

**Basi strutturali delle emoglobinopatie: instabilità
conformazionale e iper-coesione
all'interfaccia svelate tramite simulazioni AA-MD.**

*Dalla dinamica molecolare alla spiegazione della diversa
severità delle sindromi falcemiche negli eterozigoti doppi.*

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Defining global strategies to improve outcomes in sickle cell disease: a Lancet Haematology Commission



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Executive summary

All over the world, people with sickle cell disease (an inherited condition) have premature deaths and preventable severe chronic complications, which considerably affect their quality of life, career progression, and financial status. In addition, these people are often affected by stigmatisation or structural racism, which can contribute to stress and poor mental health. Inequalities affecting people with sickle cell disease are also reflected in the distribution of the disease—mainly in sub-Saharan Africa, India, and the Caribbean—whereas interventions, clinical trials, and funding are mostly available in North America, Europe, and the Middle East. Although some of these characteristics also affect people with other genetic diseases, the fate of people with sickle cell disease seems to be particularly unfair. Simple, effective interventions to reduce the mortality and morbidity associated with sickle cell disease are available. The main obstacle preventing better outcomes in this condition, which is a neglected disease, is associated with inequalities impacting the patient populations. The aim of this Commission is to highlight the problems associated with sickle cell disease and to identify achievable goals to improve outcomes both in the short and long term.

The ambition for the management of people with sickle cell disease is that curative treatments become available to every person with the condition. Although this would have seemed unrealistic a decade ago, developments in gene therapy make this potentially achievable, albeit in the distant future. Until these curative technologies are fully developed and become widely available, health-care professionals (with the support of policy makers, funders, etc) should make sure that a minimum standard of care (including screening, prophylaxis against infection, acute medical care, safe blood transfusion, and hydroxyurea) is available to all patients.

In considering what needs to be achieved to reduce the global burden of sickle cell disease and improve the quality of life of patients, this Commission focuses on five key areas: the epidemiology of sickle cell disease (Section 1); screening and prevention (Section 2); established and emerging treatments for the management of the disease (Section 3); cellular therapies with curative potential (Section 4); and training and education needs (Section 5). As clinicians, researchers,

and patients, our objective to reduce the global burden of sickle cell disease aligns with wider public health aims to reduce inequalities, improve health for all, and develop personalised treatment options. We have observed in the past few years some long-awaited momentum following the development of innovative point-of-care testing devices, new approved drugs, and emerging curative options. Reducing the burden of sickle cell disease will require substantial financial and political commitment, but it will impact the lives of millions of patients and families worldwide and the lessons learned in achieving this goal would unarguably benefit society as a whole.

Introduction

Sickle cell disease is probably the most common, serious inherited disease in the world, and one of the top 50 most common causes of non-communicable death globally, with most of these deaths occurring in African countries.¹ Furthermore, it has recently been shown to be the most common contributor to mortality globally in the 5–14 year age group.¹ Whereas the majority of common causes of death are decreasing, the number of deaths due to sickle cell disease are increasing globally.¹ Despite this, there are fewer than five effective disease-modifying agents available and most patients in the world do not have access to any of these.² Although curative treatment is possible, by haematopoietic stem-cell transplantation (HSCT) and emerging gene therapies, only a tiny minority of patients are currently treated with these because of the need for infrastructure, the cost, and associated adverse effects.¹ In the context of increasing global health inequalities, partly driven by racism, previous calls for action on sickle cell disease have been largely ineffective. There is an urgent need for the development of research, policies, and public health strategies to reduce the morbidity and mortality associated with this long-neglected condition.⁴

The first paper on sickle cell disease was published in 1910 by James Herrick in Chicago (IL, USA), who described the appearance of atypical, sickle-shaped red cells in a Black dental student from Grenada, who presented with respiratory symptoms.⁵ Following this publication, more clinical and pathophysiological observations emerged, including the identification of haemolysis as a cause of anaemia and jaundice and vaso-occlusion as a cause of pain and ischaemic tissue damage.⁶ In 1948, Janet Watson published the key

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Sickle cell disease

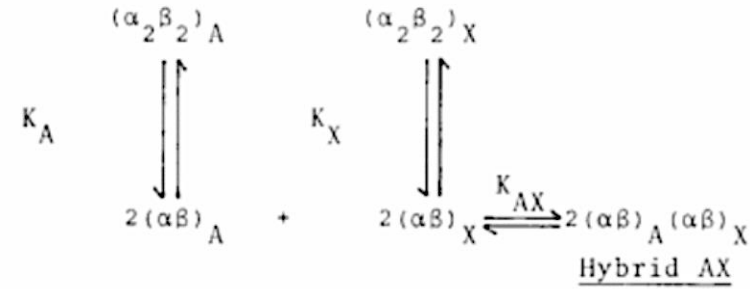
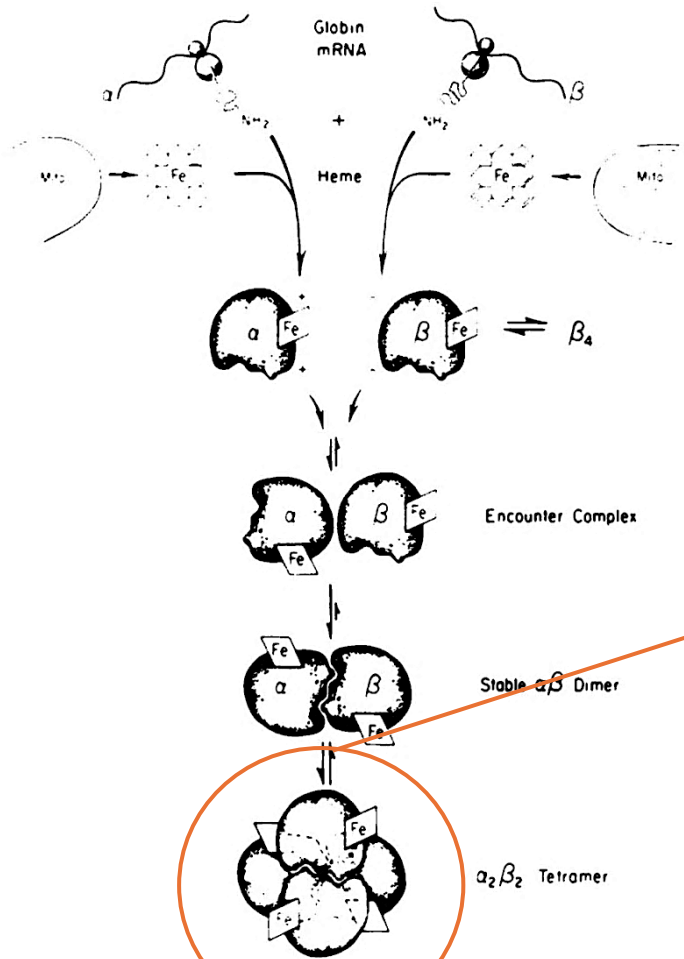
Most severe



Least severe

Sickle cell anaemia	Severe sickle cell disease	Moderately severe sickle cell disease	Mild sickle cell disease	Very mild sickle cell disease
HbSS (70 – 90%)	Severe HbS/β ⁺ thalassaemia (1-10%)	Moderate HbS/β ⁺ thalassaemia (1 – 10%)	Mild HbS/β ⁺⁺ thalassaemia (1 – 5%)	HbS/HPFH (1%)
HbS/β ⁰ thalassaemia (1-10%)	HbS/O-Arab (1-2%)	HbSC (1 – 30%)	HbSE (<1%)	HbS + other variants (<1%)
	HbS/D-Punjab (1 -2%)	HbA/S-Oman (1 – 2%)	HbA/Jamaica Plain (<0.1%)	
	HbS/C-Harlem (<0.1%)			
	HbC/S-Antilles (<0.1%)			
	HbS/Quebec-CHORI (<0.1%)			

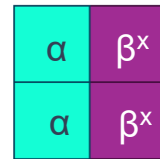
Le subunità negli eterozigoti si assemblano in tetrameri simmetrici e asimmetrici



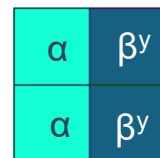
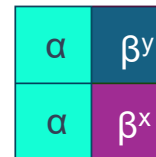
All'interno del globulo rosso i tetrameri sono in continuo equilibrio dinamico con i dimeri ($\alpha\beta$), consentendo un rapido scambio di subunità globiniche in condizioni fisiologiche

In base alle catene presenti e alla loro combinazione...

