



Fondazione
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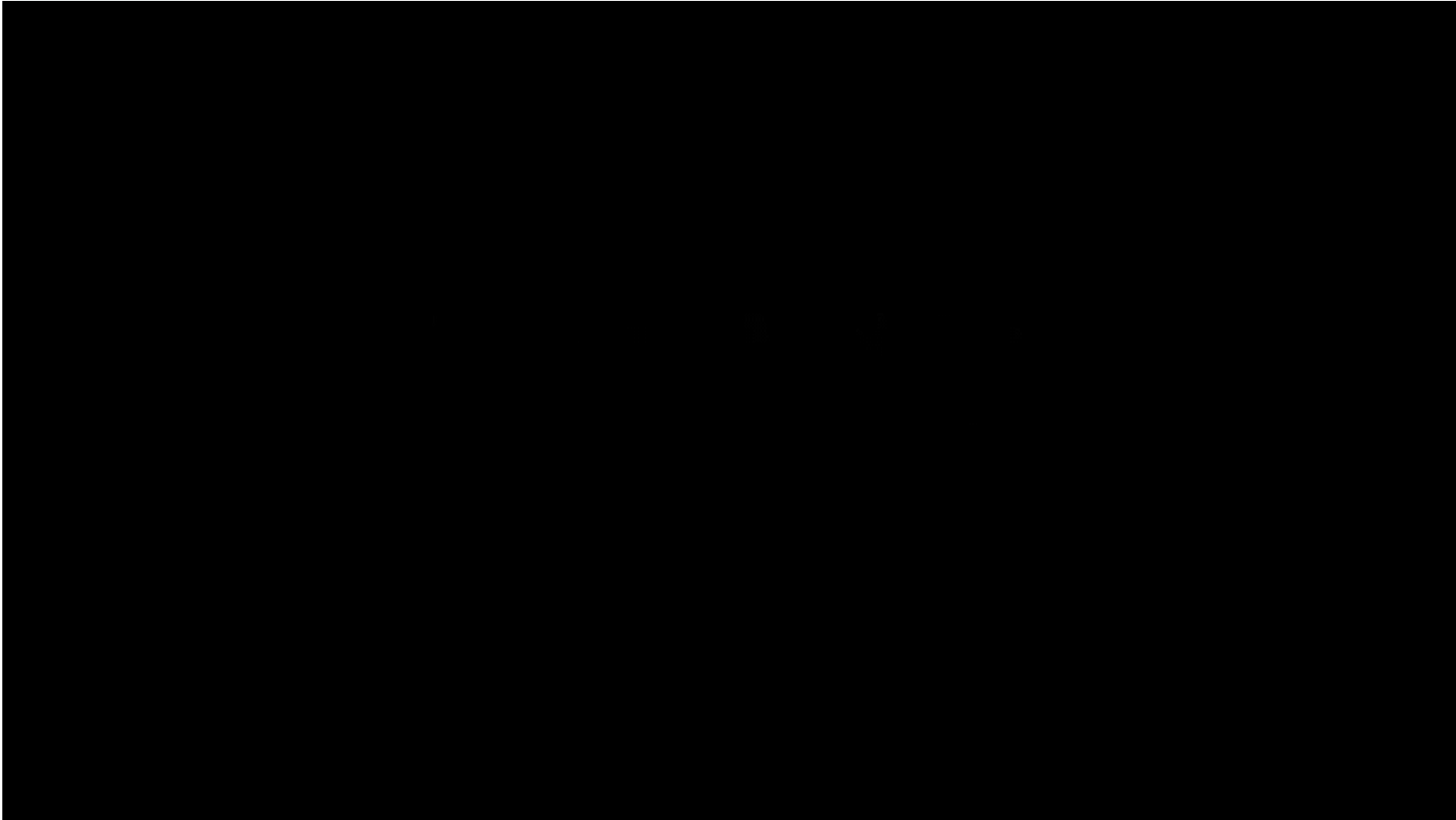
Il ruolo della Biologia Strutturale nel combattere la pandemia Covid-19: applicazioni di spettroscopia NMR nell'ambito delle scienze della vita

Caterina Alfano, PhD
Group Leader Biologia Strutturale e Biofisica
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La vita all'interno della cellula

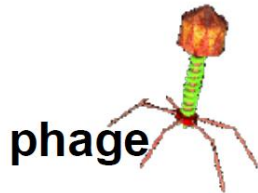
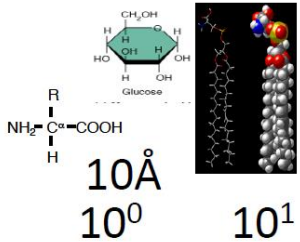




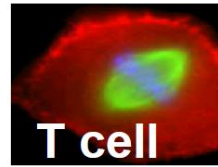
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Perchè la Biologia Strutturale?

Le biomolecole sono troppo piccolo per essere viste in dettaglio anche con i microscopi ottici più avanzati.



phage

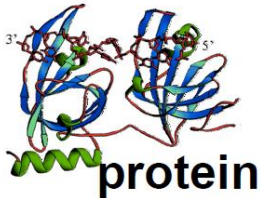


T cell

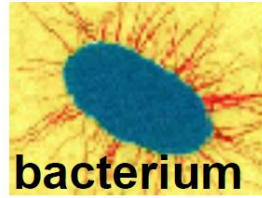


mouse

10^1 10^4 10^8 10^{12} 10^{16} 10^{20} 10^{24} 10^{28} **nm**
Daltons



protein



bacterium



fly



human

1nm = 1/1.000.000.000 m





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Perchè la Biologia Strutturale?

Le biomolecole sono troppo piccolo per essere viste in dettaglio anche con i microscopi ottici più avanzati.



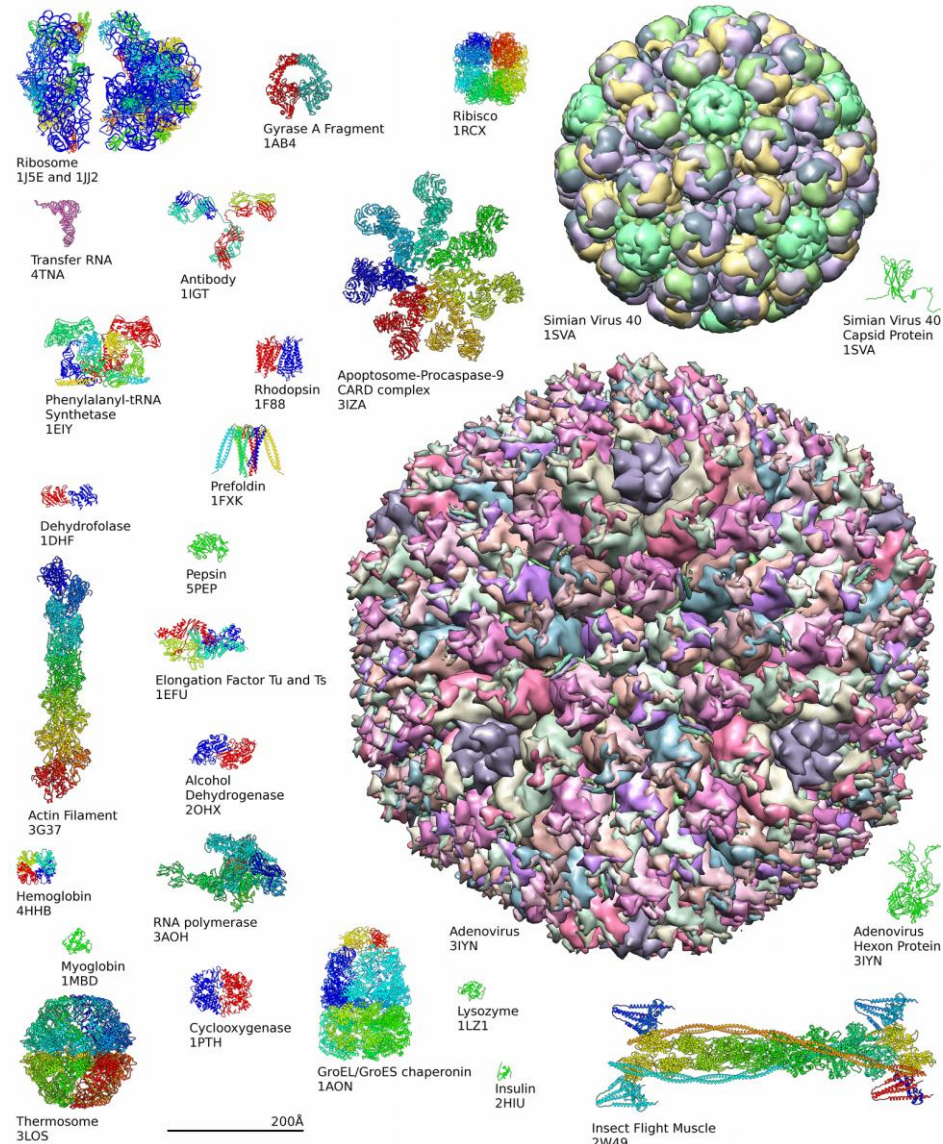


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La Biologia Strutturale

La biologia strutturale cerca di fornire un quadro completo e coerente dei fenomeni biologici a livello atomico sviluppando una comprensione completa delle forme molecolari delle macromolecole biologiche.

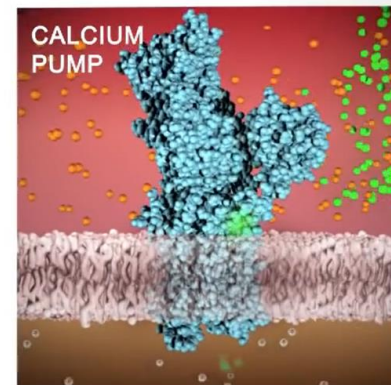
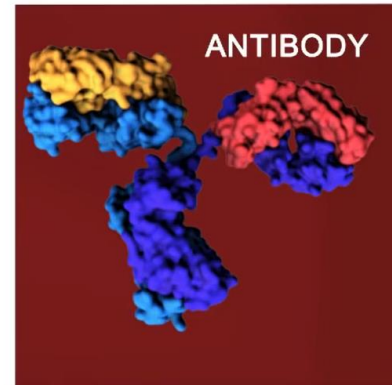
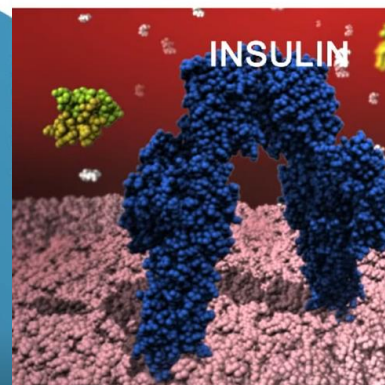
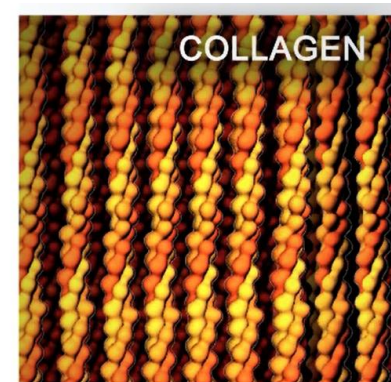
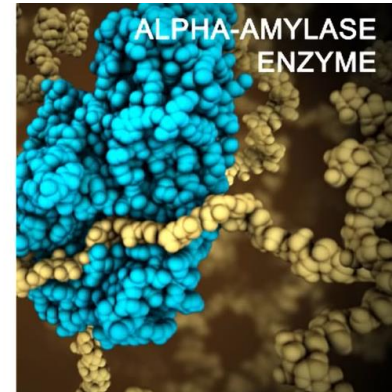
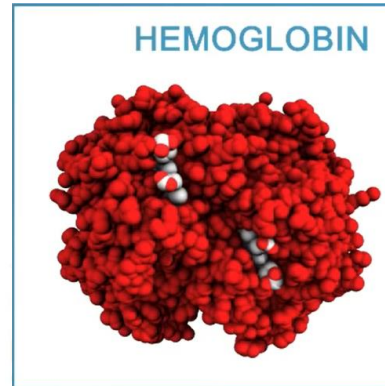
La Biologia Strutturale vede l'invisibile





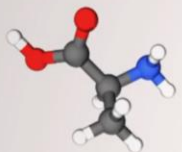
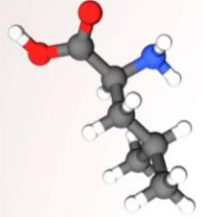
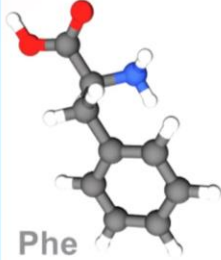
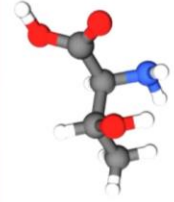
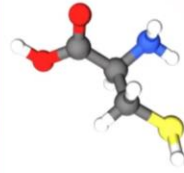
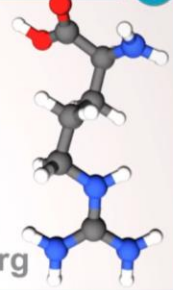
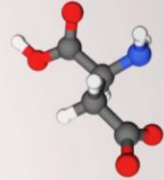
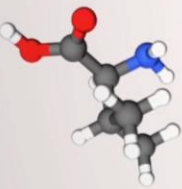
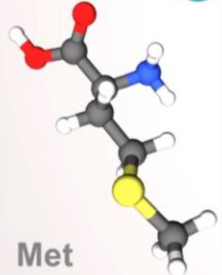
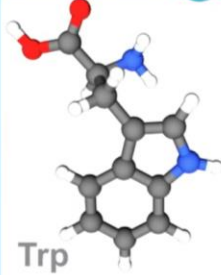
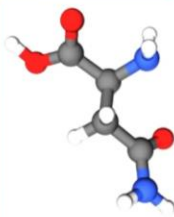
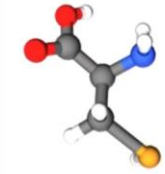
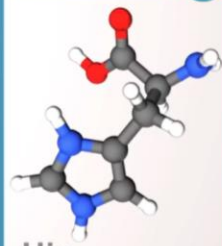
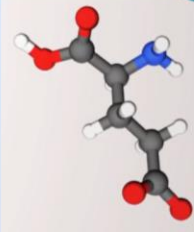
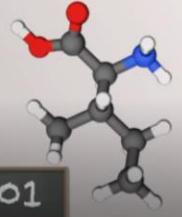
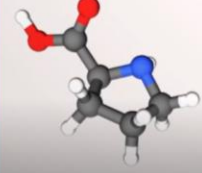
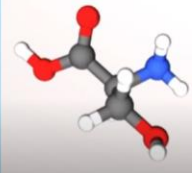
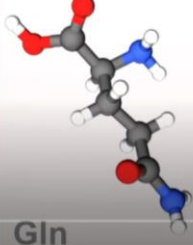
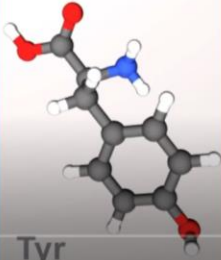
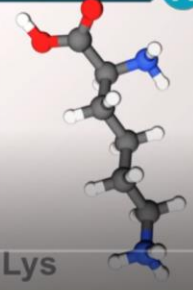
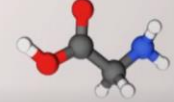
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Molte proteine per molte funzioni





