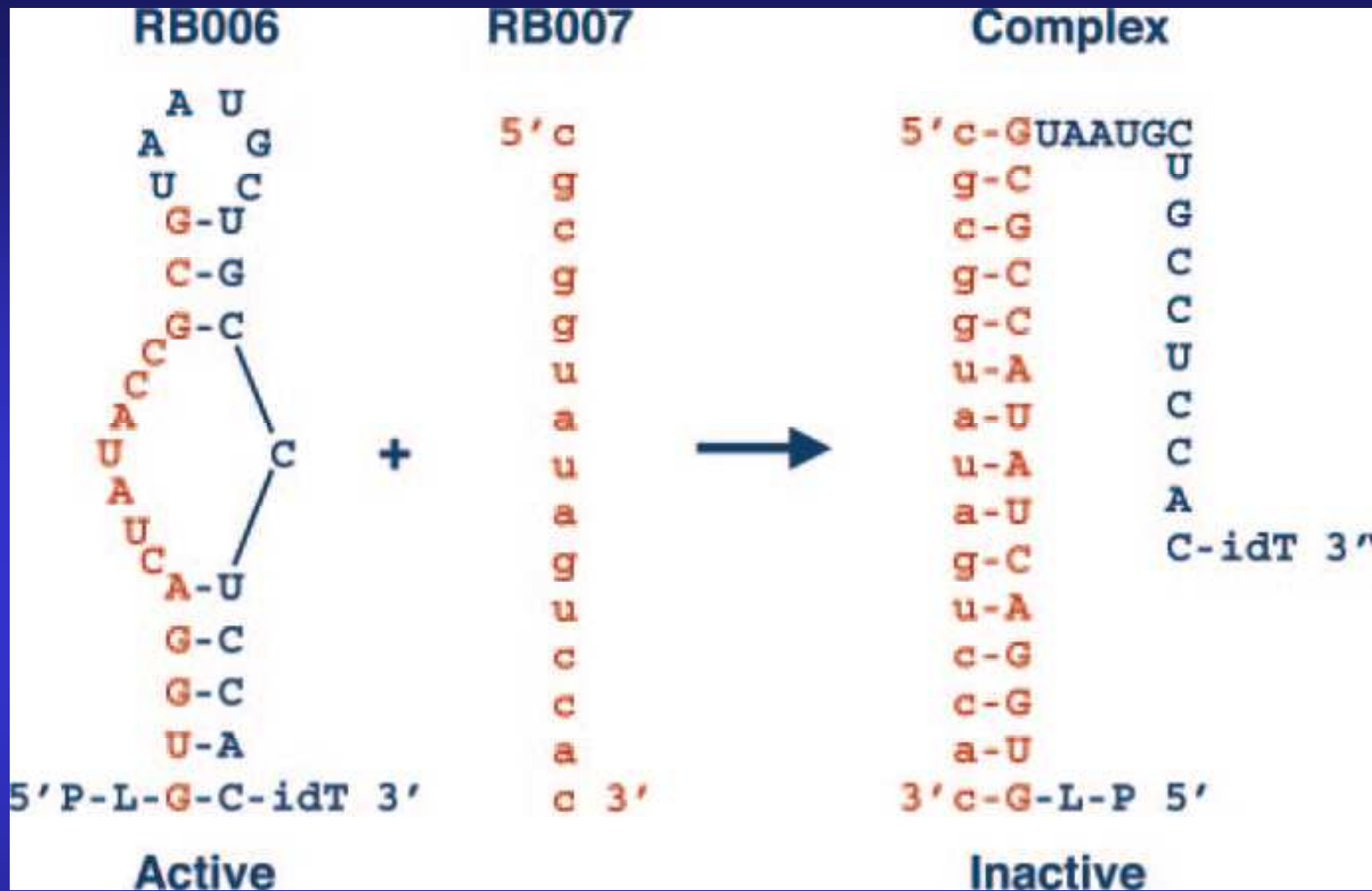
6 Pazienti con trombosi → pazienti non sottoponibili ai normali trattamenti anticoagulanti