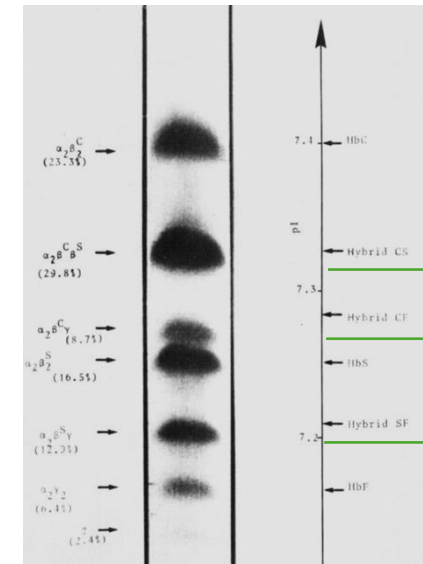
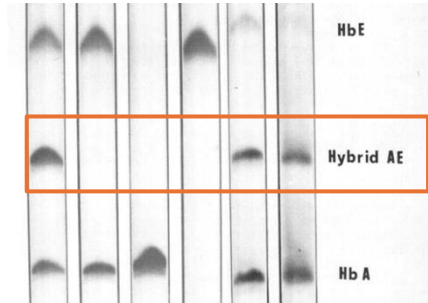
TETRAMERI SIMMETRICI



TETRAMERO ASIMMETRICO



Low Temperature Isoelectrofocusing

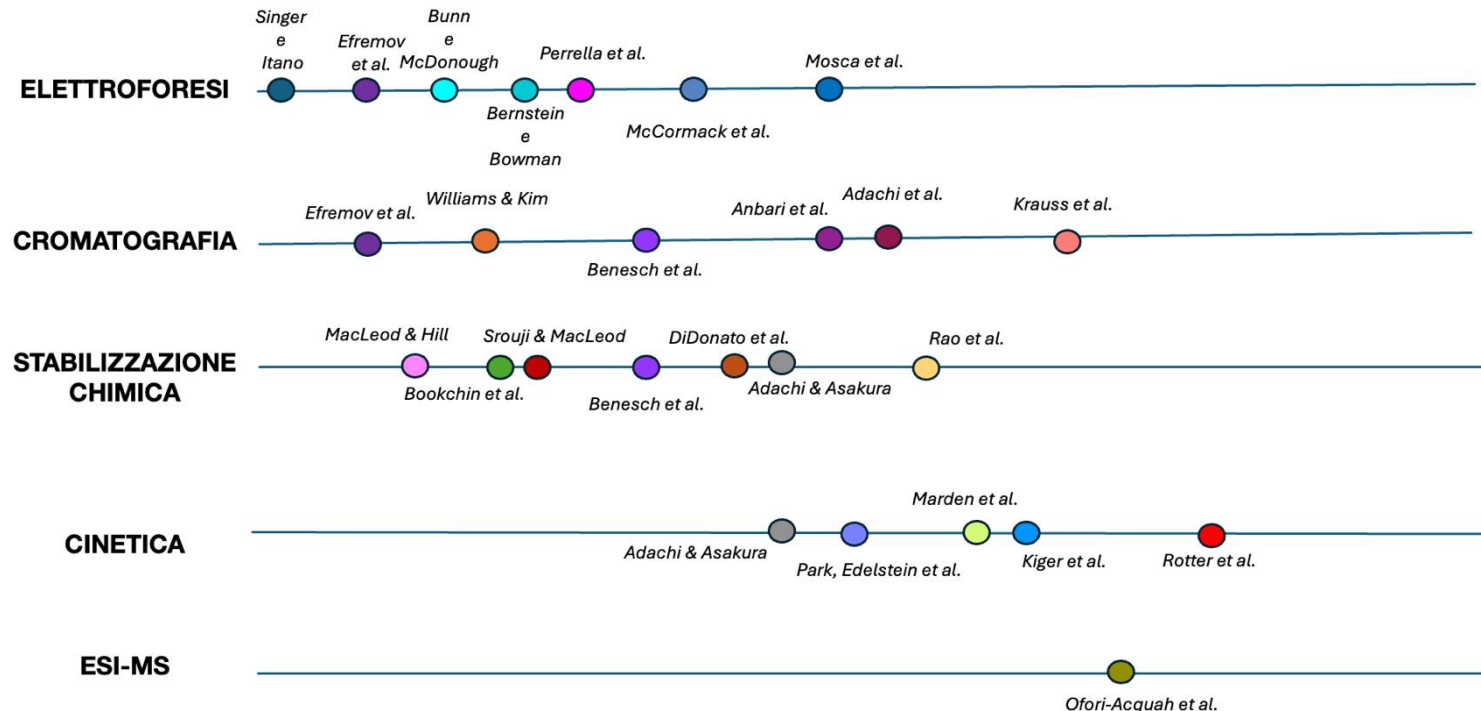


Ibrido CS

Ibrido CF

Ibrido SF

Possono oggi i modelli computazionali aiutare a migliorare la comprensione dei tetrameri asimmetrici?



▲ LIMITI SPERIMENTALI STORICI (1959–2000)

La labilità intrinseca dell'asimmetria

Scambio subunitario ultrarapido

L'equilibrio dinamico tetramero-dimero fa collassare le specie eterologhe prima della separazione.

Alterazioni da Cross-linking

I fissativi chimici bloccano la dissociazione ma distorcono le proprietà allosteriche native.

Invisibilità degli stati di transizione

Le cinetiche rapide e la spettrometria catturano popolazioni medie, non conformazioni asimmetriche isolate.