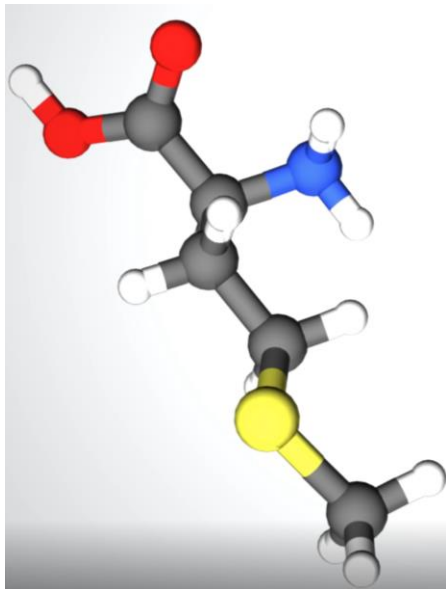
I mattoncini delle proteine

<p>Alanine A</p>  <p>Ala</p>	<p>Leucine L</p>  <p>Leu</p>	<p>Phenylalanine F</p>  <p>Phe</p>	<p>Threonine T</p>  <p>Thr</p>	<p>Cysteine C</p>  <p>Cys</p>	<p>Arginine R</p>  <p>Arg</p>	<p>Aspartic Acid D</p>  <p>Asp</p>
<p>Valine V</p>  <p>Val</p>	<p>Methionine M</p>  <p>Met</p>	<p>Tryptophan W</p>  <p>Trp</p>	<p>Asparagine N</p>  <p>Asn</p>	<p>Selenocysteine U</p>  <p>Sec</p>	<p>Histidine H</p>  <p>His</p>	<p>Glutamic Acid E</p>  <p>Glu</p>
<p>Isoleucine I</p>  <p>Ile</p>	<p>Proline P</p>  <p>Pro</p>	<p>Serine S</p>  <p>Ser</p>	<p>Glutamine Q</p>  <p>Gln</p>	<p>Tyrosine Y</p>  <p>Tyr</p>	<p>Lysine K</p>  <p>Lys</p>	<p>Glycine G</p>  <p>Gly</p>

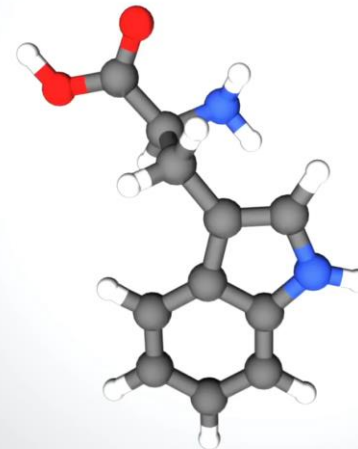


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I mattoncini delle proteine



- Carbon
- Oxygen
- Nitrogen
- Hydrogen
- Sulfur

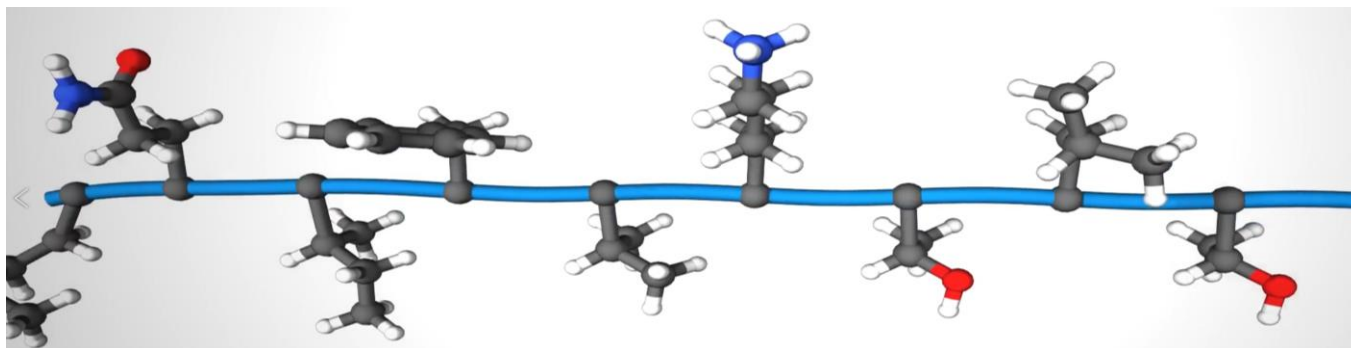
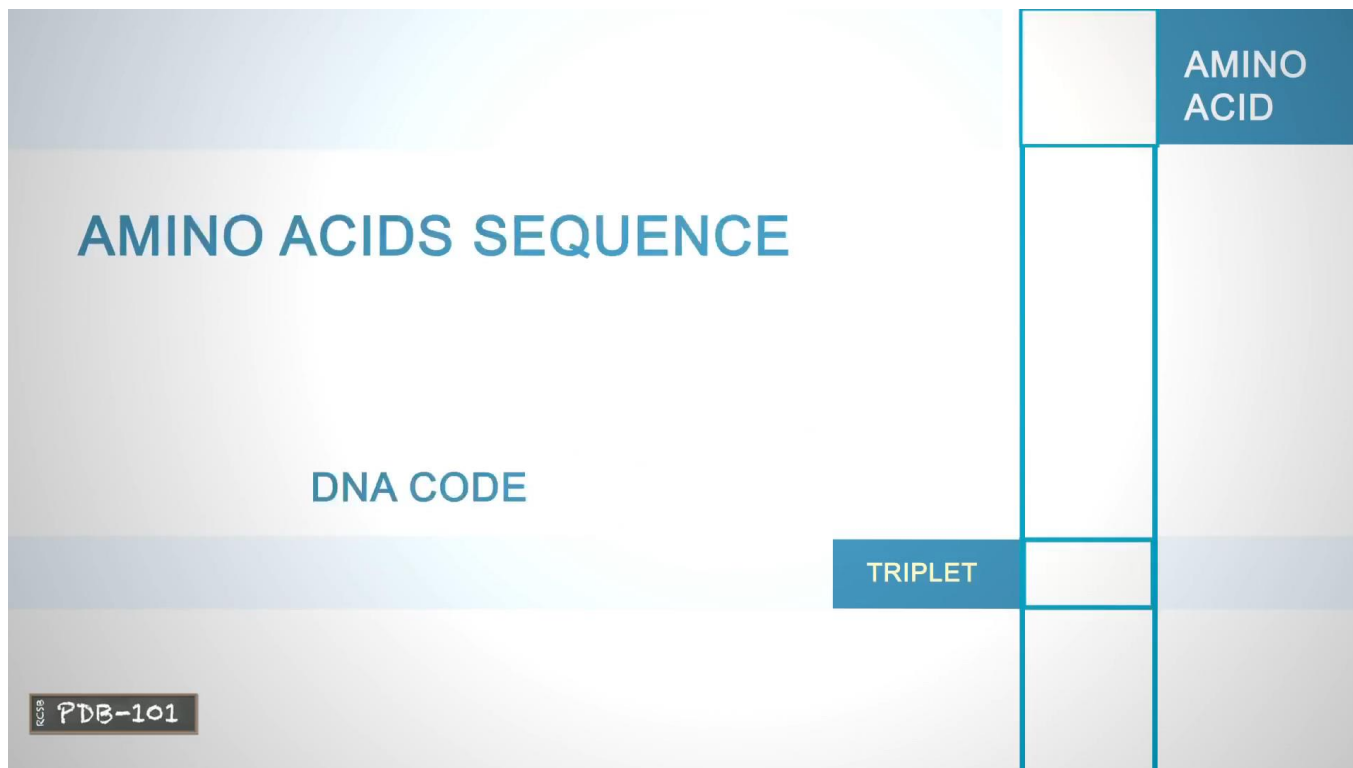


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La struttura primaria





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La necessità della Biologia Strutturale

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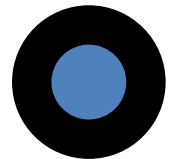
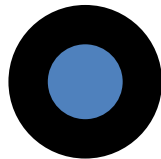
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La necessità della Biologia Strutturale

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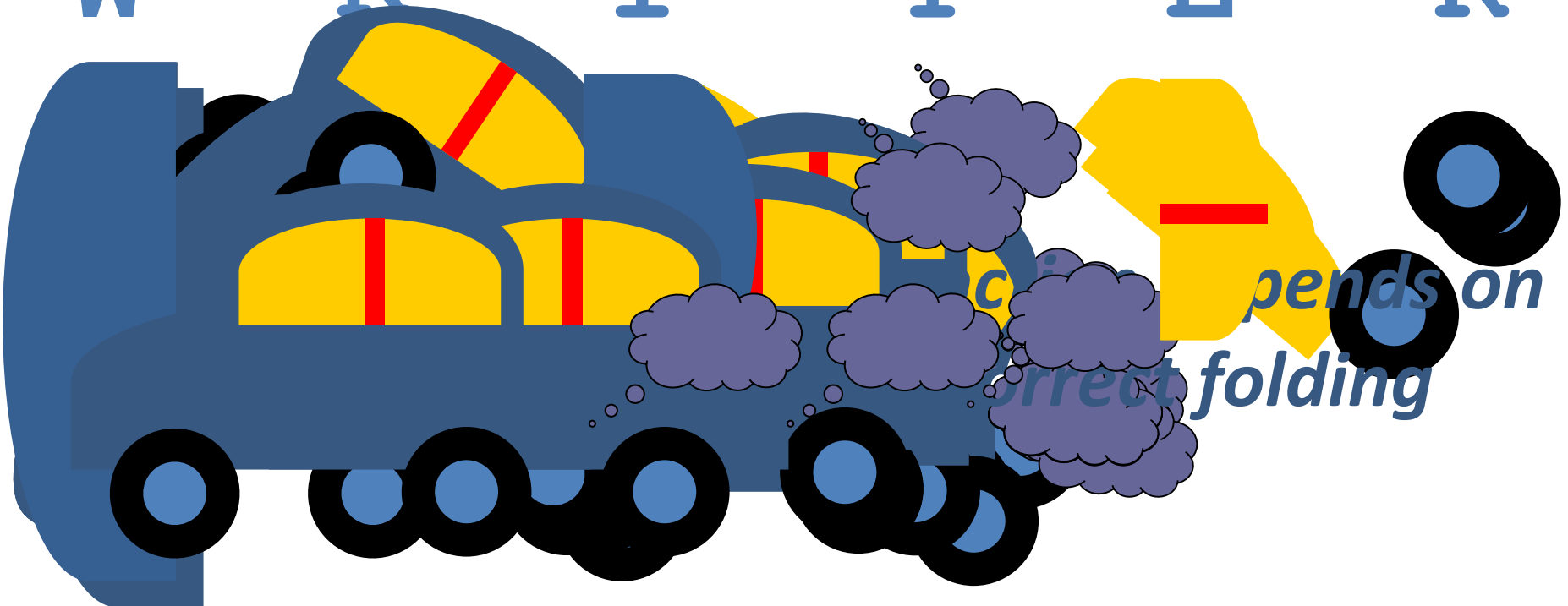
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The need of Structural Biology

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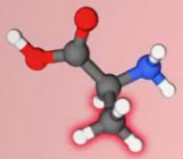
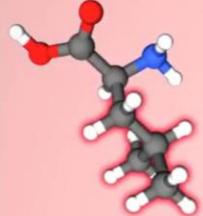
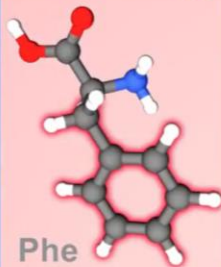
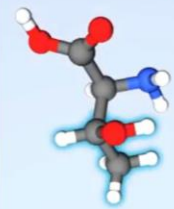
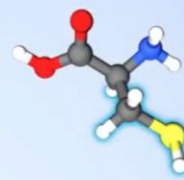
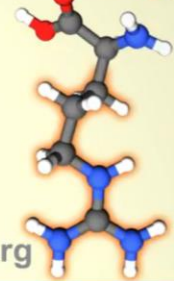
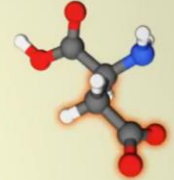
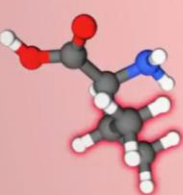
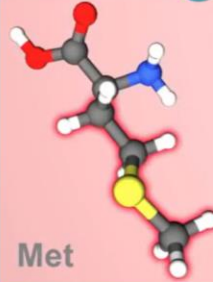

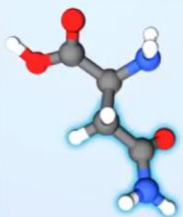
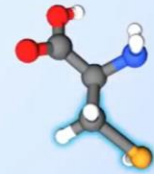
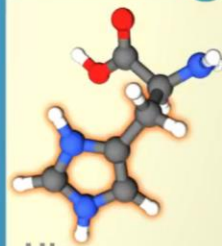
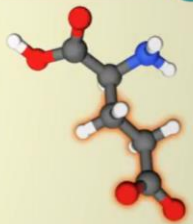
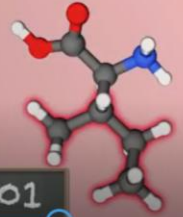
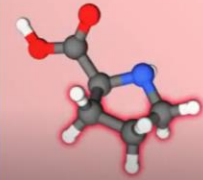
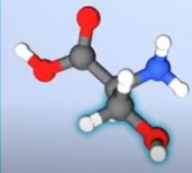
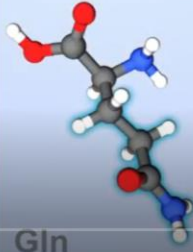
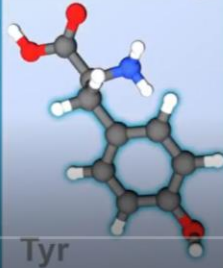
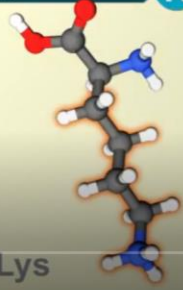
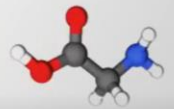


*Function depends on
correct folding*



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I mattoncini delle proteine

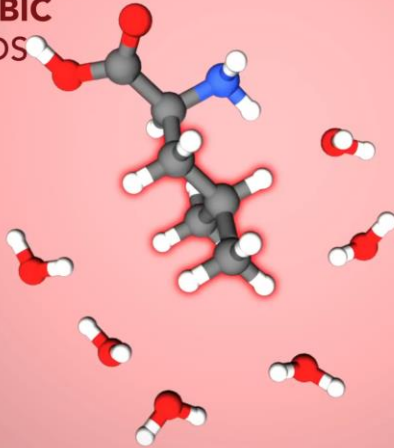
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<p>Valine V</p>  <p>Val</p>	<p>Methionine M</p>  <p>Met</p>	<p>Tryptophan W</p>  <p>Trp</p>	<p>Asparagine N</p>  <p>Asn</p>	<p>Selenocysteine U</p>  <p>Sec</p>	<p>Histidine H</p>  <p>His</p>	<p>Glutamic Acid E</p>  <p>Glu</p>
<p>Isoleucine I</p>  <p>Ile</p>	<p>Proline P</p>  <p>Pro</p>	<p>Serine S</p>  <p>Ser</p>	<p>Glutamine Q</p>  <p>Gln</p>	<p>Tyrosine Y</p>  <p>Tyr</p>	<p>Lysine K</p>  <p>Lys</p>	<p>Glycine G</p>  <p>Gly</p>



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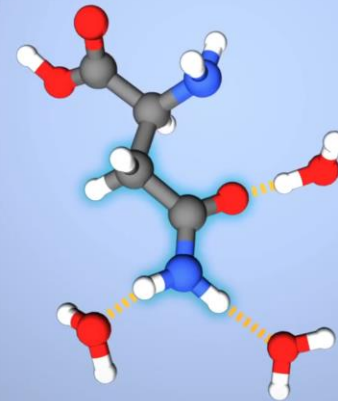
I mattoncini delle proteine