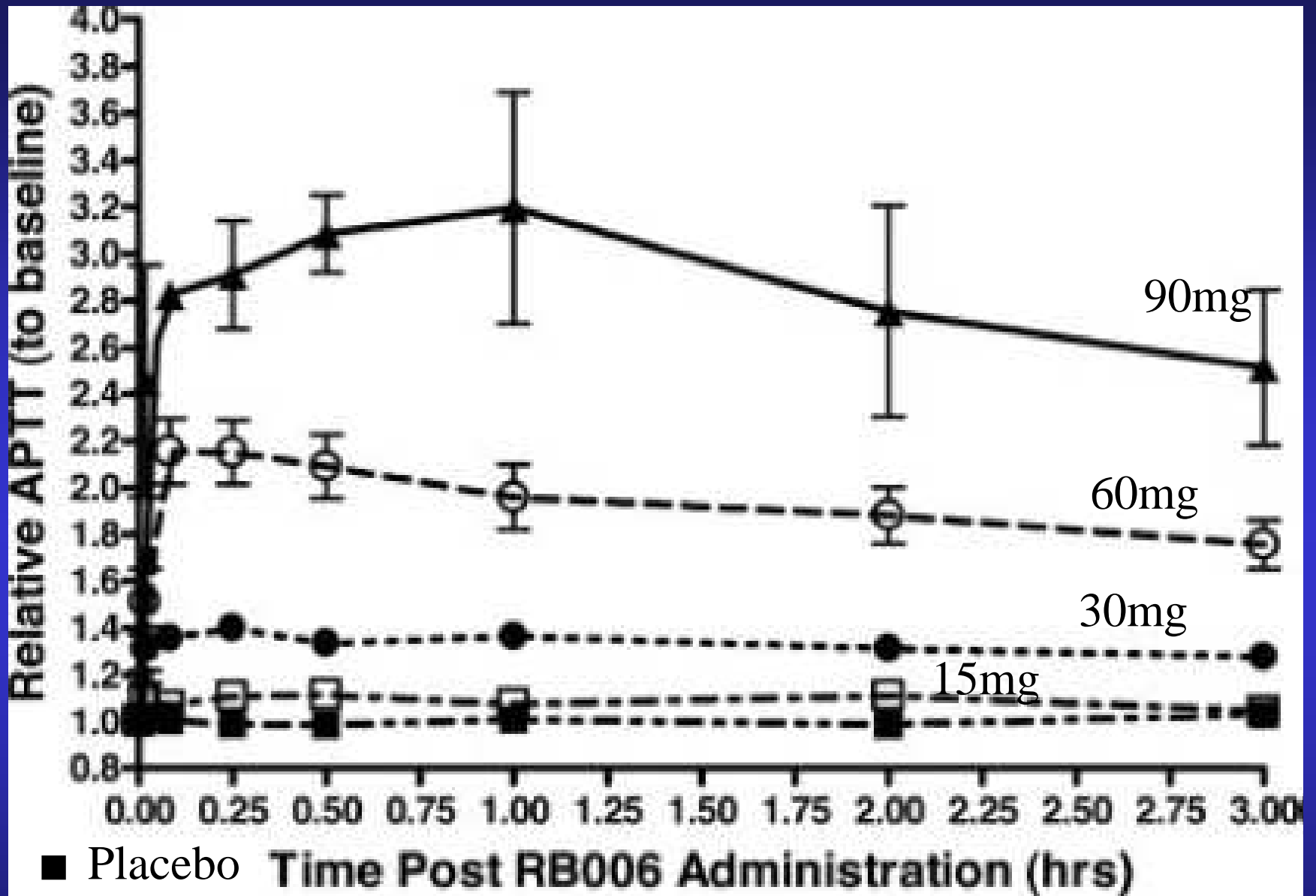
# First-in-human experience of an antidote-controlled anticoagulant using RNA aptamer technology: a phase 1a pharmacodynamic evaluation of a drug-antidote pair for the controlled regulation of factor IXa activity.