1959 – 1972
Origini & Ibridi

1973 – 1979
Isolamento & Cross-linking

1980 – 1989
Studi di Legame & Cinetica

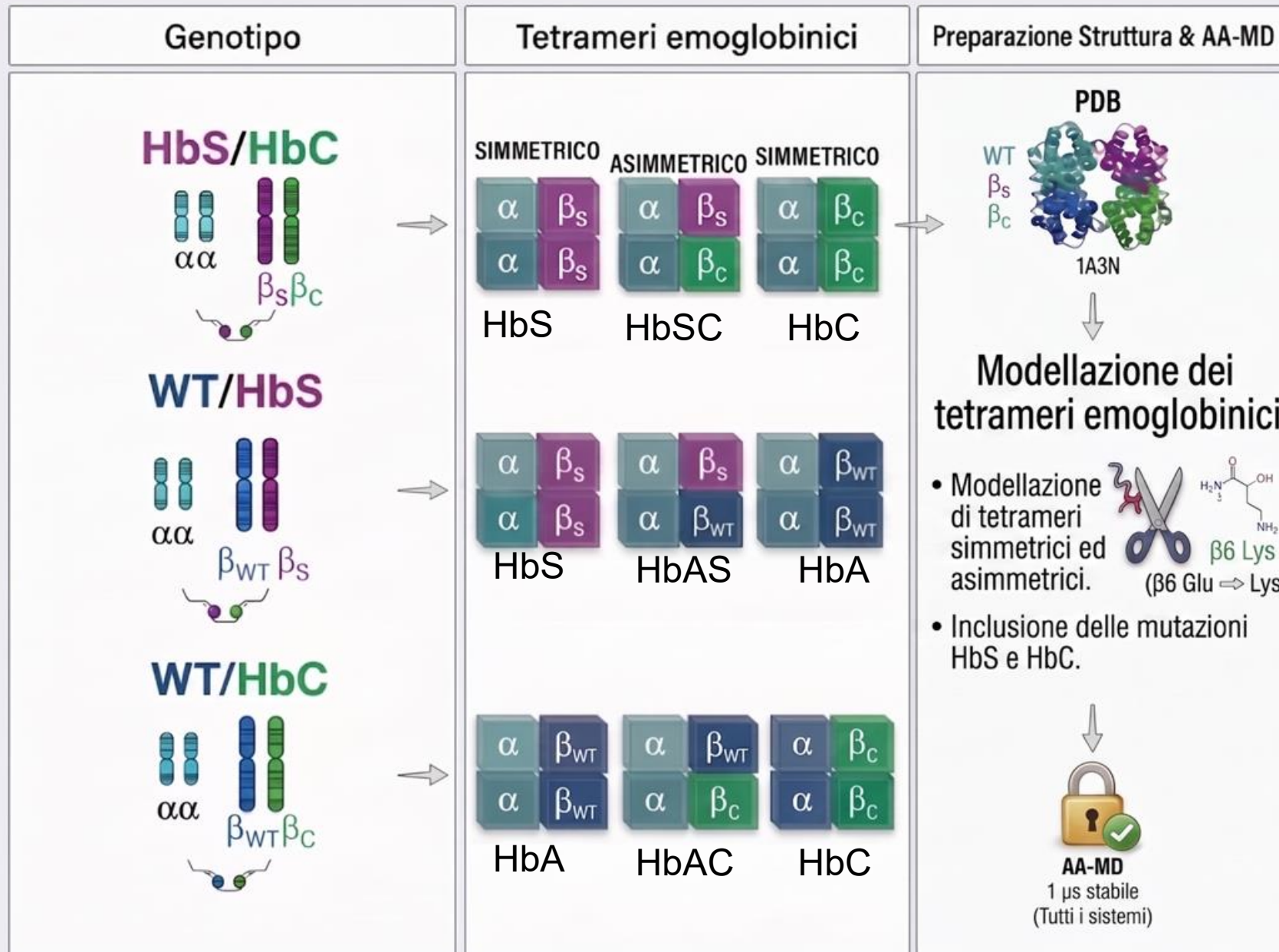
1990 – 2011
Spettrometria Nativa

2011 – 2026
Risoluzione Computazionale ????

✓ VANTAGGI COMPUTAZIONALI OGGI

- Tracciamento in microsecondi delle asimmetrie senza necessità di congelamento o modifiche chimiche.
- Quantificazione termodinamica della cooperatività allosterica tra subunità mutanti e wild type.
- Modellazione ad alta fedeltà di complessi eterooligomerici anche in assenza di strutture cristallografiche.

Razionale dello studio



Analisi computazionale condotta in **condizioni di deossigenazione (T state)**

Modellazione in silico di simmetrici e asimmetrici variando la **posizione 6 delle catene β**

- β_{WT_6Glu}
- β_{S_6Val}
- β_{C_6Lys}



QUESITO FONDAMENTALE:
le sostituzioni asimmetriche producono un fenotipo intermedio lineare, o scatenano distorsioni mecano-allosteriche non-additive all'interfaccia?

Il Vincolo della Simmetria C2

CONFRONTO DEI MODELLI DI COOPERATIVITÀ

MODELLO MWC (Monod-Wyman-Changeux) - Transizione Concertata Simmetrica



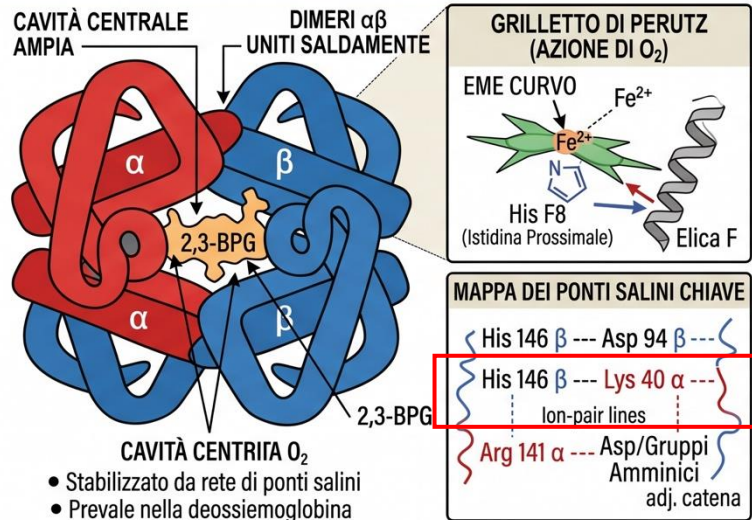
TUTTE le subunità cambiano contemporaneamente da T a R (Transizione CONCERTATA)

MODELLO KNF (Koshland-Némethy-Filmer) - Modello Sequenziale per Induzione



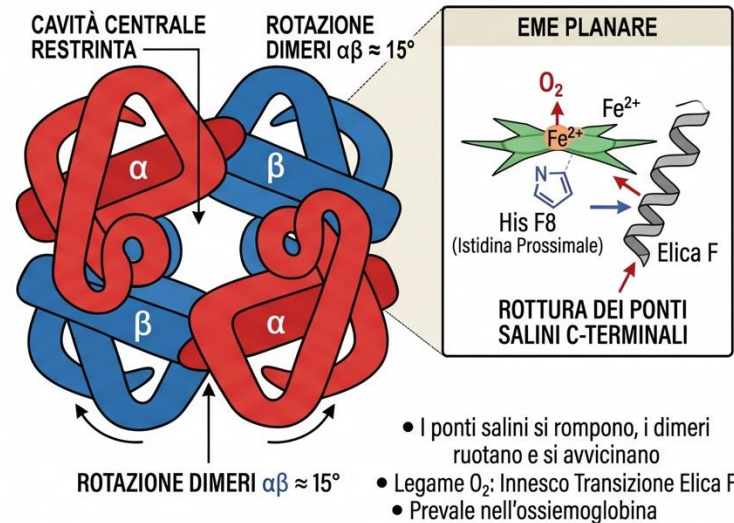
UNA subunità **alla volta** cambia da T a R **INDOTTA** dal legame (Modello SEQUENZIALE)