**HYDROPHOBIC
AMINO ACIDS**

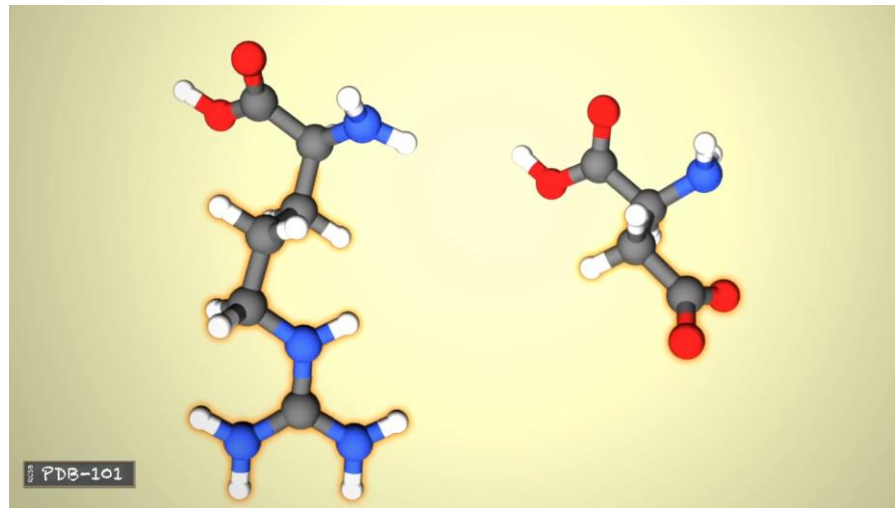


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**HYDROPHILIC
AMINO ACIDS**



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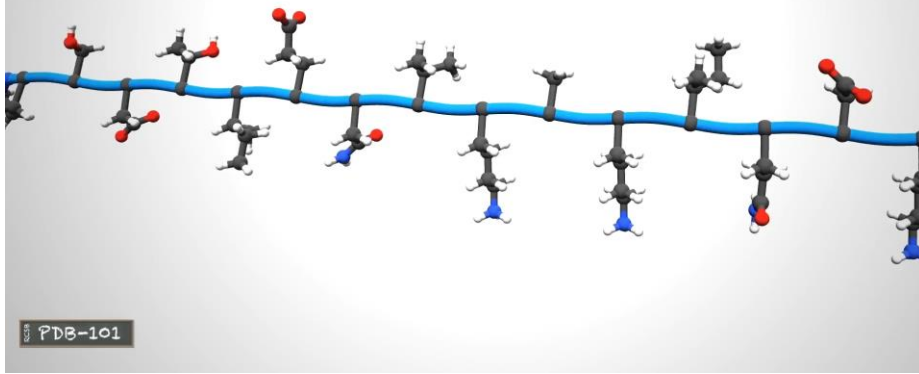
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La struttura secondaria

ALPHA
HELIX

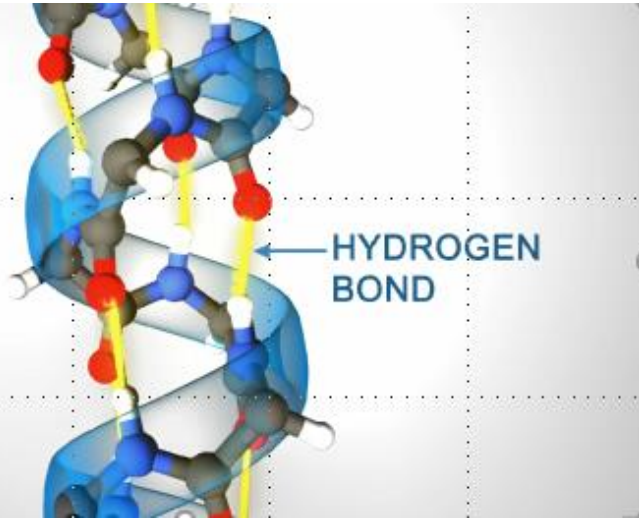




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La struttura secondaria

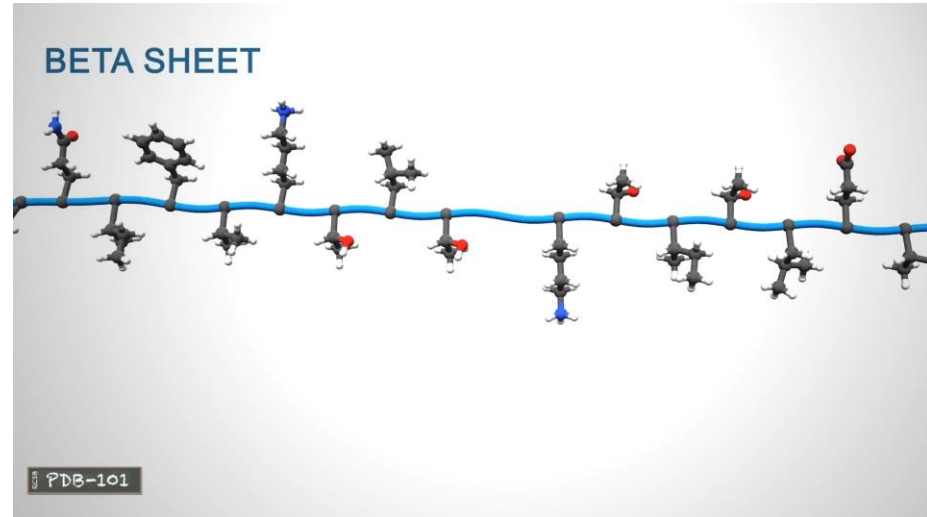
ALPHA
HELIX



HYDROGEN
BOND

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BETA SHEET

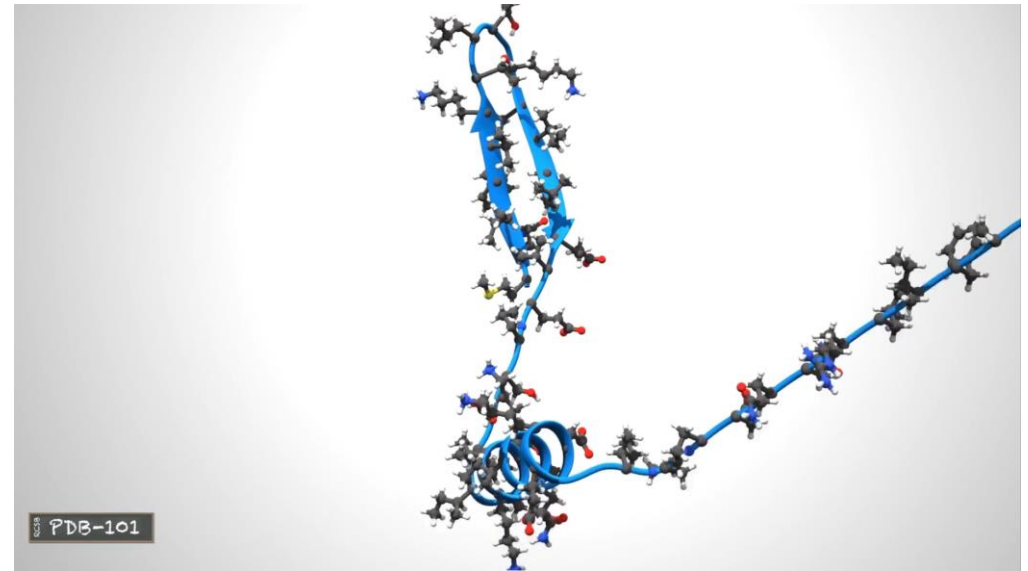
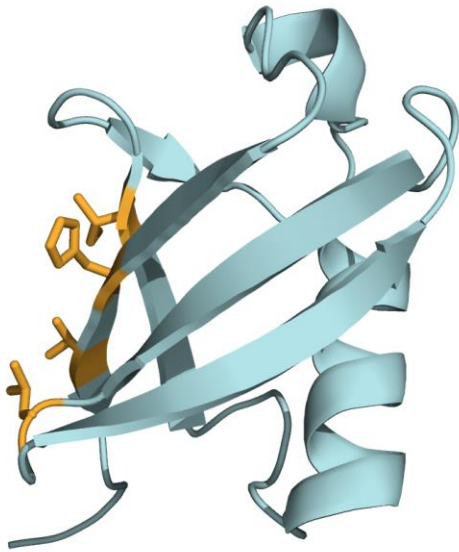


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La struttura terziaria

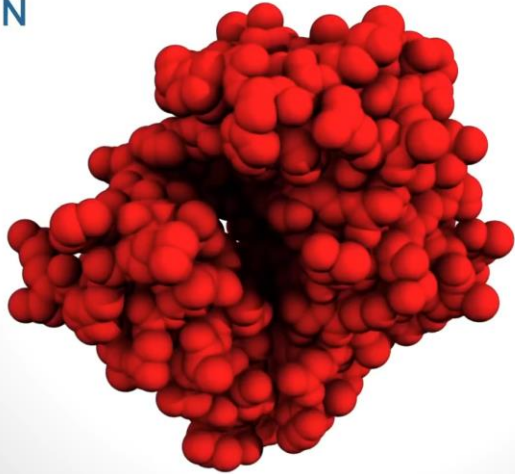




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Forma della proteina → Funzione della proteina

HEMOGLOBIN

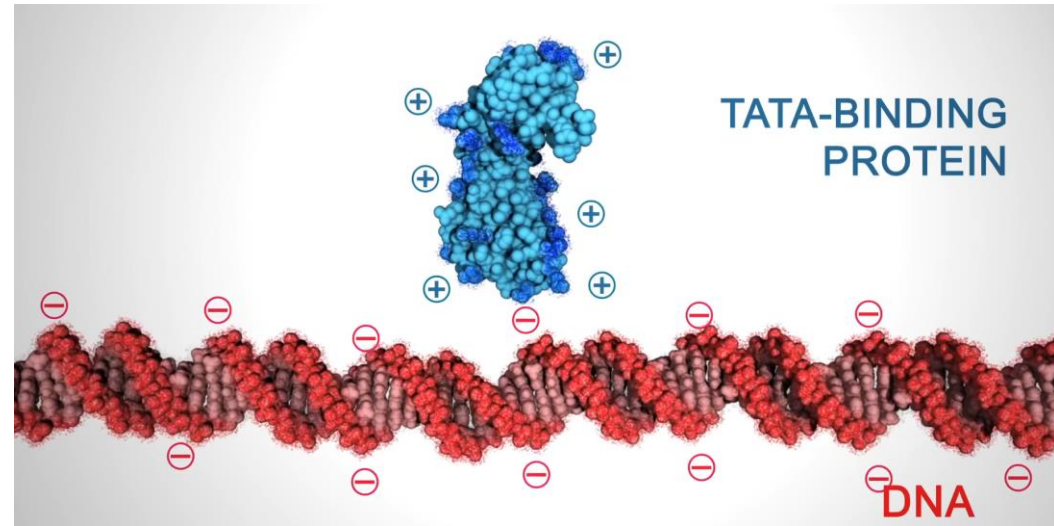
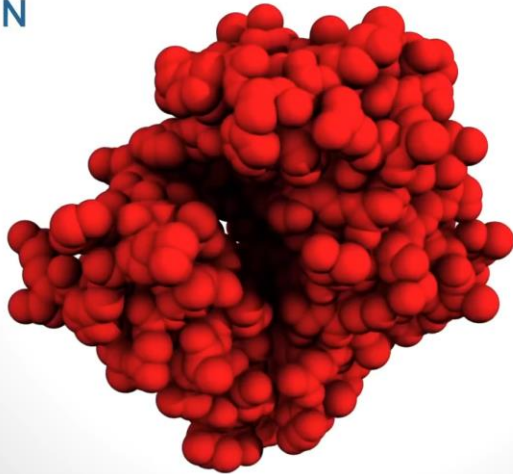




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Forma della proteina → Funzione della proteina

HEMOGLOBIN

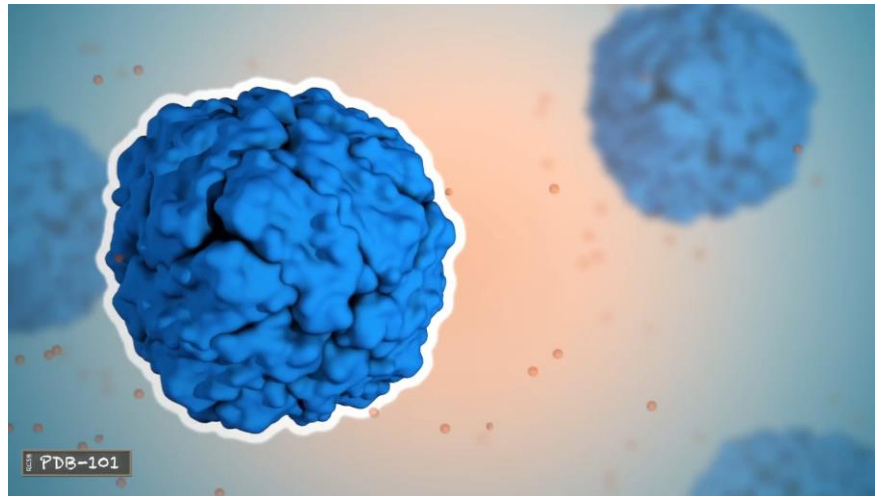
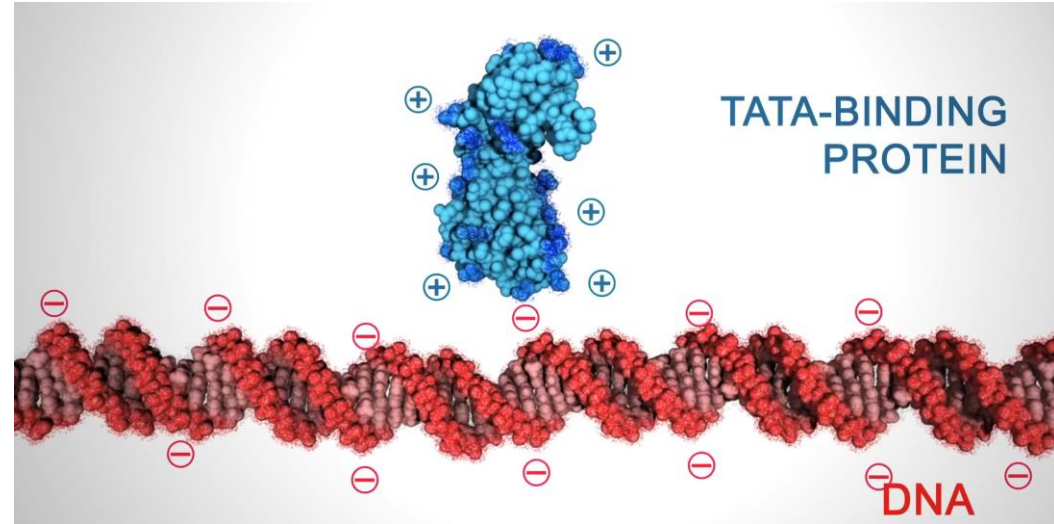
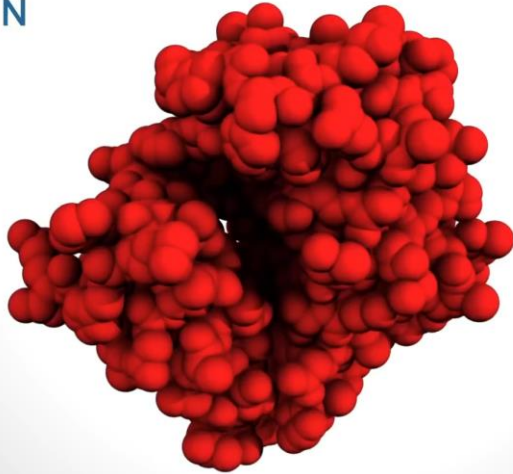




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Forma della proteina → Funzione della proteina

HEMOGLOBIN





Fondazione
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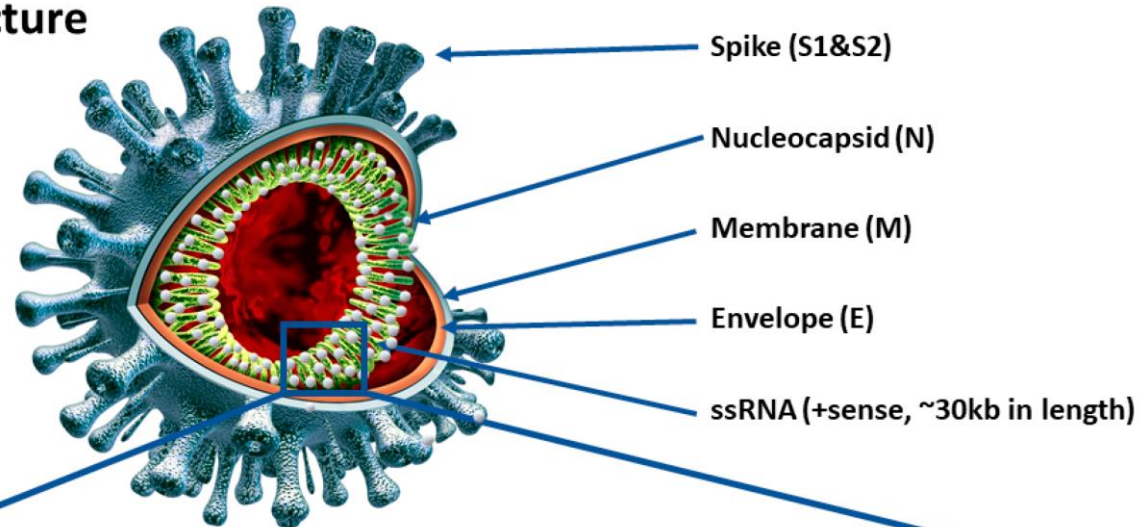
Il Covid-19 è una malattia pandemica?