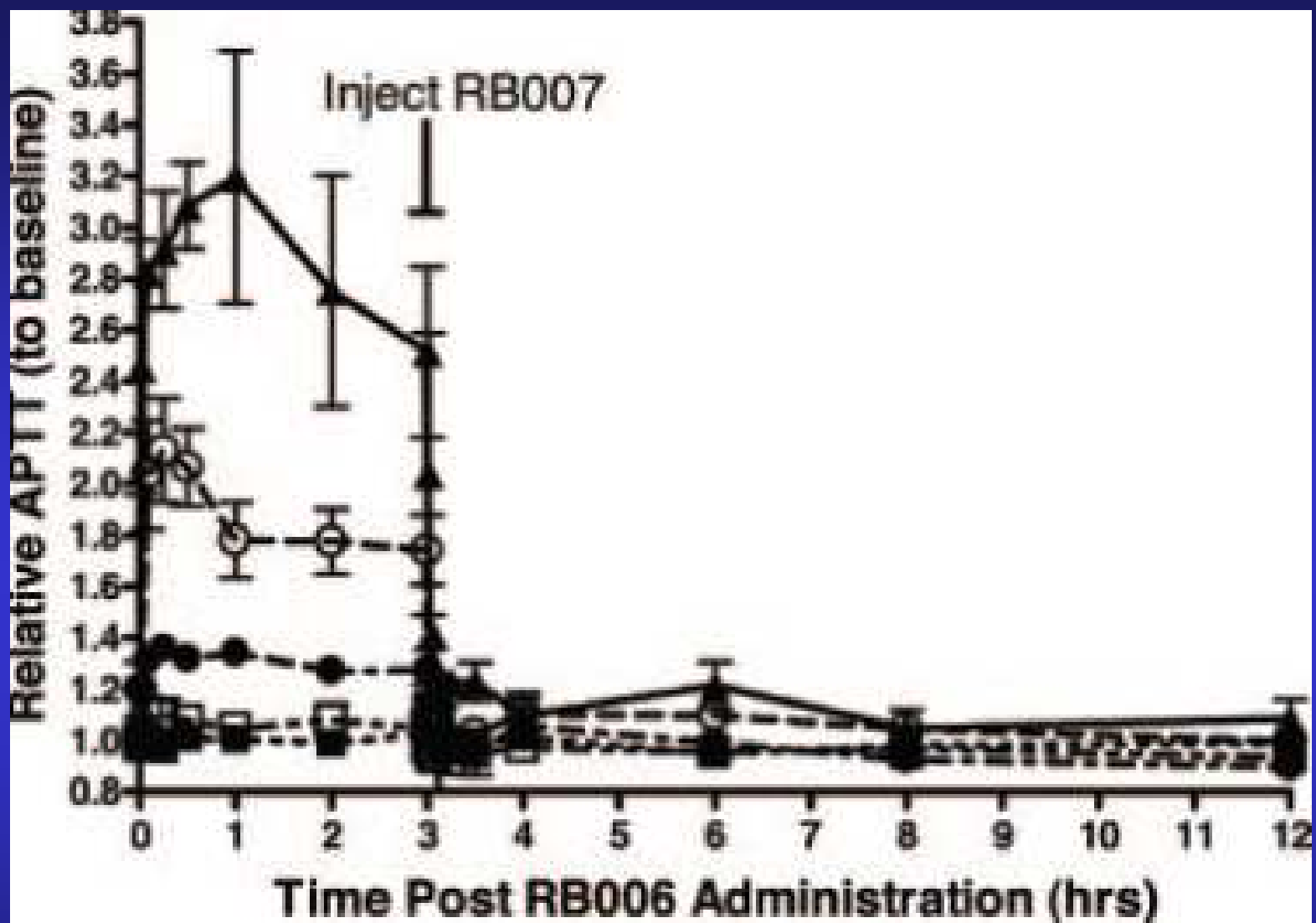
- Selectivity, titratability, rapidity of onset, and active reversibility are desirable pharmacological properties of anticoagulant therapy administered for acute indications and collectively represent an attractive platform to maximize patient safety. A novel anticoagulation system (REG1, Regado Biosciences), developed using a protein-binding oligonucleotide to factor IXa (drug, RB006) and its complementary oligonucleotide antidote (RB007), was evaluated in healthy volunteers. The primary objective was to determine the safety profile and to characterize the pharmacodynamic responses in this first-in-human study. **METHODS AND RESULTS:** Regado 1a was a subject-blinded, dose-escalation, placebo-controlled study that randomized 85 healthy volunteers to receive a bolus of drug or placebo followed 3 hours later by a bolus of antidote or placebo. Pharmacodynamic samples were collected serially. Subject characteristics were the following: median age, 32 years (interquartile range, 23 to 39 years); female gender, 35%; and median weight, 79 kg (interquartile range, 70 to 87 kg). No significant differences were found in median hemoglobin, platelet, creatinine, or liver function studies. There were no significant bleeding signals associated with RB006, and overall, both drug and antidote were well tolerated. One serious adverse event, an episode of transient encephalopathy, occurred in a subject receiving the low intermediate dose of RB006. The subject's symptoms resolved rapidly, and no further sequelae occurred. A predictable dose-pharmacodynamic response, reflected in activated partial thromboplastin time measurements, was seen after administration of the bolus of drug, with a clear correlation between the peak posttreatment activated partial thromboplastin time and post hoc weight-adjusted dose of drug (correlation coefficient, 0.725;  $P < 0.001$ ). In subjects treated with drug, antidote administration reversed the pharmacological activity of the drug, with a rapid (mean time, 1 to 5 minutes across all dose levels) and sustained return of activated partial thromboplastin time to within the normal range. The activated clotting time followed a similar anticoagulant response and reversal pattern. As anticipated, prothrombin time remained unchanged compared with baseline. **CONCLUSIONS:** These observations represent a first-in-human experience of an RNA aptamer and its complementary oligonucleotide antidote used as an anticoagulant system. The findings contribute to an emerging platform of selective, actively reversible anticoagulant drugs for use among patients with thrombotic disorders of the venous and arterial circulations.





P polyethylene glycol; idT, inverted deoxythymidine





Pharmacodynamic effects of RB006 at 0 to 3 hours after RB006 administration.

The relative increase in APTT over baseline for each subject receiving RB006 before RB007 or placebo administration (all subjects assigned to arms 2 and 3) is shown vs subjects receiving placebo. Data represent the mean SEM for all subjects receiving treatment at each dose level.

# CONCLUSIONI

- 1. Aptameri contro il FIXa sono potenti anticoagulanti**
- 2. Oligonucleotidi complementari agli aptameri possono agire da antidoti e neutralizzare l'azione anticoagulante**