STATO T (TESO): BASSA AFFINITÀ O₂

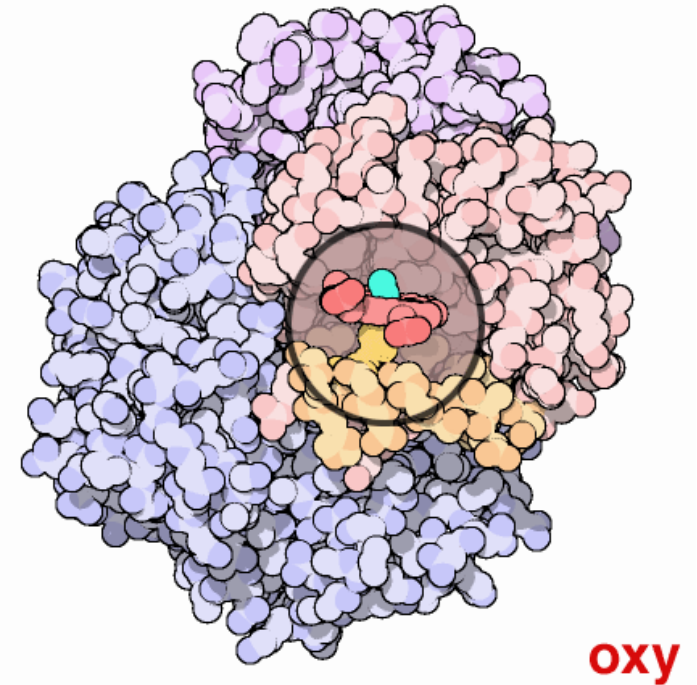


Stabilizzato dal **lucchetto di Perutz** (fitta rete di ponti salini)

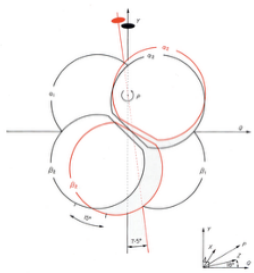
STATO R (RILASSATO): ALTA AFFINITÀ O₂



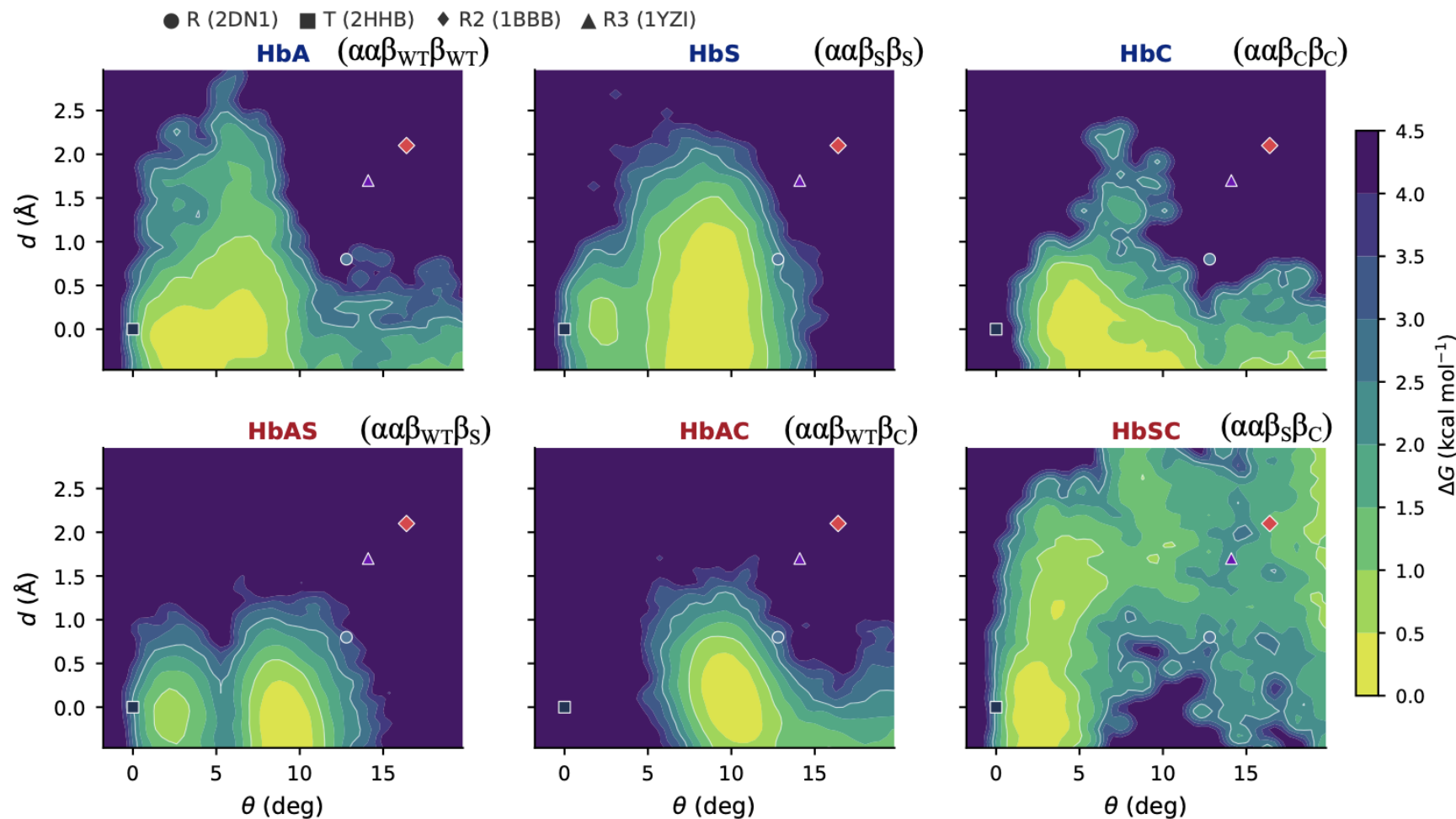
Rottura coordinata dei ponti salini e rotazione di 15° di un dimero



Stereochemistry of Cooperative Effects in Haemoglobin – Perutz (1970); Baldwin e Cyrus Chothia (1979) (J. Mol. Biol. 129: 175-220); Monod, J; Wyman, J; Changeux, J.-P. (1965). "On the Nature of Allosteric Transitions — a Plausible Model". J. Mol. Biol. 12 (1): 88–118.; Koshland, D.E., Némethy, G. and Filmer, D. (1966) Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochemistry 5, 365–385



Instabilità conformazionale: HbSC perde la stabilità dello Stato T



HbA, HbS, HbC:

La molecola è rigidamente confinata nel minimo energetico dello stato T.