Gennaio 2020: pubblicazione dell'intero genoma di SARS-CoV-2

SARS-CoV-2 Structure



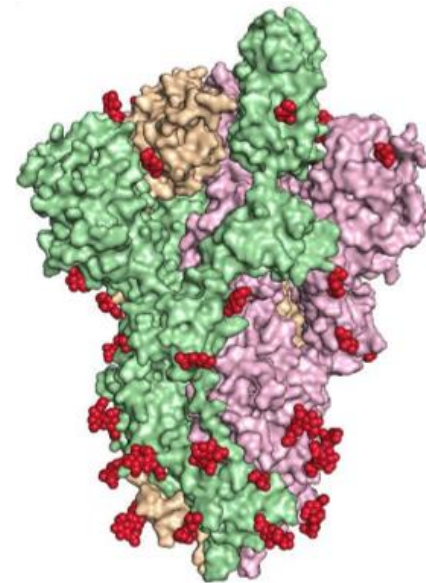
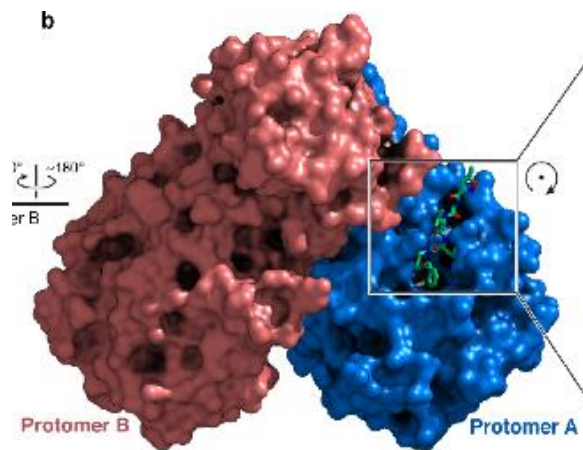


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I Biologi Strutturali a lavoro

A circa due settimane dalla pubblicazione del genoma...

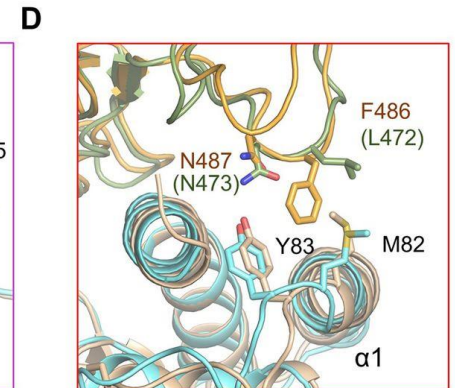
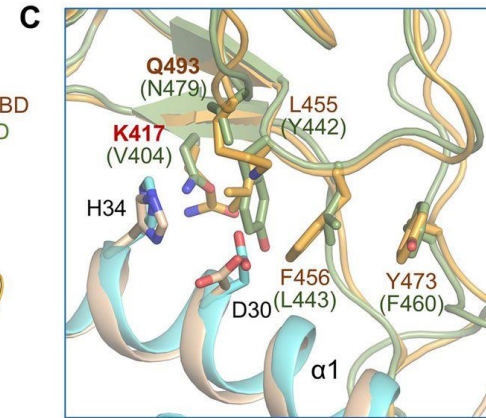
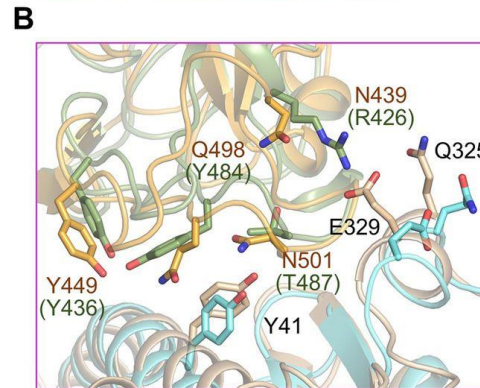
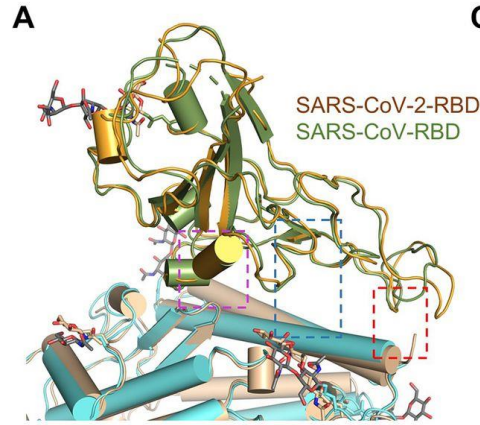
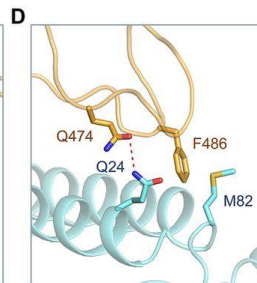
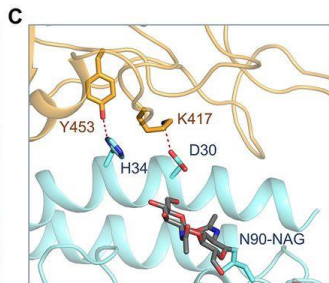
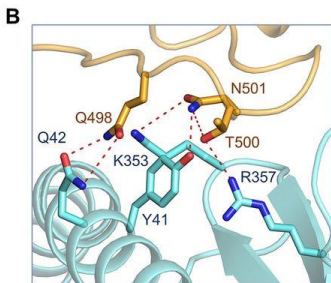
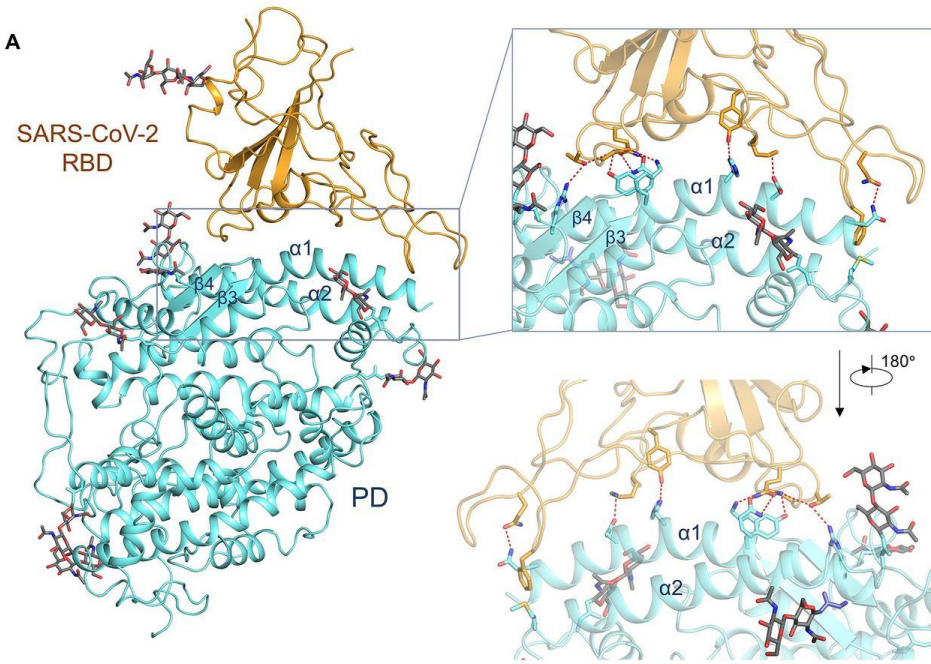
- E' stata pubblicata la prima struttura tridimensionale della Main Protease
- E' stata pubblicata la prima struttura tridimensionale della proteina Spike





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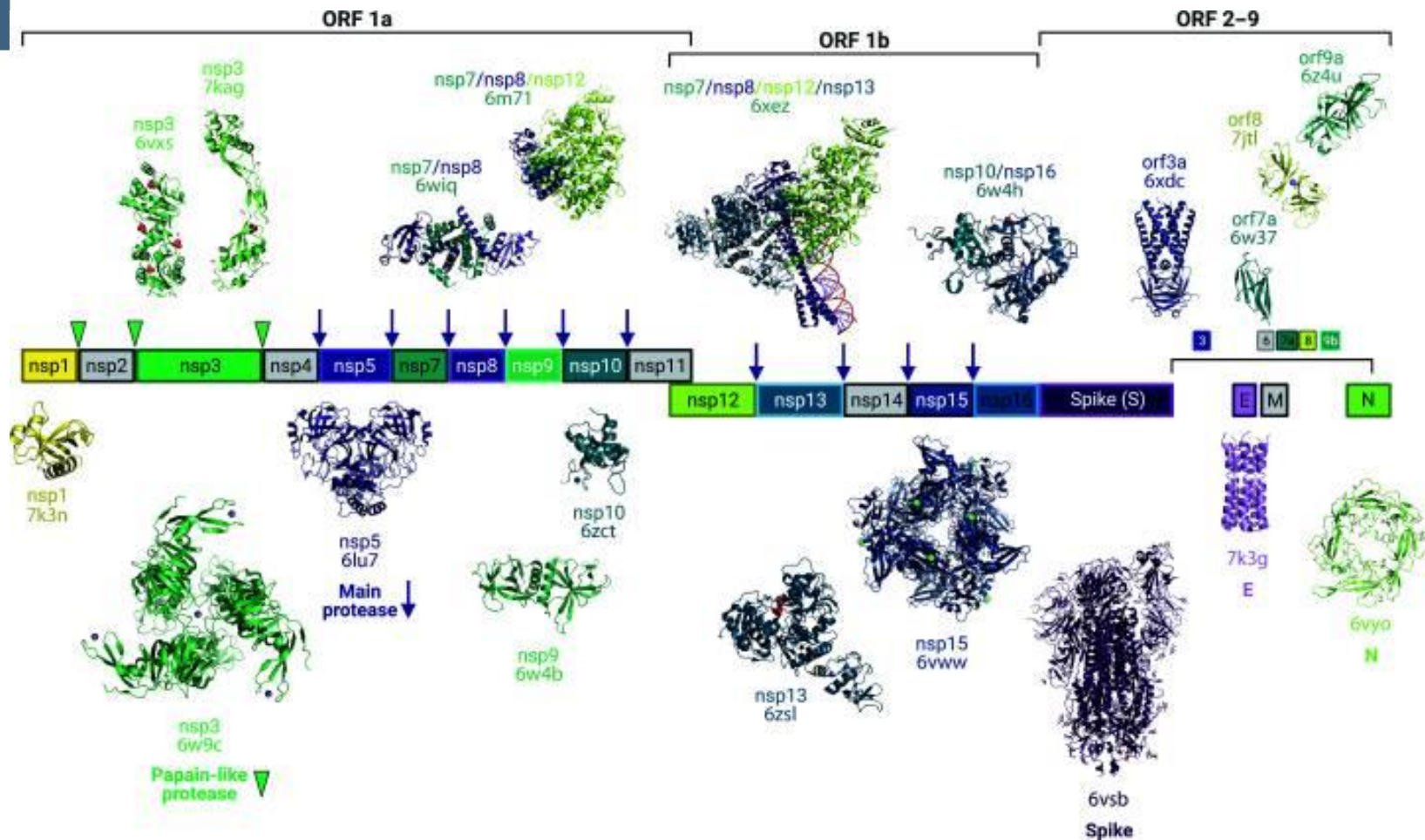
I Biologi Strutturali a lavoro





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I Biologi Strutturali a lavoro



La scoperta delle strutture delle proteine SARS-CoV-2 ha aiutato gli scienziati a sviluppare piccole molecole, anticorpi e altre terapie che possono distruggere il virus



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I Biologi Strutturali a lavoro

I biologi strutturali hanno studiato un numero sostanziale di proteine codificate dal genoma virale, che hanno fornito dettagli essenziali su come funziona il virus.

La biologia strutturale ha svolto ruoli cruciali nell'influenzare la nostra comprensione di come funzionano i meccanismi virali, di come le diverse proteine virali interagiscono con le proteine dell'ospite umano e di come i ligandi si legano alle proteine virali.