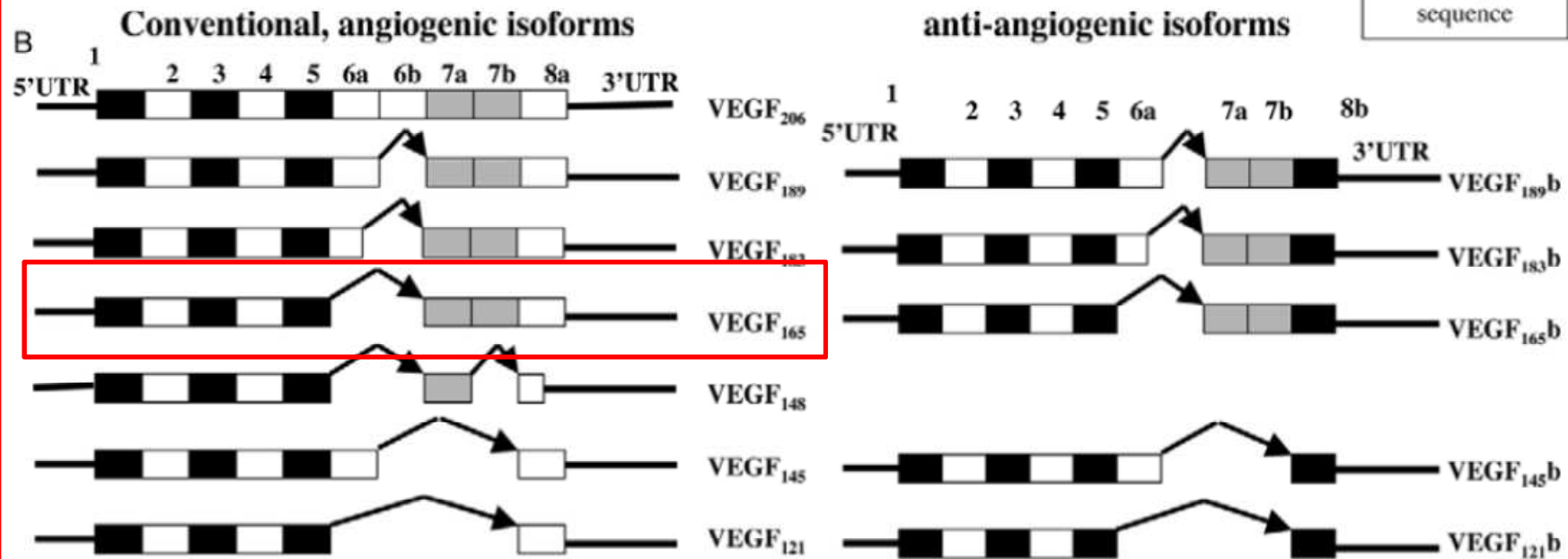
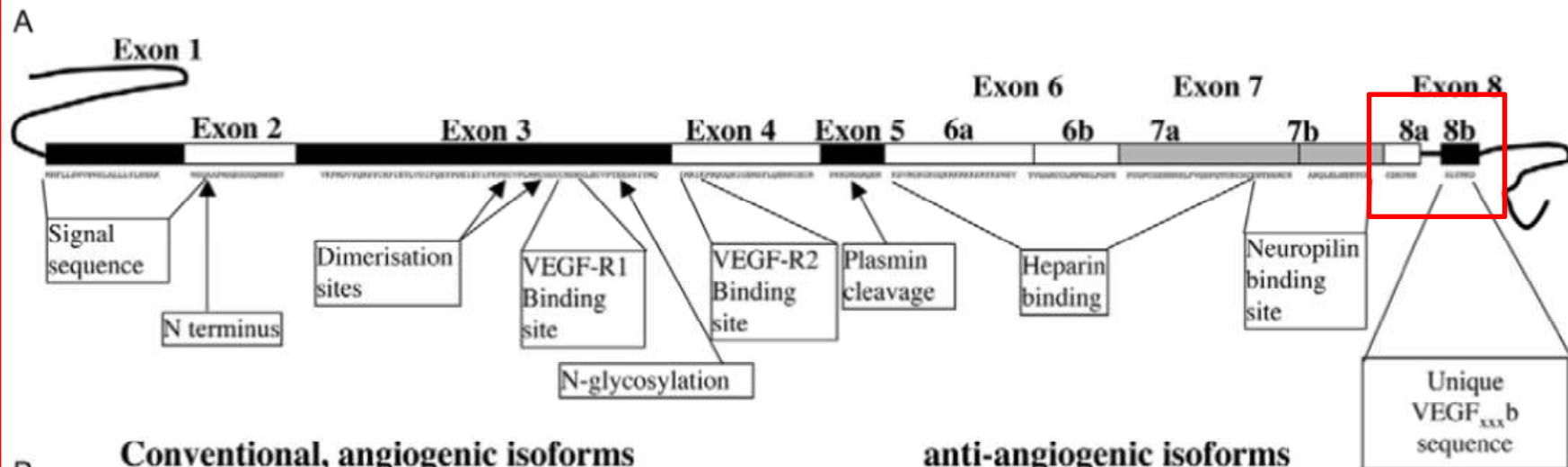
La maculopatia senile umida, che costituisce è causata dalla crescita di vasi sanguigni anomali, che danneggiano l'area dell'occhio responsabile della visione centrale, che è essenziale per la maggior parte delle attività visive



# VEGF and Macula Degeneration

- Both clinical and preclinical findings have implicated vascular endothelial growth factor (VEGF) in the pathophysiology macular edema and degeneration.
- \*VEGF is both a potent enhancer of vascular permeability and a key inducer of angiogenesis.
- \*VEGF levels are elevated in the eyes of patients.
- Injection of VEGF (the VEGF165 isoform in particular) into healthy eyes of animals can induce associated ocular pathologies