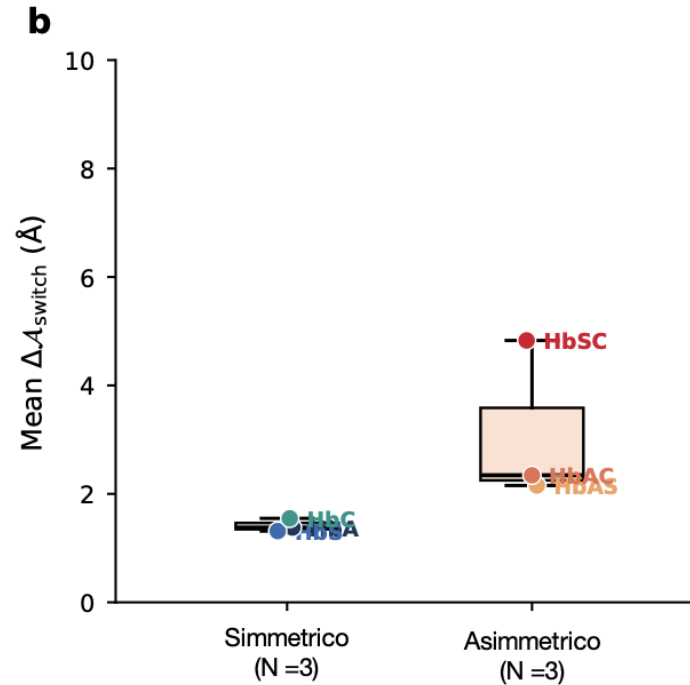
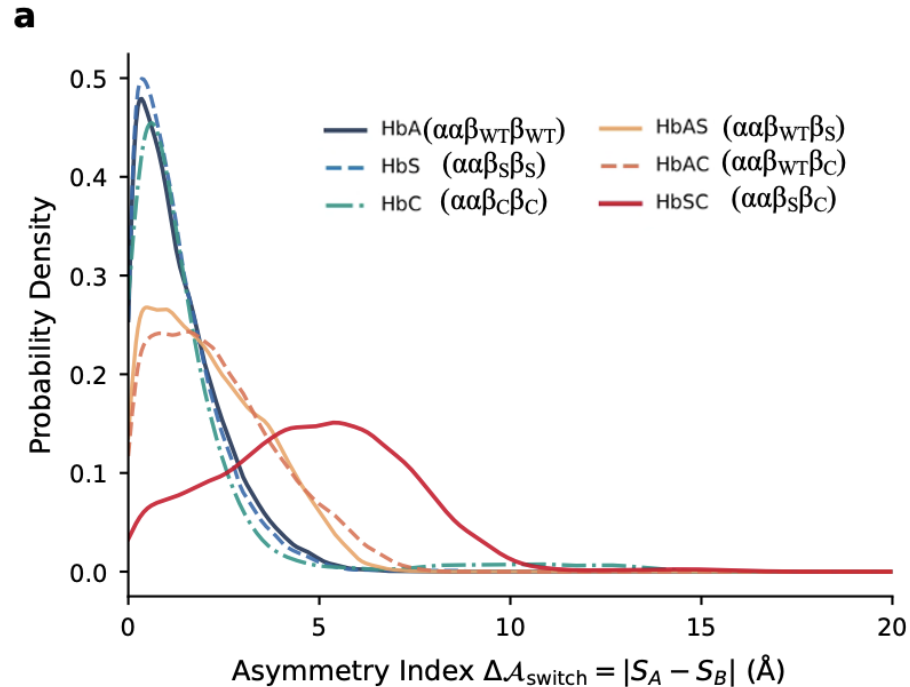
HbAS e HbAC:

Lieve flessibilità rotazionale, mantenendo una traslazione d quasi nulla.

HbSC:

Marcata dispersione conformazionale. Il doppio eterozigote perde la capacità biofisica di mantenere stabilmente la conformazione dello stato T.

Rottura del dogma di simmetria: l'eterozigosi doppia rompe la simmetria dell'emoglobina



Metrica: ΔA_{switch} = differenza di scorrimento tra le due interfacce $\alpha_1\beta_2$ e $\alpha_2\beta_1$

Simmetrici (N = 3):

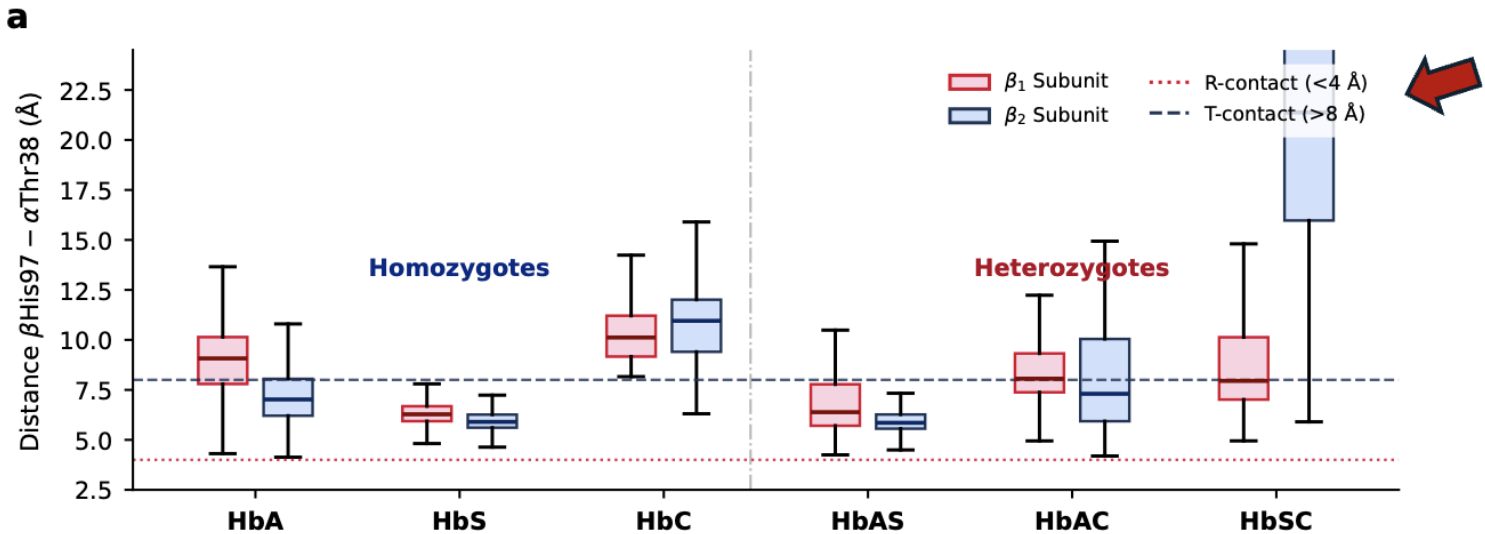
Distribuzione unimodale stretta centrata a < 0.8 Å → Conservazione rigorosa della simmetria rotazionale C_2 .

Asimmetrici (N= 3):

Asimmetria lieve in HbAS e HbAC (~ 2.5 Å), Marca asimmetria HbSC (~ 4.9 Å).

HbSC rompe la simmetria della molecola, le due interfacce si disaccoppiano lavorando in modo asimmetrico

L'asimmetria in HbSC fa collassare il "lucchetto" di Perutz

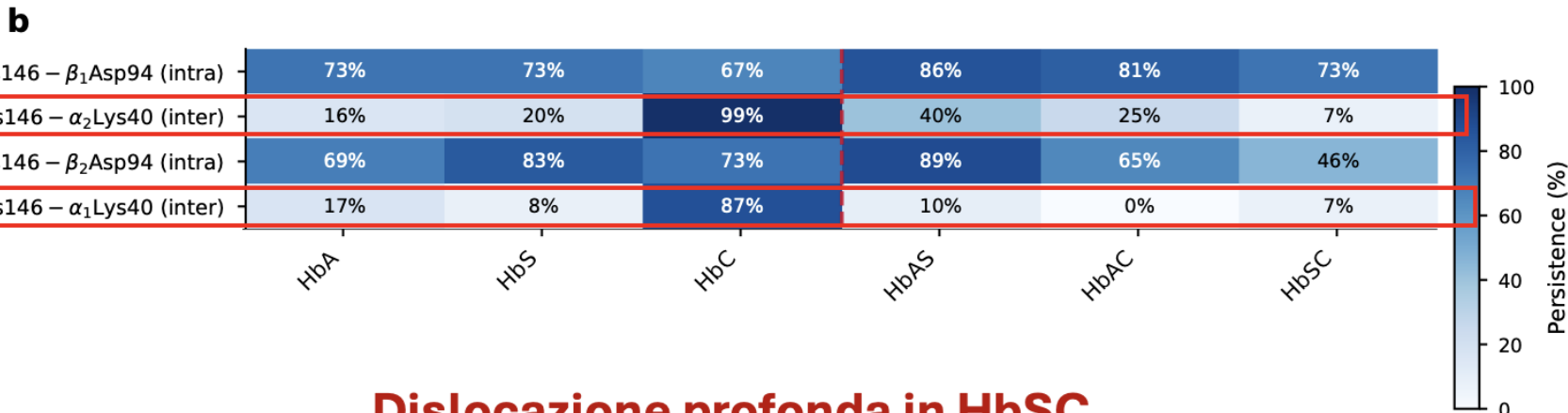


HbA, HbS, HbC, HbAS/HbAC:

La distanza resta confinata nei valori di contatto T (~6–10 Å) e rete elettrostatica globale preservata e tamponata (~ 80 – 99%).