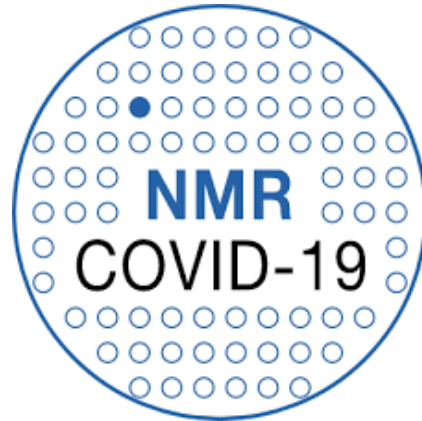
L'importanza della condivisione

- **I biologi strutturali hanno una lunga storia di condivisione dei dati in database pubblici e in condivisione delle risorse;**
- **La tendenza alla condivisione e le piattaforme già predisposte dalla comunità per la condivisione dei dati hanno favorito una diffusione ancora più rapida dei dati strutturali durante la pandemia.**



Fondazione
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L'importanza della condivisione

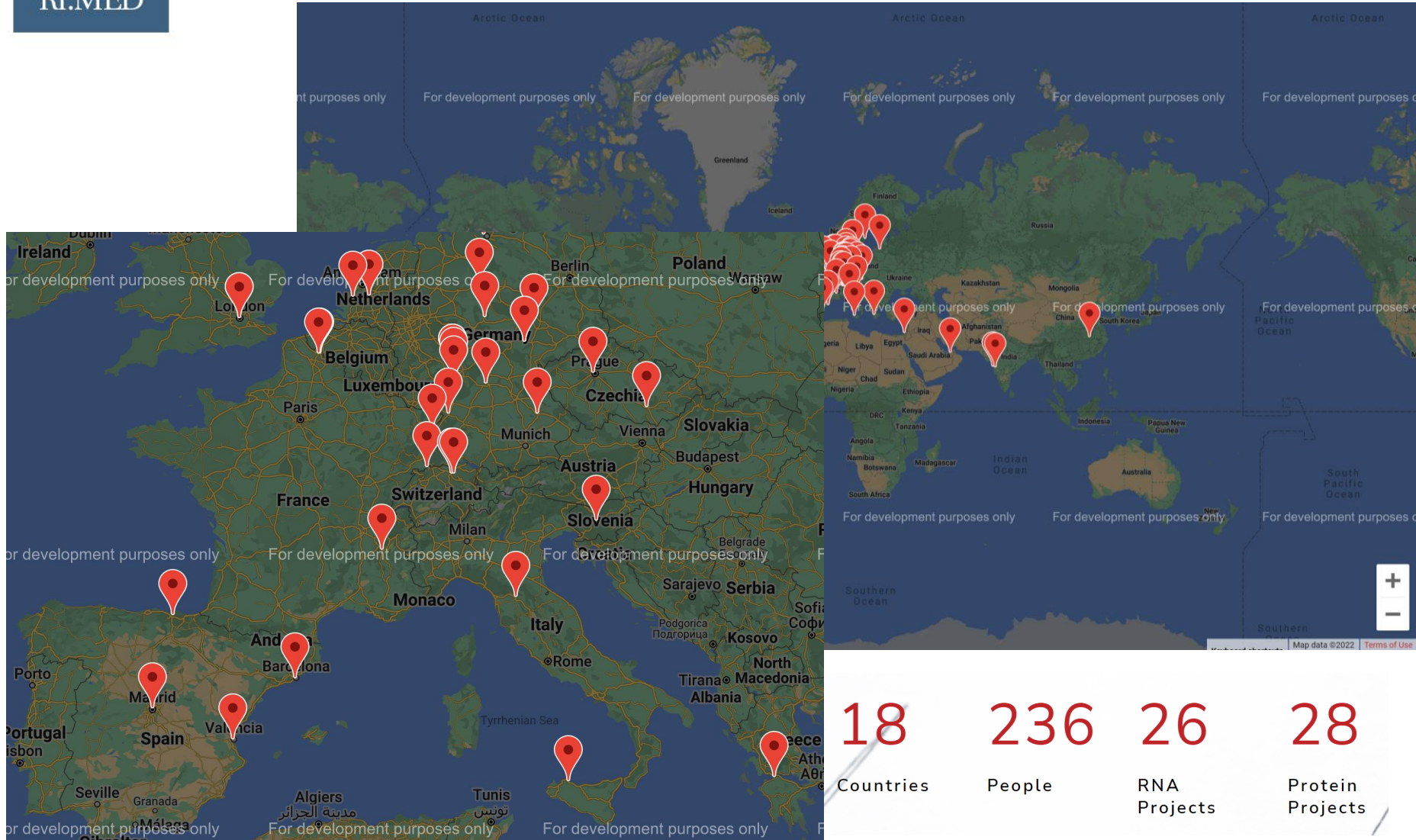


Il Consorzio Covid19-NMR è stato avviato presso l'Università Goethe di Francoforte nel marzo 2020 e da allora è rapidamente cresciuto fino a diventare un consorzio internazionale. Oggi, scienziati di tutto il mondo stanno collaborando in uno sforzo unico per studiare SARS-CoV2 utilizzando la spettroscopia NMR, basata su principi di scienza aperta.



Fondazione
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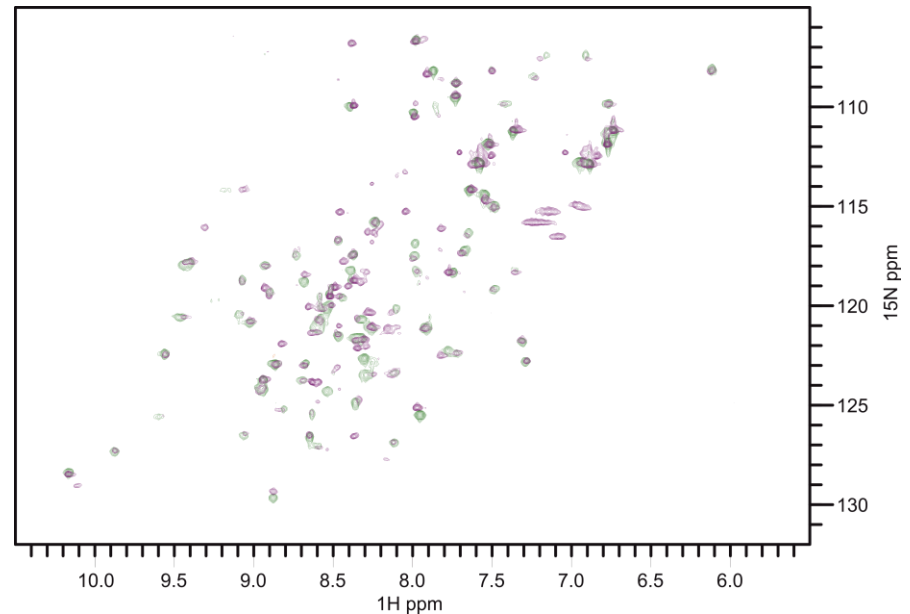
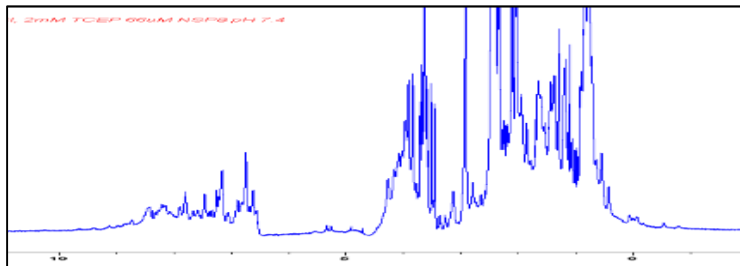
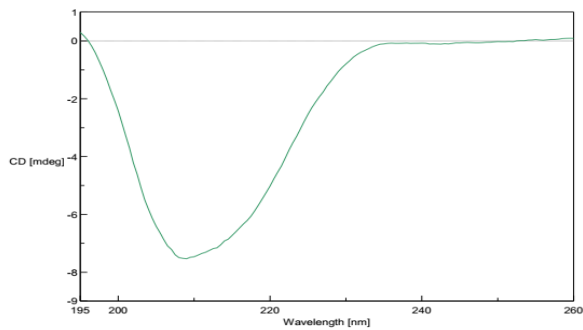
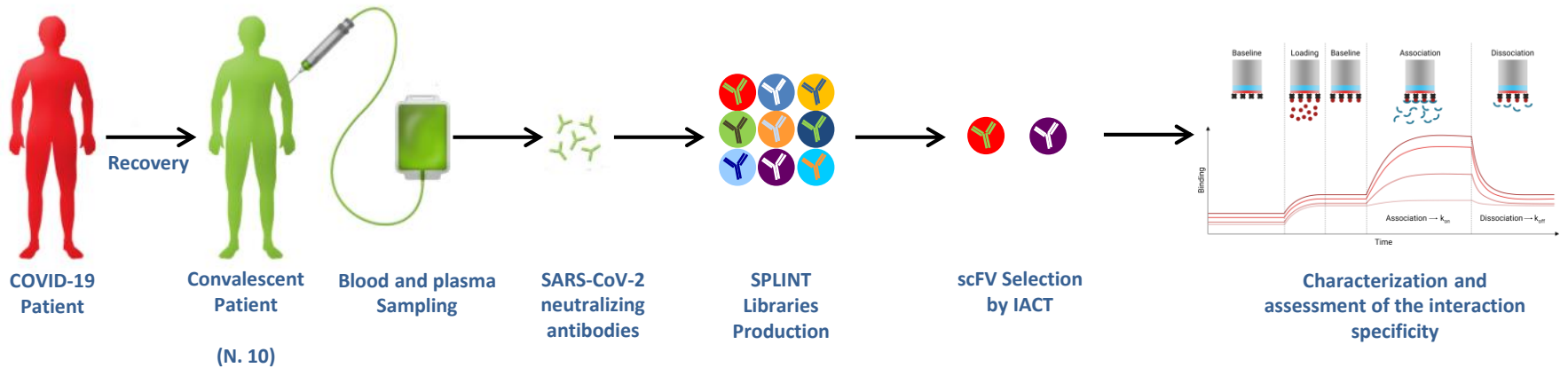
L'importanza della condivisione





Fondazione
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Development of neutralizing antibodies against SARS-CoV-2





I metodi utilizzati in Biologia Strutturale

I biologi strutturali utilizzano diversi metodi per determinare le strutture proteiche che generalmente implicano misurazioni su un gran numero di molecole identiche allo stesso tempo.

- ✓ X-Ray crystallography
- ✓ Nuclear magnetic resonance (NMR)
- ✓ Cryo-electron microscopy (cryo-EM)

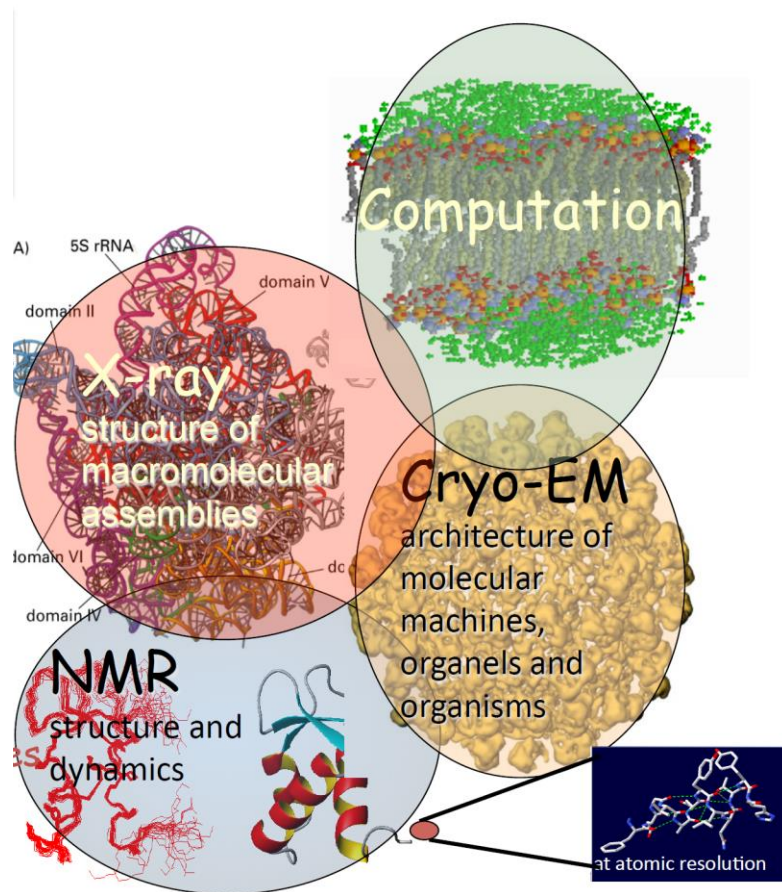
- ✓ Mass spectrometry
- ✓ Electron paramagnetic resonance (EPR)
- ✓ Multiangle light scattering
- ✓ Small angle scattering
- ✓ Circular dichroism



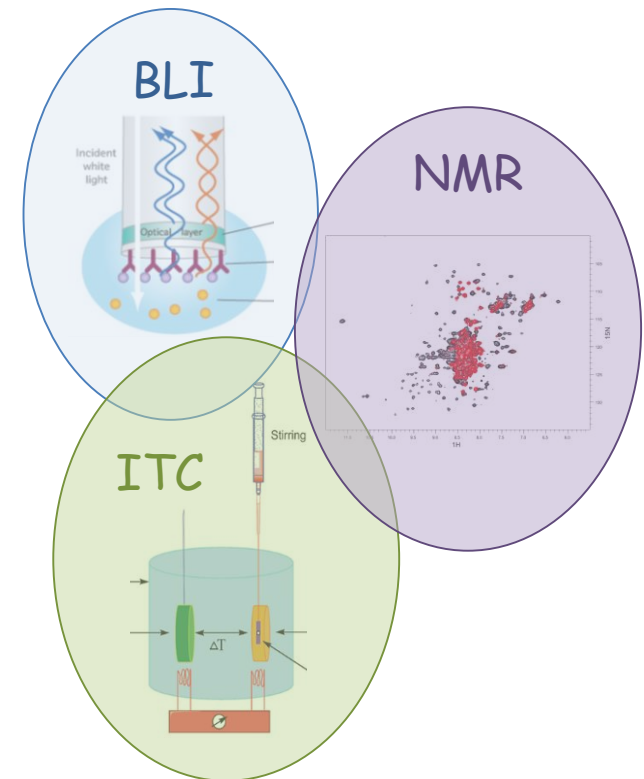
Fondazione
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Approccio Integrato

STRUTTURA DELLA PROTEINE



INTERAZIONI FRA PROTEINE E PROTEINE E PICCOLE MOLECOLE





Fondazione
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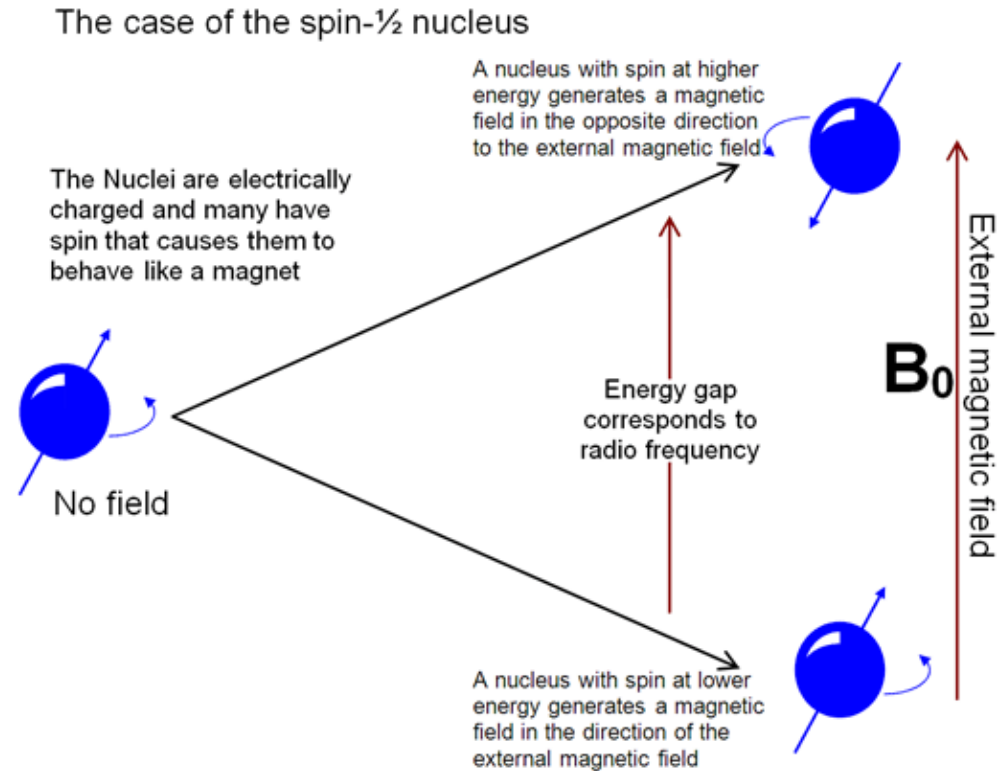
La Risonanza Magnetica Nucleare

L' NMR è un campo della spettroscopia basato sull'assorbimento di radiazioni elettromagnetiche nella regione delle radiofrequenze, 10 MHz-1 GHz

I nuclei hanno spin e sono caricati elettricamente.