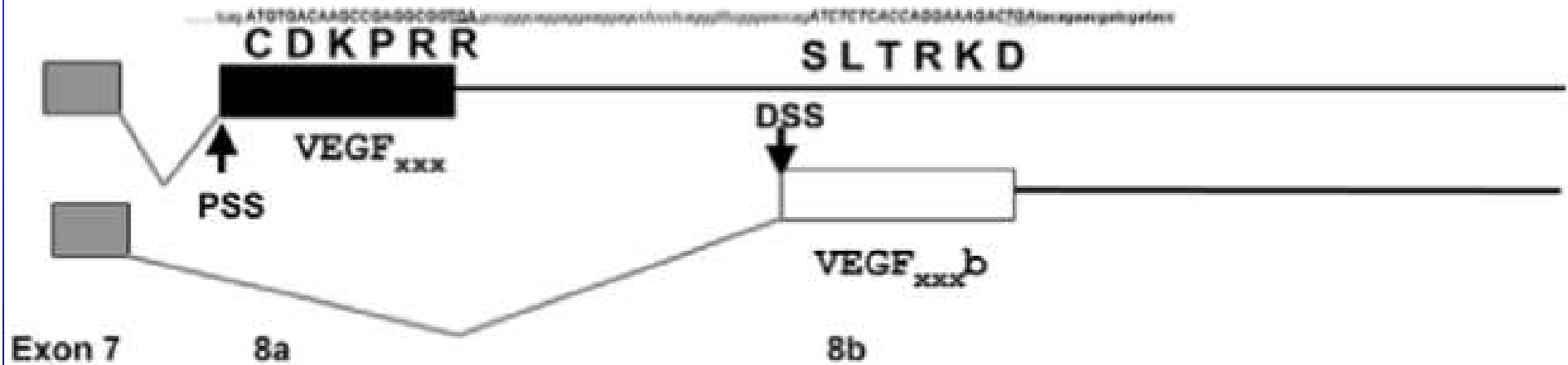


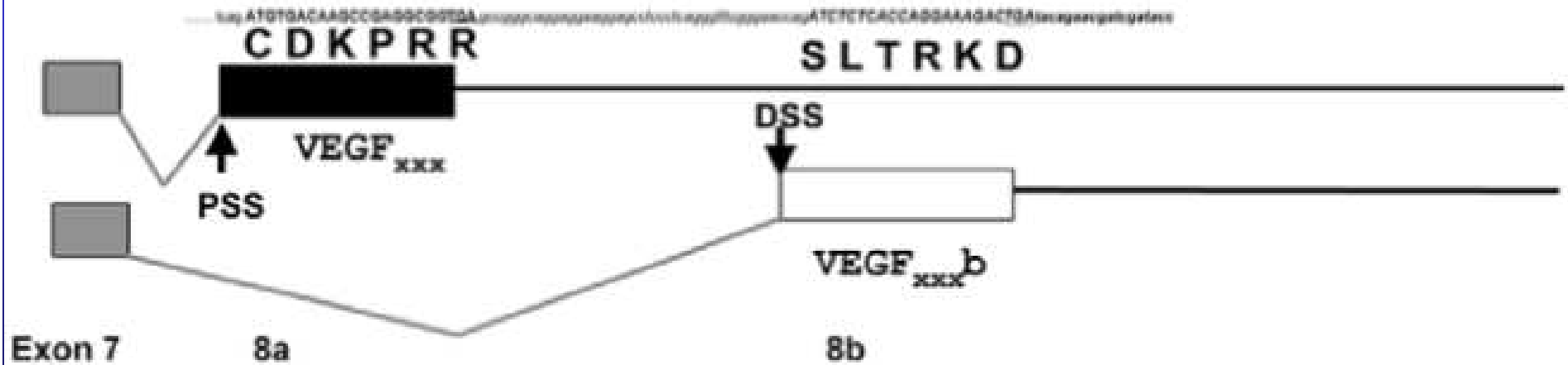
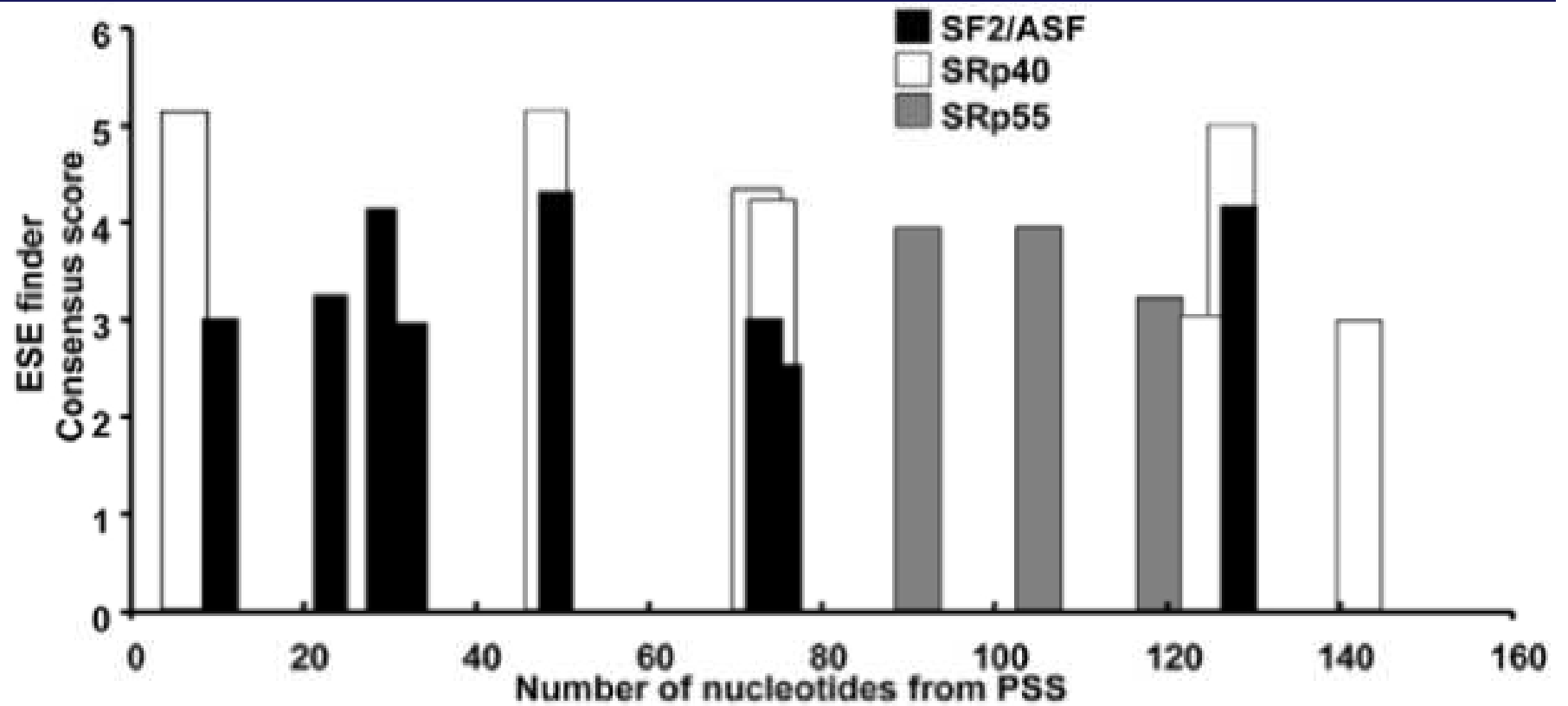