HbC: Marcata iper-coesione (ponti inter-catena stabili all'87–99%)

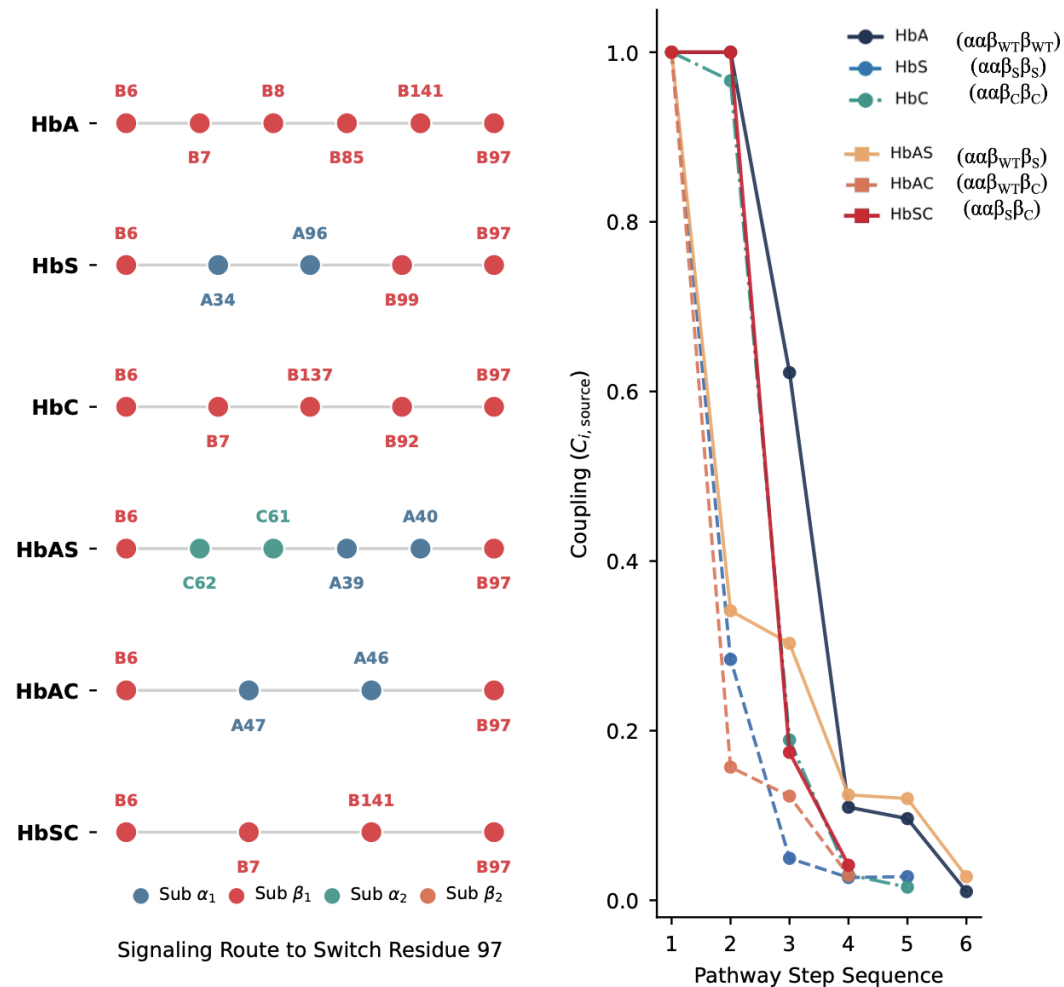
HbSC: Collasso ponte $\rightarrow \beta_1$ e β_2 (His146 – Lys40) preservato solo nel 7% del tempo; instabilità bilaterale dello stato T dove la subunità β_2 subisce una dislocazione profonda (supera i 16–22 Å).



Dislocazione profonda in HbSC

I due dimeri tendono fisicamente ad allontanarsi e separarsi nello spazio

La mutazione in superficie trasmette il disturbo direttamente al centro dell'emoglobina in HbSC



HbA, HbS, HbC, HbAS, HbAC

Via a Dispersione

Il segnale allosterico compie un percorso lungo (5–6 nodi) e attraversa le subunità α , in presenza di catene β^S



Protezione Allosterica: le subunità α assorbono la tensione, preservando la cerniera centrale.

Doppio Eterozigote HbSC

Cortocircuito Diretto

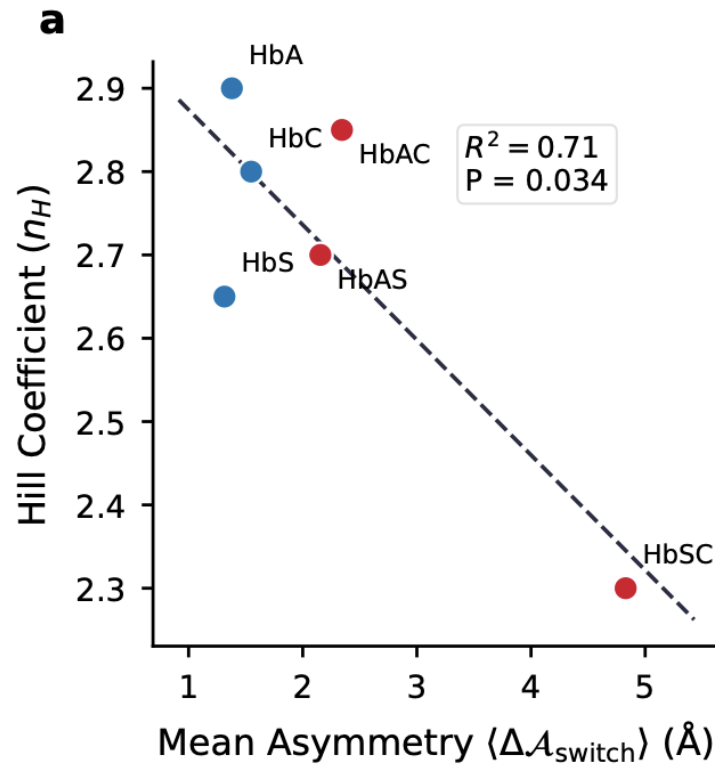
Circuito ultracorto a soli 4 residui: il segnale non si disperde e resta confinato nella catena β .



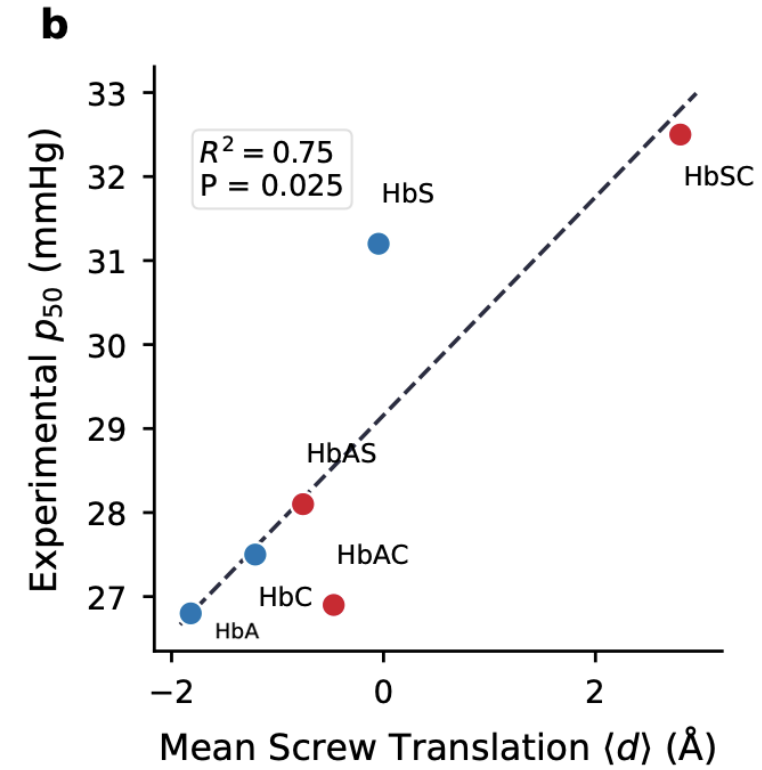
Iper-Coesione Rigida: la tensione scarica integra sulla cerniera, forzando la dislocazione dei dimeri.