Se viene applicato un campo magnetico esterno, è possibile un trasferimento di energia tra l'energia di base a un livello di energia superiore (generalmente un singolo intervallo di energia).

Quando la rotazione ritorna al suo livello di base, l'energia viene emessa alla stessa frequenza.





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L'effetto dell'intorno chimico

La precisa frequenza di risonanza della transizione energetica dipende dal campo magnetico effettivo nel nucleo.

Questo campo è influenzato dalla schermatura elettronica che a sua volta dipende dall'ambiente chimico.

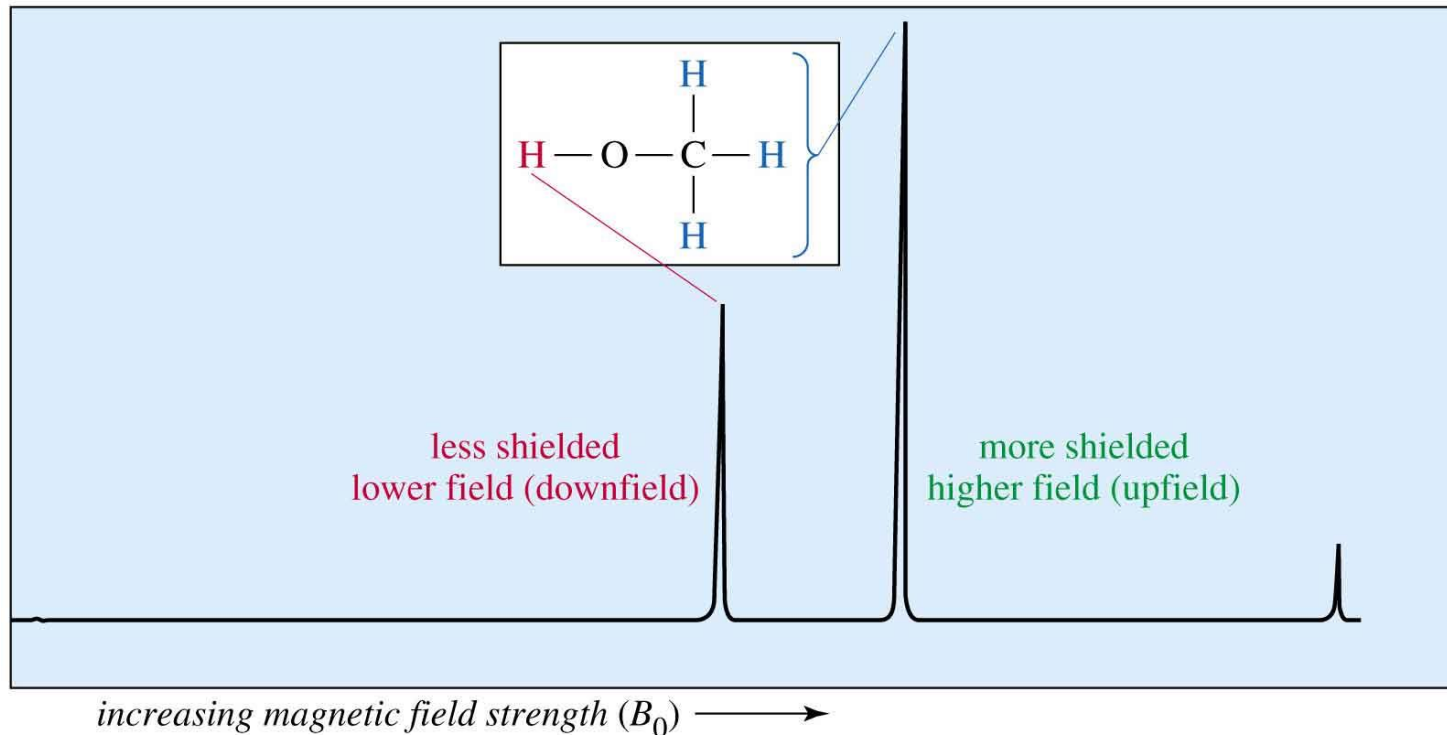
Di conseguenza, le informazioni sull'ambiente chimico del nucleo possono essere derivate dalla sua frequenza di risonanza.

In generale, più elettronegativo è il nucleo, maggiore è la frequenza di risonanza. Altri fattori come le correnti d'anello (anisotropia) e la deformazione del legame influenzano lo spostamento di frequenza.



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L'effetto dell'intorno chimico



Il gruppo metilico (CH₃) è quello che dona meno elettroni e quindi risuona al minimo spostamento chimico.

Il gruppo idrossile (OH) è il più donatore di elettroni, quindi ha il più alto spostamento chimico.



Principali applicazioni dell'NMR

1) Structural (chemical) elucidation

- Natural product chemistry
- Synthetic organic chemistry
 - analytical tool of choice of synthetic chemists
 - used in conjunction with MS and IR

2) Study of dynamic processes

- reaction kinetics
- study of equilibrium (chemical or structural)

3) Structural (three-dimensional) studies

- Proteins, Protein-ligand complexes
- DNA, RNA, Protein/DNA complexes
- Polysaccharides

4) Drug Design

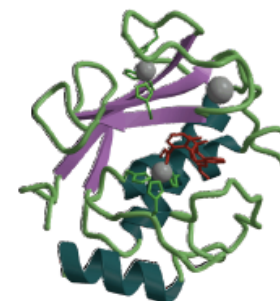
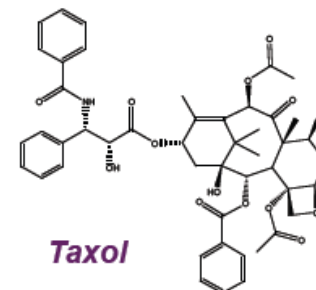
- Structure Activity Relationships by NMR

5) Solid State NMR

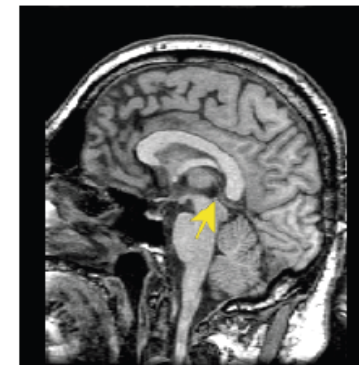
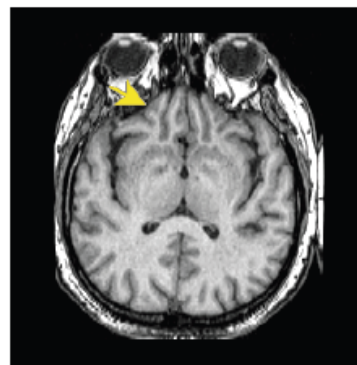
6) Medicine

- MRI
- Metabolomics

MRI images of the Human Brain



NMR Structure of MMP-13 complexed to a ligand





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Routine o non routine

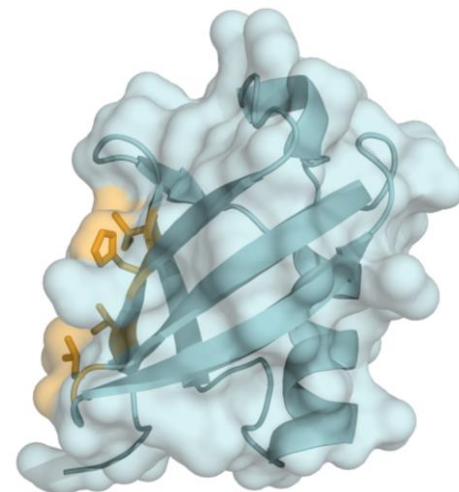
NMR è una tecnica di routine in chimica organica

Tecnica di chimica analitica utilizzata nel controllo di qualità, per determinare il contenuto e la purezza di un campione, nonché la sua struttura molecolare.

NMR NON è una routine per le macromolecole

```
      10           20           30           40  
MQIFVKTLTG  KTITLEVEPS  DTIENVKAKI  QDKEGIPPDQ  
  
      50           60           70  
QRLIFAGKQL  EDGRTLSDYN  IQKESTLHLV  LRLRGG
```

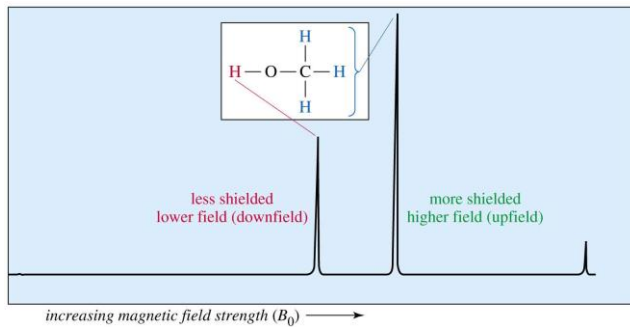
Formula: $C_{378}H_{629}N_{105}O_{118}S_1$
Total number of atoms: 1231



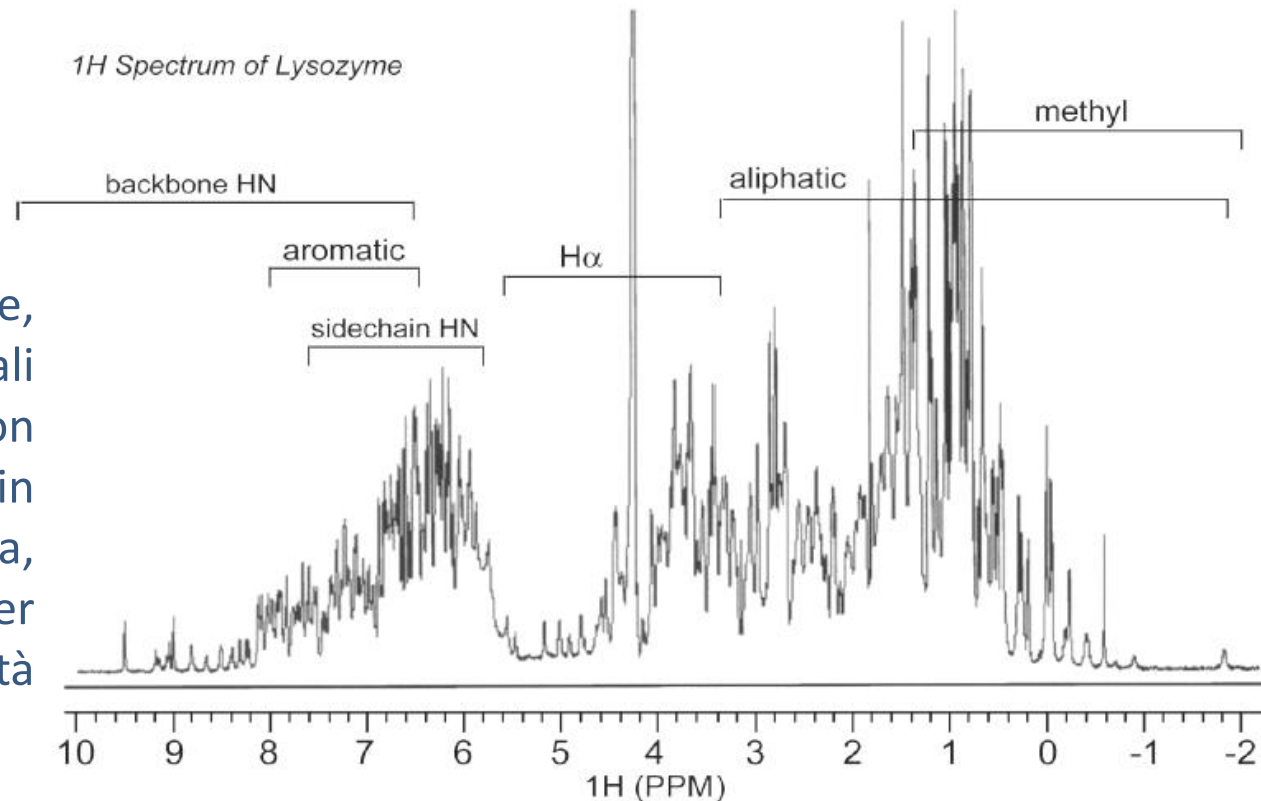


Fondazione
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Esperimento NMR monodimensionale di una proteina



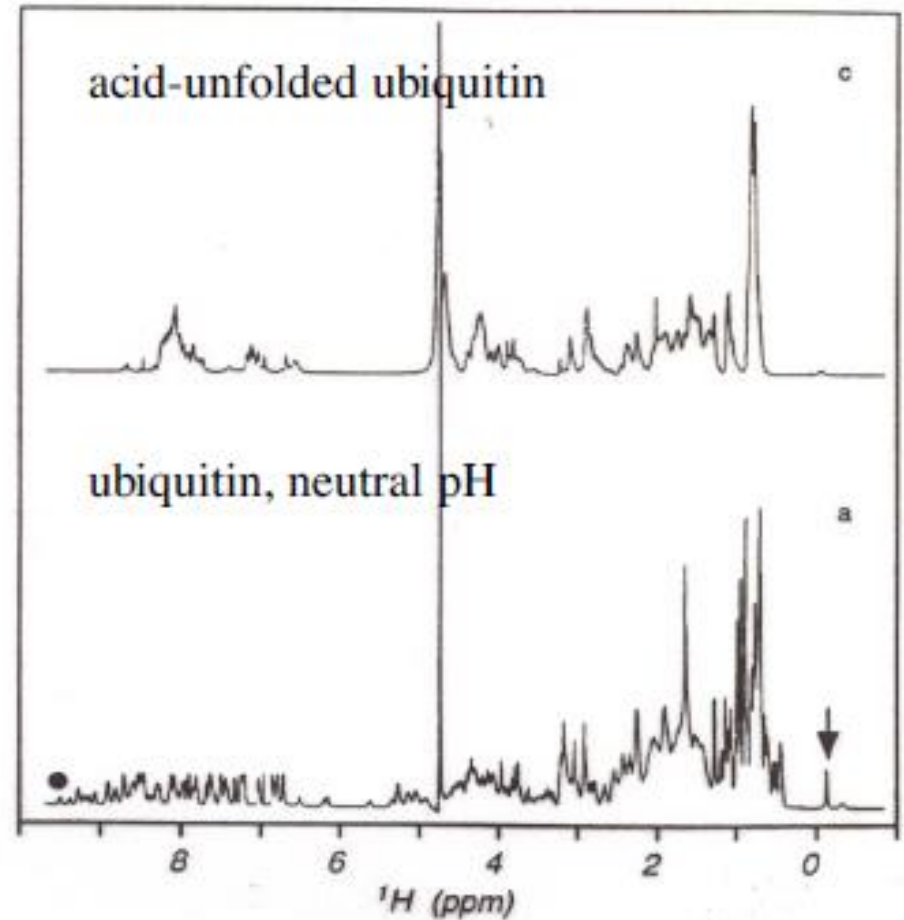
Anche per le piccole proteine, gli spettri monodimensionali sono complicati e non possono essere analizzati in modo completo. Tuttavia, possono essere utili per valutare l'idoneità/stabilità del campione proteico





NMR in solution to solve the 3D structure of proteins: 1D EXPERIMENTS MATTER TOO

- ✓ Per le proteine prive di struttura, i picchi sono generalmente ampi (molti protoni in ogni picco) e c'è una scarsa dispersione dello spostamento chimico
- ✓ Per le proteine correttamente ripiegate i picchi dovrebbero essere nitidi e lo spettro dovrebbe mostrare una buona dispersione dei segnali