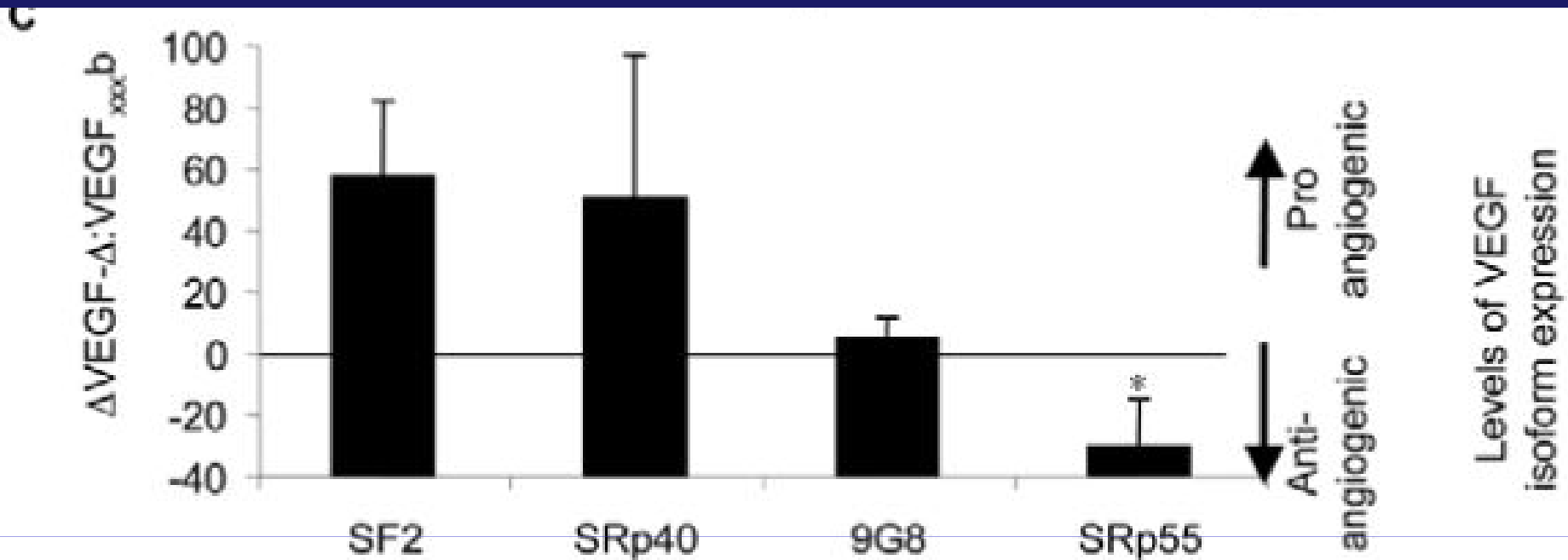
# Proximal splice-site selection (PSS)

# Distal splice-site selection DSS

10







Effect of overexpression of splicing factors on VEGF isoform production.

# Vascular Endothelial Growth Factor and the Potential Therapeutic Use of Pegaptanib (Macugen®) in Diabetic Retinopathy

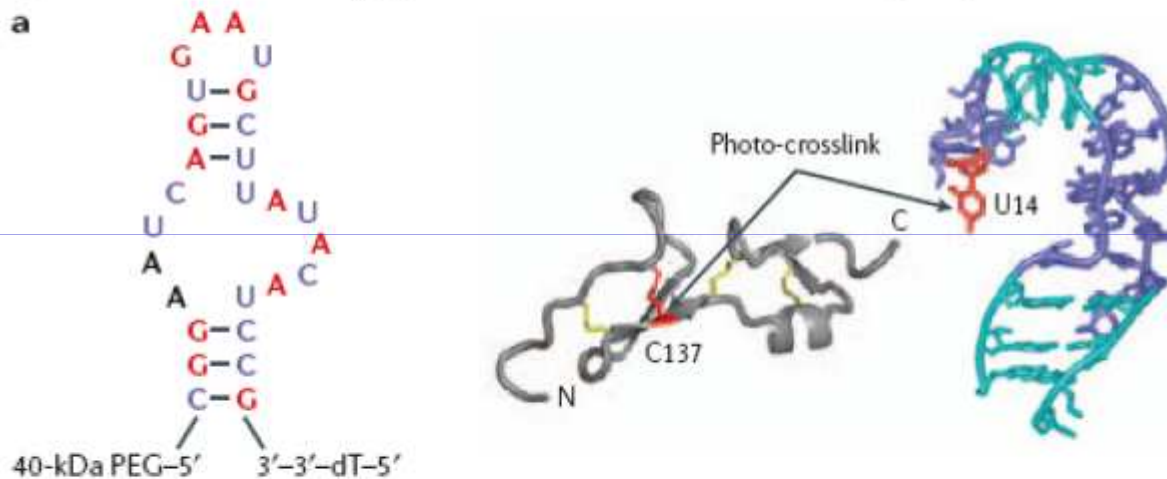
Starita C, Patel M, Katz B, Adamis A

- .
- Pegaptanib, a novel RNA aptamer currently used in the treatment of age-related macular degeneration, binds and inactivates VEGF165 and has been shown in animal models to reverse the blood-retinal barrier breakdown.