L'assenza di dispersione sulle catene alfa potrebbe spiegare l'instabilità conformazionale dell'HbSC

L'asimmetria conformazionale e lo scorrimento assiale correlano con i parametri funzionali dell'emoglobina



L'**asimmetria** dinamica dello switch allosterico spiega il 71% della varianza nella **perdita di cooperatività** di legame dell'ossigeno.



I sistemi che mantengono d compatto o negativo (HbA, HbC, HbAC) presentano p_{50} fisiologici normali (~26–27 mmHg), mentre i **complessi che scivolano assialmente** (HbS e soprattutto HbSC) mostrano l'elevato p_{50} tipico della **bassa affinità per l'ossigeno**.

Conclusioni

Rottura del dogma della simmetria (C_2)

L'asimmetria non rappresenta una media fenotipica lineare. La compresenza di catene β dissimili distrugge l'armonia quaternaria, inducendo un disaccoppiamento meccano-funzionale tra le due interfacce $\alpha_1\beta_2$ e $\alpha_2\beta_1$.

Comportamento dell'asimmetrico HbSC ($\alpha\alpha\beta_s\beta_c$), che si discosta maggiormente dagli altri

A differenza dei portatori sani (che ammortizzano le tensioni), in HbSC il circuito allosterico scarica direttamente sullo switch $\beta 97$ dove i ponti salini cardine di Perutz crollano al 7% su entrambi i lati, provocando la dislocazione fisica dei dimeri ($>16 \text{ \AA}$) e l'evasione dallo stato deossi T.

Ponte predittivo tra atomi e clinica

La perdita di cooperatività correla con la rottura di simmetria (ΔA_{switch}), mentre l'affinità ($p50$) correla con lo scorrimento assiale (d). La dinamica molecolare diventa un potenziale biomarcatore prognostico in silico.



Prospettive future e potenziali sviluppi traslazionali

Estensione alle Varianti Rare del Globulo Rosso

- Applicare la pipeline di analisi alle altre varianti emoglobiniche, anche rare, creando un atlante predittivo biofisico-fenotipico, utile per la correlazione genotipo-fenotipo per varianti note, ed estenderlo da un punto di vista predittivo anche per combinazioni genotipiche che coinvolgono varianti poco descritte o non riscontrate.
 - Studio del comportamento dell'emoglobina fetale HbF ($\alpha\alpha\gamma\gamma$)
 - Studio degli asimmetrici combinati con HbF ($\alpha\alpha\gamma\gamma$)

Drug Discovery e Allosteric Modulation Mirata

Sviluppo di modulatori allosterici di nuova generazione che interferiscono con la cerniera $\alpha_1\beta_2$



RINGRAZIAMENTI



Basi strutturali delle emoglobinopatie: instabilità conformazionale e iper-coesione all'interfaccia svelate tramite simulazioni AA-MD

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RINGRAZIAMENTI



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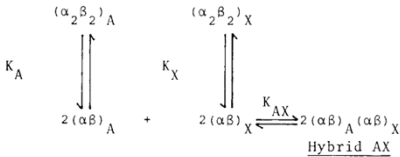
⁵ CREO, Laboratorio Globulo Rosso, Università degli studi di Perugia.



SHORT COMMUNICATION

DIRECT DETECTION OF OXY ASYMMETRICAL HEMOGLOBIN
HYBRIDS IN BLOOD SAMPLES

It has been previously demonstrated that, in most of the subjects carrying hemoglobinopathies, normal and abnormal hemoglobin variants coexist in the same red cell (1,2). It is also known that, if two different hemoglobins are mixed and incubated under proper conditions of temperature, buffer and ionic strength, a hybrid can be formed, owing to the dimerization and reassociation reactions, as illustrated by the following scheme:



The existence of hybrids of this type has been postulated by Itano (3) and Guidotti (4) and later proved directly by McLeod (5), who stabilized the hybrid between HbA and HbS with a particular cross-linking reagent and isolated it by electrophoresis.

The isolation of these asymmetrical hybrids by conventional electrophoretic techniques is however technically difficult, because of the rate of the

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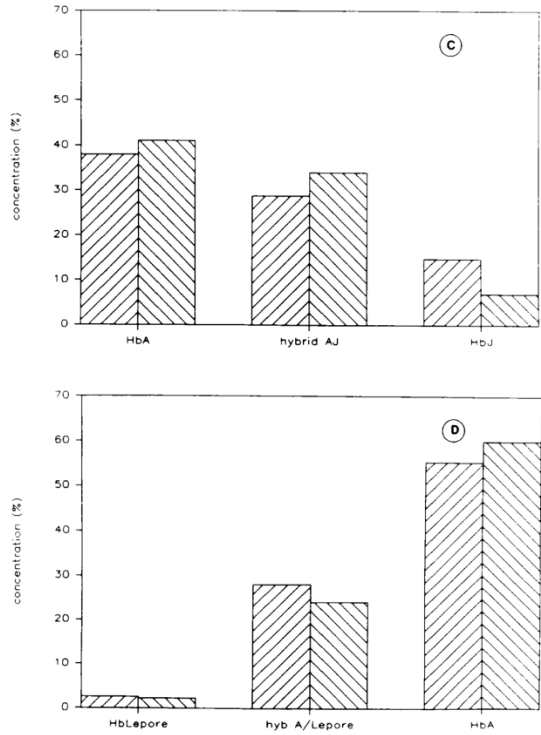


Fig. 2 Cont.

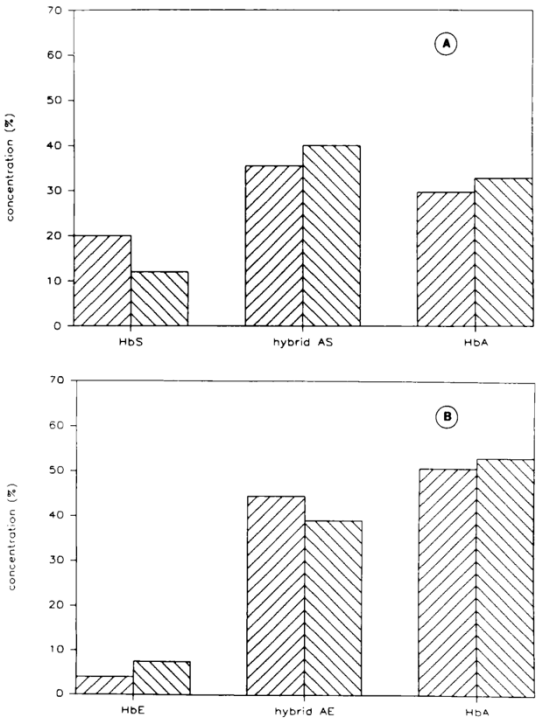


FIGURE 2

Distribution of the main hemoglobin components in the analyzed samples. The concentrations measured with the IEF analysis (▨) are reported in comparison with those calculated (■) assuming a binomial distribution of the parent and the hybrid species, according to Bunn (7). A: blood with HbS; B: blood with HbE; C: blood with HbJ Paris; D: blood with Hb Lepore.