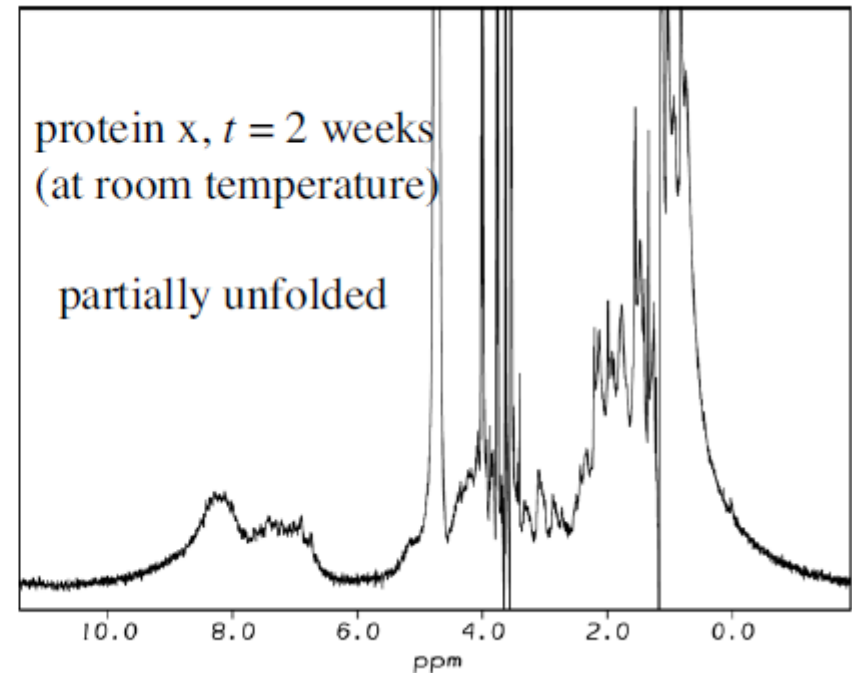
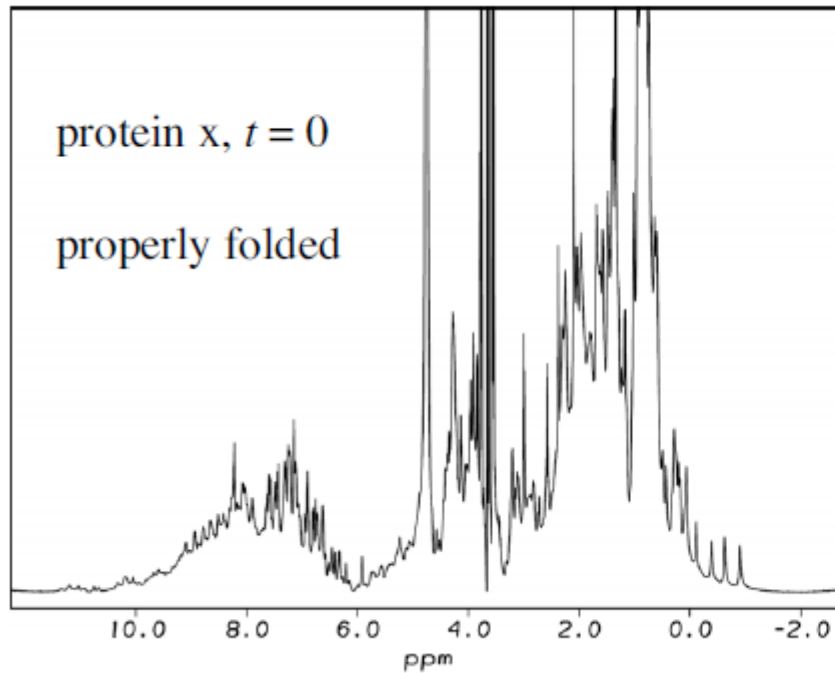


Fondazione
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NMR in solution to solve the 3D structure of proteins: 1D EXPERIMENTS MATTER TOO

✓ E' fondamentale controllare la stabilità di una proteina

Possono essere necessarie settimane di tempo dello strumento per acquisire tutti i dati per la determinazione della struttura → IL CAMPIONE DEVE ESSERE STABILE PER IL TEMPO NECESSARIO E ALLA TEMPERATURA DI ACQUISIZIONE DEI DATI

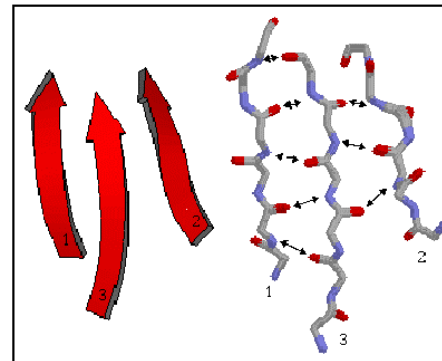
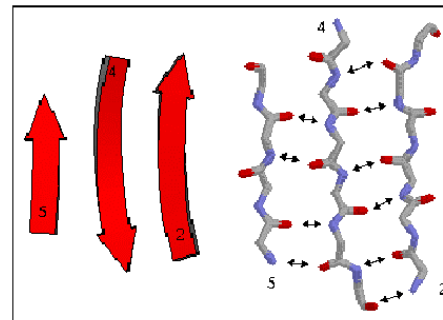
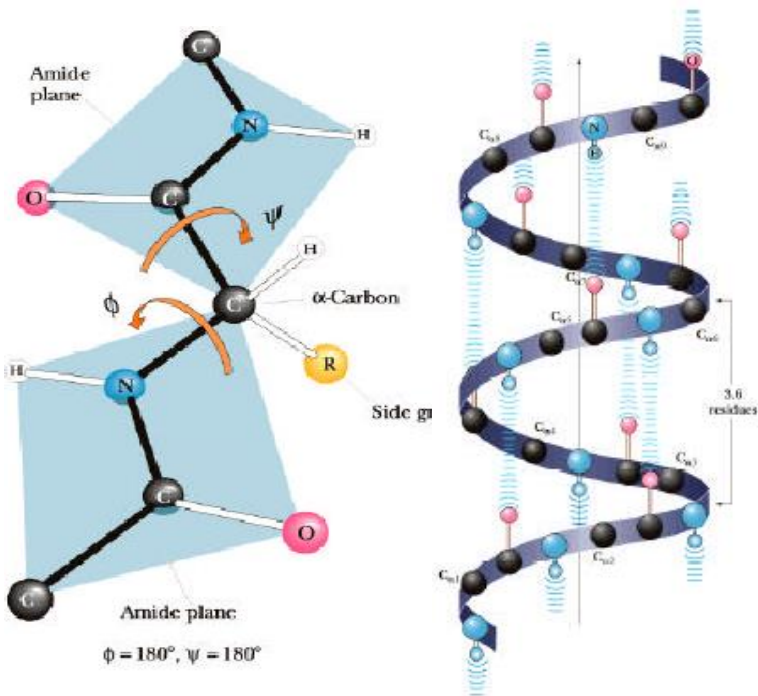




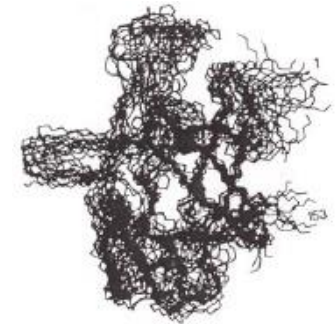
Fondazione
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NMR in solution to solve the 3D structure of proteins

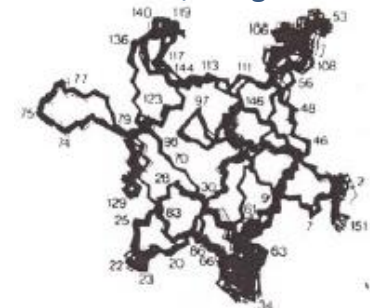
Il processo di determinazione di una struttura mediante NMR consiste nel misurare il maggior numero possibile (centinaia/migliaia) di brevi distanze e angoli protone-protone.



536 distance restraints
RMSD = 2 Angstroms



2780 distance restraints
RMSD = 0,4 Angstroms



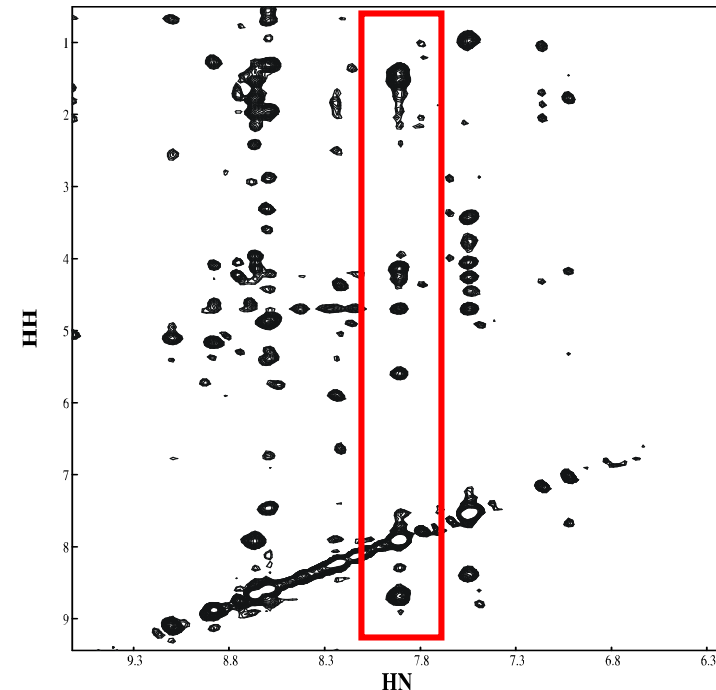


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NMR in solution to solve the 3D structure of proteins

L'NMR fornisce un mezzo per determinare se due protoni in una proteina sono distanti ≤ 5 Angstrom. L'esperimento di base utilizzato si chiama NOESY

L'esperimento NOESY si basa sul Nuclear Overhauser Effect, il cambiamento (aumento) dell'intensità del segnale da un dato nucleo come risultato dell'eccitazione o della saturazione della frequenza di risonanza di un altro nucleo → L'entità dell'effetto dipende dalla distanza.





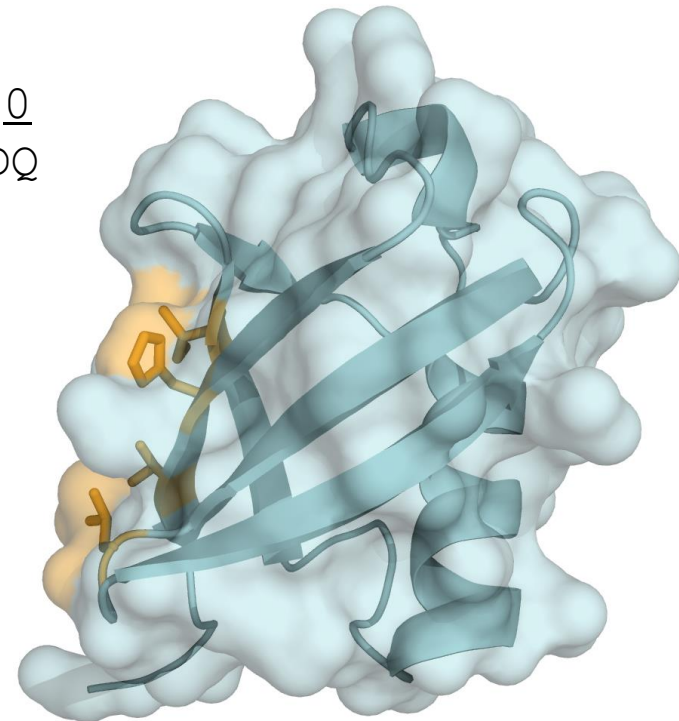
Fondazione
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NMR in solution to solve the 3D structure of proteins

Per misurare le distanze, dobbiamo scoprire quali atomi danno origine ai segnali negli spettri → DOBBIAMO ASSEGNARE LA PROTEINA (determinare le risonanze per tutti gli atomi).

<u>10</u>	<u>20</u>	<u>30</u>	<u>40</u>
MQIFVKTLTG	KTITLEVEPS	DTIENVKAKI	QDKEGIPPDQ
<u>50</u>	<u>60</u>	<u>70</u>	
QRLIFAGKQL	EDGRTLSDYN	IQKESTLHLV	LRLRGG

Formula: $C_{378}H_{629}N_{105}O_{118}S_1$
Total number of atoms: 1231



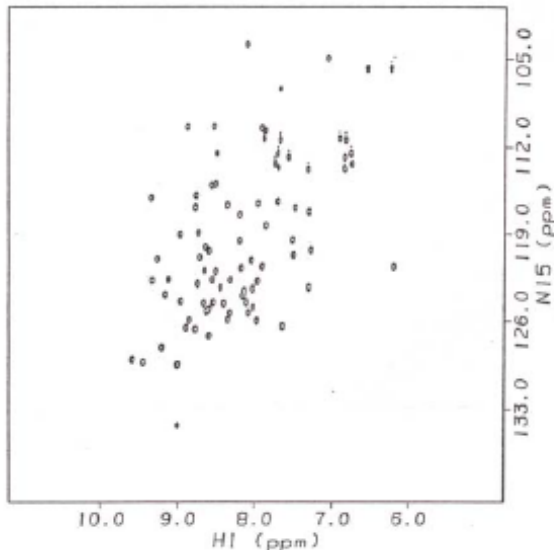


Fondazione
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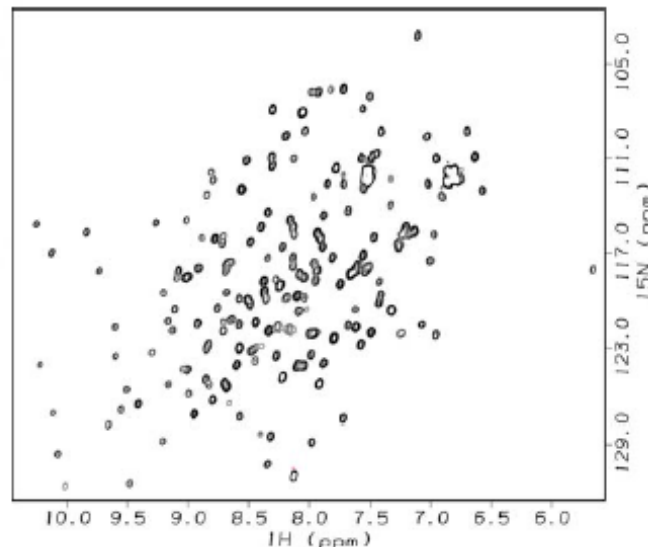
NMR in solution to solve the 3D structure of proteins: SIZE OF THE PROTEIN MATTERS

- ✓ Il tempo di correlazione rotazionale scala con la dimensione della proteina con conseguente ampliamento del picco e diminuzione del rapporto S/N
- ✓ Le proteine più grandi hanno più atomi → più picchi negli spettri → più sovrapposizioni

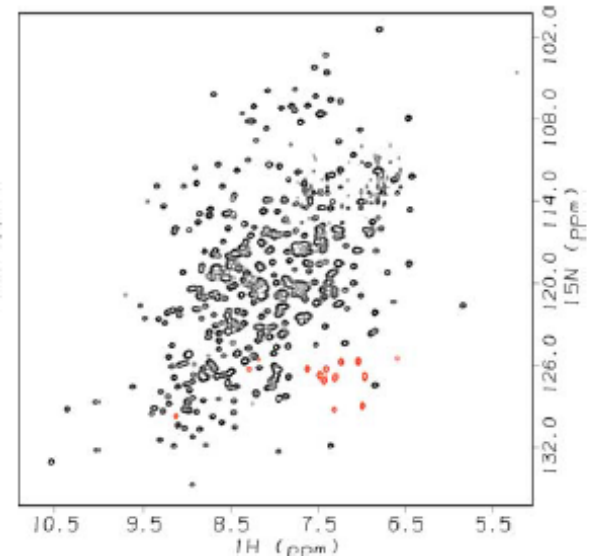
ubiquitin (76 amino acids, 8.5 kDa)