## Il pegaptanib e' un antagonista selettivo del VEGF<sub>165</sub>

Il Pegaptanib e' un aptamero a filamento singolo di **RNA** formato da 28 nucleotidi legato a 2 molecole di 20-kDa di glicole polietilenico (PEG)

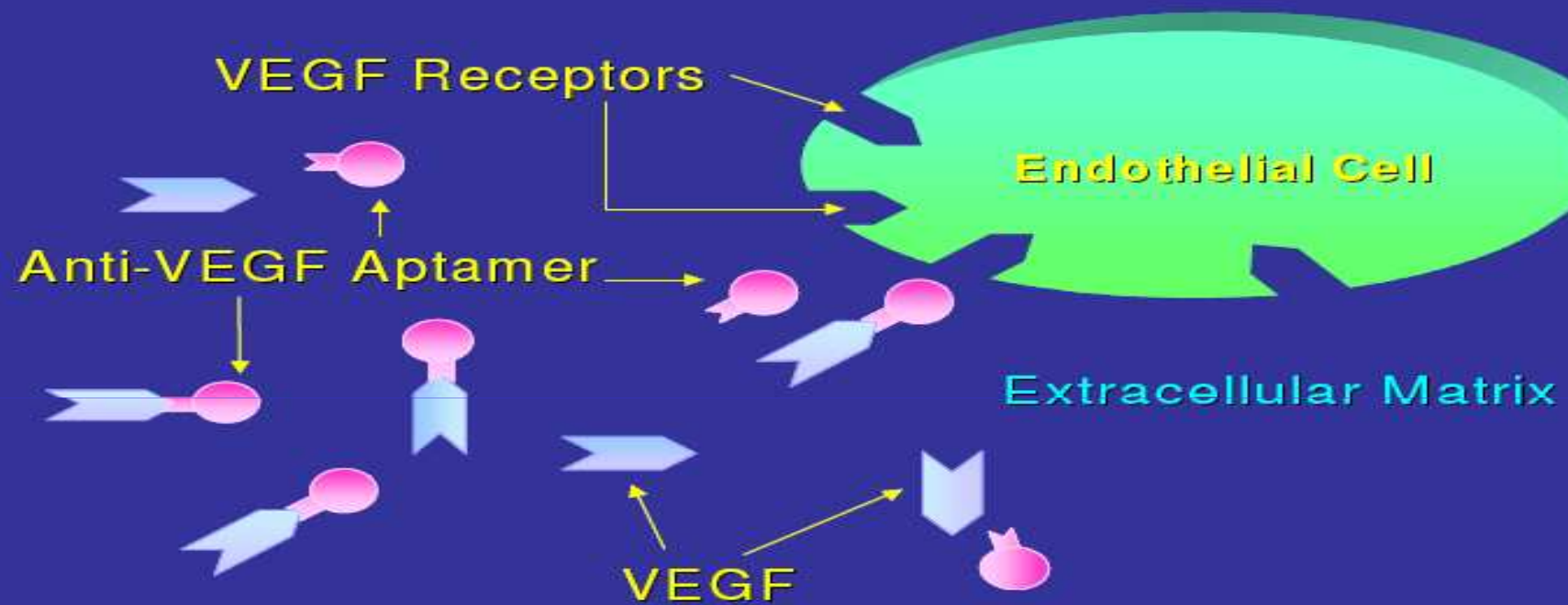
E' dotato di alta affinita' per il VEGF<sub>165</sub> (vascularizzazione patologica) e nessun legame con il VEGF<sub>121</sub> (vascularizzazione fisiologica)



a | Sequenza e struttura secondaria del pegaptanib.