BRIEF REVIEW

Subunit Assembly of Hemoglobin: An Important Determinant
of Hematologic Phenotype

By H. Franklin Bunn

Hemoglobin's physiologic properties depend on the orderly assembly of its subunits in erythropoietic cells. The biosynthesis of α - and β -globin polypeptide chains is normally balanced. Heme rapidly binds to the globin subunit, either during translation or shortly thereafter. The formation of the $\alpha\beta$ -dimer is facilitated by electrostatic attraction of a positively charged α -subunit to a negatively charged β -subunit. The $\alpha\beta$ -dimer dissociates extremely slowly. The difference between the rate of dissociation of $\alpha\beta$ - and $\alpha\gamma$ -dimers with increasing pH explains the well-known alkaline resistance of Hb F. Two dimers combine to form the functioning $\alpha_2\beta_2$ -tetramer. This model of hemoglobin assembly explains the different levels of positively charged and negatively charged mutant hemoglobins that are encountered in heterozygotes and the effect of α -thalassemia and heme deficiency states in modifying the level of the

variant hemoglobin as well as Hb A₂. Electrostatic interactions also affect the binding of hemoglobin to the cytoplasmic surface of the red cell membrane and may underlie the formation of target cells. Enhanced binding of positively charged variants such as S and C trigger a normally dormant pathway for potassium and water loss. Thus, the positive charge on β^+ is responsible for the two major contributors to the pathogenesis of Hb SC disease: increased proportion of Hb S and increased intracellular hemoglobin concentration. It is likely that electrostatic interactions play an important role in the assembly of a number of other multisubunit macromolecules, including membrane receptors, cytoskeletal proteins, and DNA binding proteins.

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JUST as the accuracy and durability of a fine watch depend on the well-engineered fit of its moving parts, many regulatory events in biology require dynamic interaction between the "moving parts" of multisubunit proteins. Hemoglobin is probably the most thoroughly studied example; its physiologic function depends on gear- and ratchet-like movement between its subunits.¹⁻³ Our understanding of how hemoglobin works at the molecular and atomic levels is based on contributions from many disciplines, including structural and physical biochemistry, molecular genetics, and clinical medicine. Lessons learned from hemoglobin can be applied with profit to a number of other multisubunit proteins, some of which are of special interest to hematologists.

A rigorous set of constraints is involved in the orderly construction of a multisubunit protein. In most instances, including hemoglobin, the unlike subunits are products of different genes. (Exceptions include insulin and haptoglobin, whose subunits are cleaved from a precursor polypeptide and linked by disulfide bonds.) Efficient biosynthesis of hemoglobin requires balanced production of α and β polypeptide chains. Moreover, enough heme must be synthesized in the mitochondria to provide each of the newly made globin chains with its all-important prosthetic group. The reaction between heme and apoglobin is rapid.^{4,5} Therefore, it is likely that heme is inserted into its proper niche either during translation of the nascent globin chain or shortly thereafter. A slight mismatch in globin chain biosynthesis can be tolerated because erythroid precursor cells have an efficient

adenosine triphosphate-dependent proteolytic system⁶⁻⁸ that attacks free subunits^{9,10} but leaves assembled dimers and tetramers unscathed. Likewise, excess heme can be catabolized by an inducible microsomal oxygenase.¹¹ Despite intensive experimental scrutiny, the mechanisms responsible for the precise coordination between the synthesis of globin subunits and that of heme are unclear. These good house-keeping practices are essential to normal red cell viability. The thalassemias are prototypes of chain imbalance. Most of the phenotypic features of both the β -thalassemias and the α -thalassemias can be explained by either the deleterious effect of free subunits on the red cell membrane or the accumulation of these relatively insoluble and noncooperative subunits in the cytosol.

SUBUNIT ASSEMBLY OF HEMOGLOBIN

Once equivalent amounts of α - and β -subunits have been translated on their respective polyribosomes, and once heme has been inserted, the hemoglobin subunits are now faced

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SUBUNIT ASSEMBLY OF HEMOGLOBIN

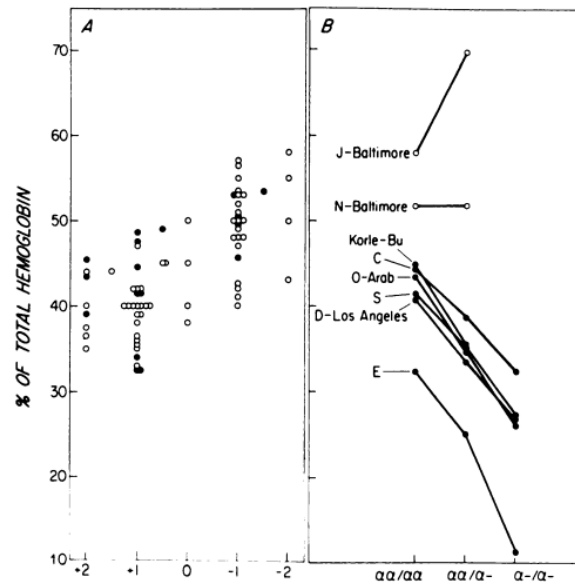


Fig 2. (A) Effect of charge on the proportion of abnormal hemoglobin in individuals heterozygous for 72 stable β -globin variants. Each data point represents a mean value for a given variant. The solid points (●) denote measurements of Huisman^{20a} using high-resolution chromatography. Substitutions involving a histidine residue were scored as a change of one half charge. The "-1" group differs significantly from the "+1" group ($P < .001$) and from the "0" group ($P < .05$). (B) Effect of α -thalassemia on the proportion of six positively charged β -chain variants (●) and two negatively charged variants (○) (data from Bunn and McDonald²⁰).

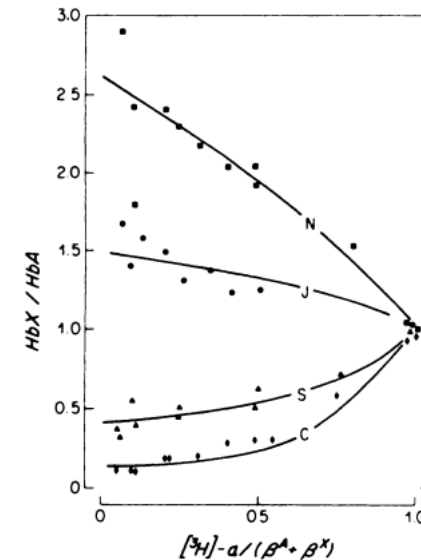


Fig 3. Subunit competition. Equimolar mixtures of normal (β^A) and a variant β -subunit (β^N , β^J , β^S , β^C) were mixed with increasing amounts of tritium-labeled α -subunits. At intracellular pH (7.2) these variant β -subunits in their native heme-intact state have net surface charges of -4.5, -3.5, -1.5, and -0.5, respectively. The products (Hb A and Hb X) were measured by high-performance liquid chromatography. The solid lines represent the computed theoretical fit for competitive recombination reactions between β^A and β^X , assuming that at low α -chain concentrations, the Hb X-Hb A ratio is a measure of the relative rates of assembly of $\alpha\beta^A$ and $\alpha\beta^X$ (data from Mrabet et al²⁹).