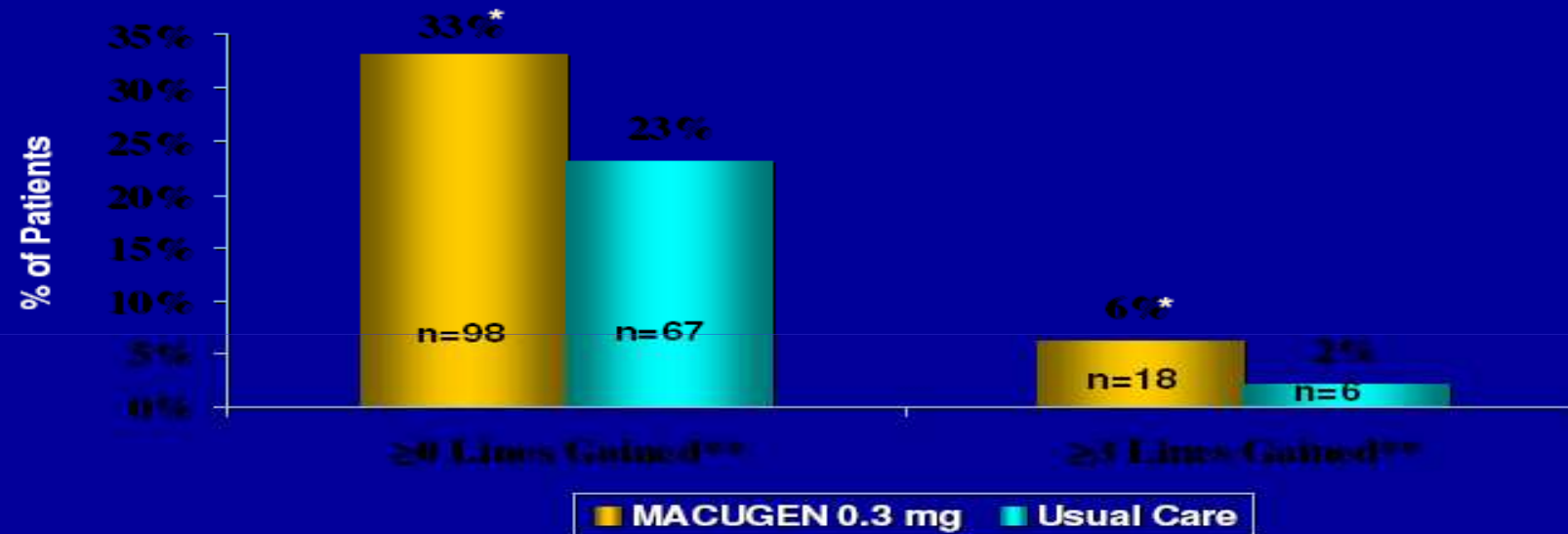
Il legame avviene tra la cisteina - 137 del VEGF<sub>165</sub> e l'uridina-14 dell'aptamero<sub>14</sub> (in rosso).

## Extracellular Neutralization of VEGF



- Il Pegaptanib si lega specificamente al VEGF-165, impedendone l'aggancio con il suo recettore

## Mantenimento o miglioramento del visus



\*Nominal  $P < 0.05$  vs Usual Care

\*\*Prespecified endpoint

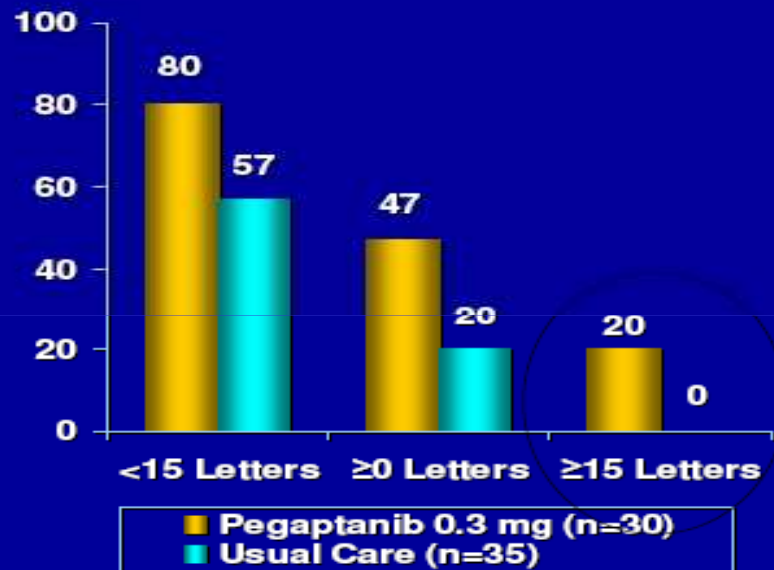
Gragoudas ES et al. *N Eng J Med*. 2004;351:2805-2816.



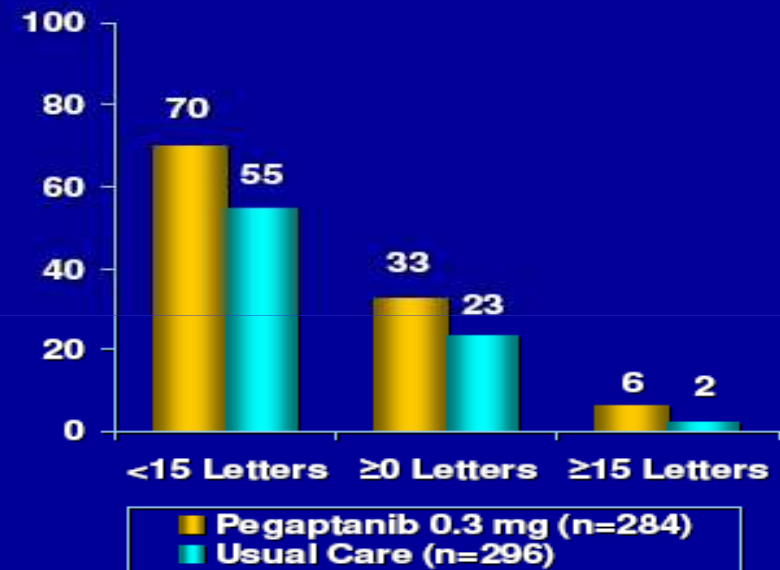
## Responders nello studio V.I.S.I.O.N. Lesioni iniziali vs tutti I Pazienti

\* Lesione iniziale definita come: occulta, senza essudati, e occhio controlaterale con visus migliore

*Lesione iniziale \**



*Tutti i Pazienti*



V.I.S.I.O.N. Clinical Trial Group. *Retina* 2005;25:815-827.