AlgH (189 amino acids, 20.2 kDa)



EPSP synthase (427 amino acids, 46.2 kDa)



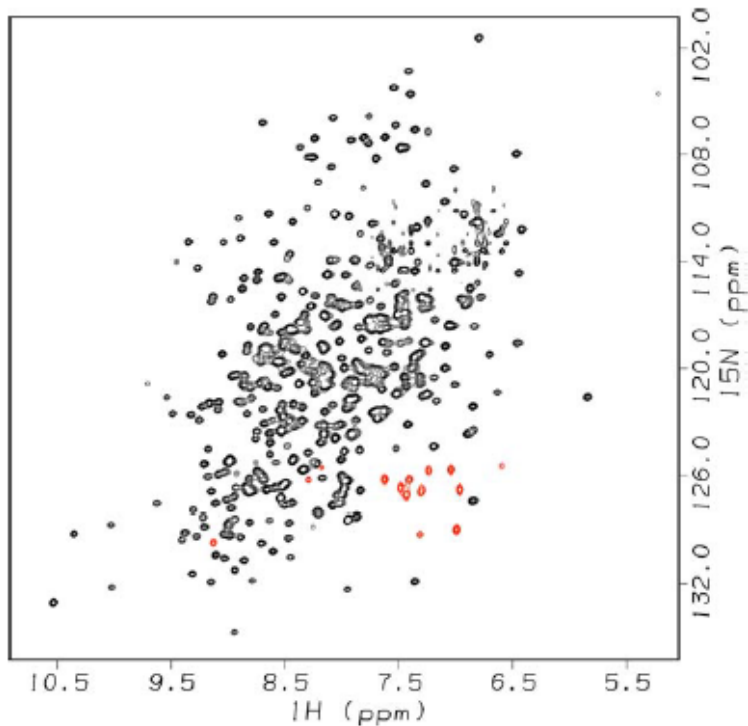


NMR in solution to solve the 3D structure of proteins: SIZE OF THE MAGNET MATTERS

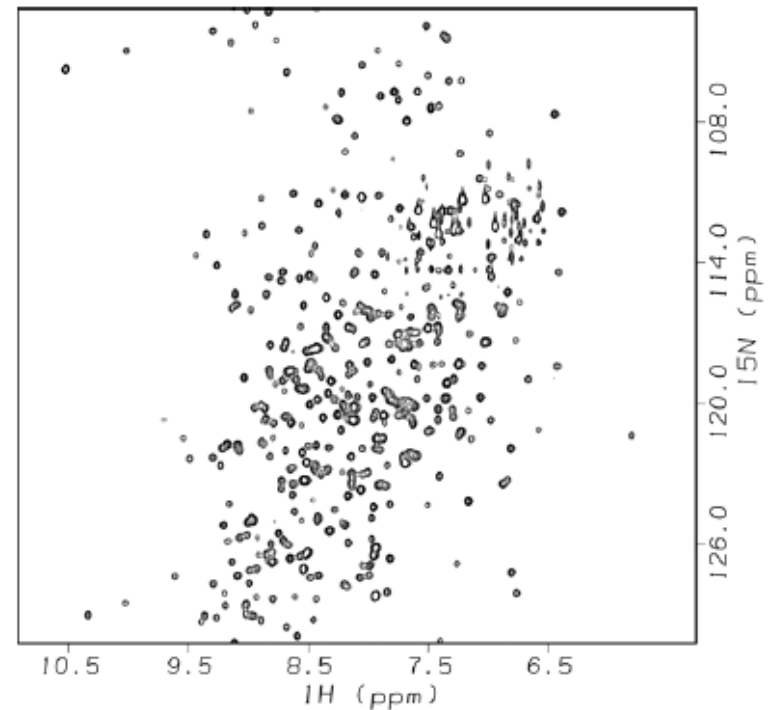
Campo magnetico più alto...

- Maggiore sensibilità (aumento S/N)
- Aumento della risoluzione (diminuzione della sovrapposizione dei picchi)

600 MHz



800 MHz

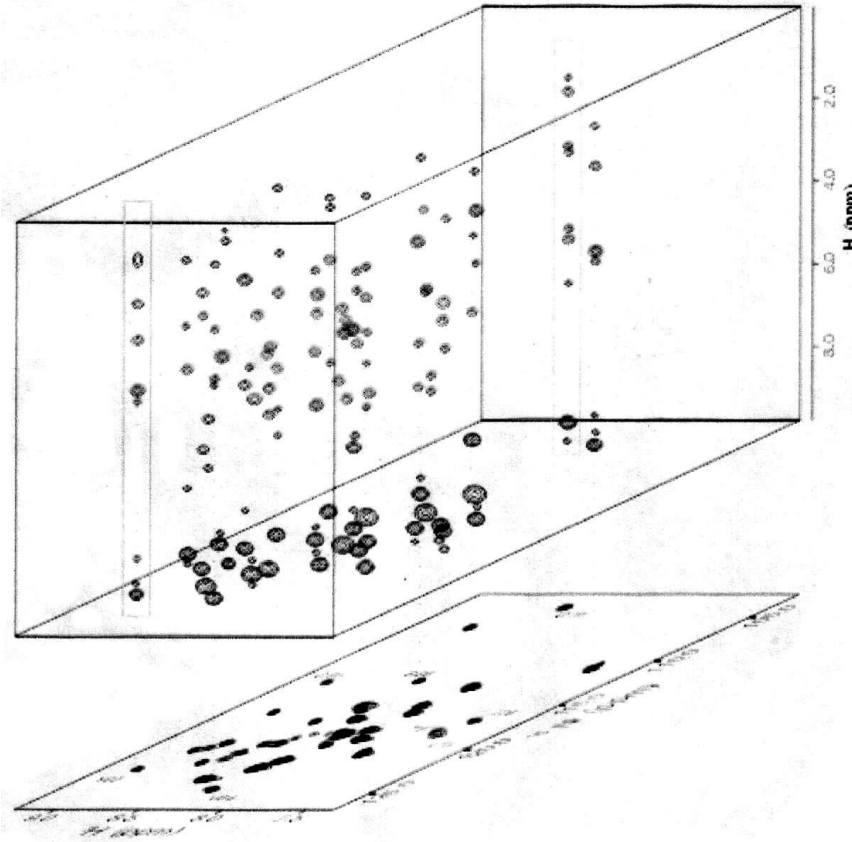




Fondazione
Ri.MED

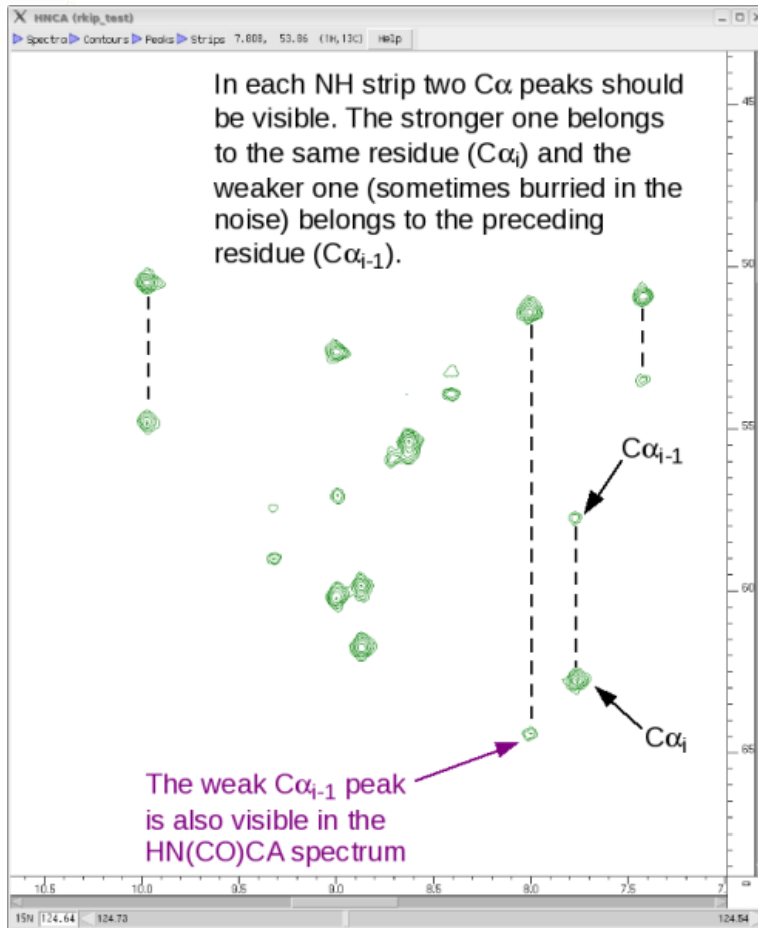
NMR in solution to solve the 3D structure of proteins: NEEDS ISOTOPICALLY LABELLED PROTEINS

I moderni studi NMR delle proteine si basano su esperimenti multidimensionali ed eteronucleari che coinvolgono nuclei ^1H , ^{13}C e ^{15}N . Questi metodi prevedono la selezione del segnale e mezzi per ridurre la sovrapposizione del segnale

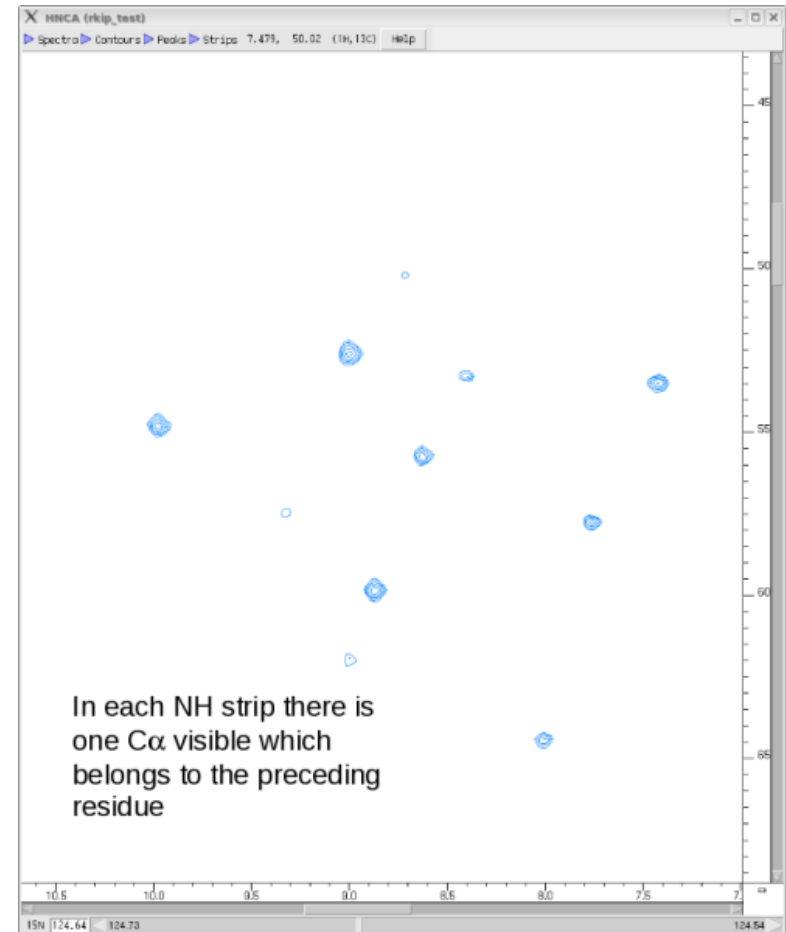




NMR in solution to solve the 3D structure of proteins: SEQUENTIAL ASSIGNMENT



HNCA



HN(CO)CA

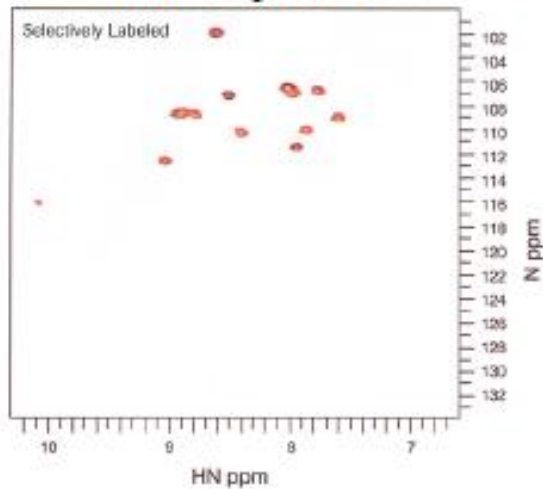


Fondazione
Ri.MED

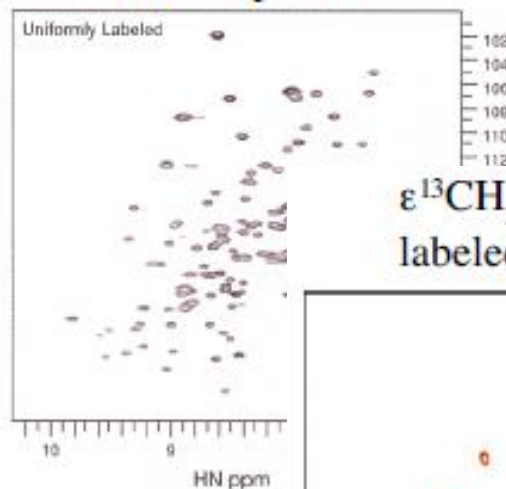
NMR in solution to solve the 3D structure of proteins: NEEDS ISOTOPICALLY SELECTIVELY LABELLED PROTEINS

Marcatura isotopica selettiva per identificare specifici tipi di amminoacidi, gruppi o per assegnare stereospecificità

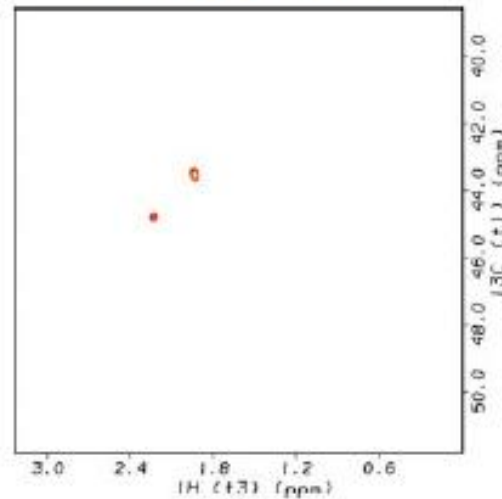
^{15}N -Gly only
labeled protein



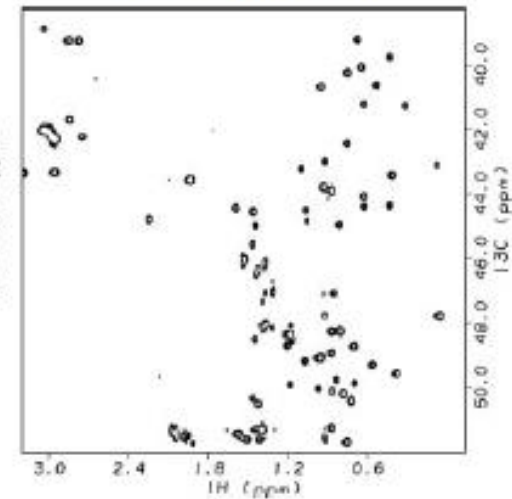
uniformly ^{15}N
labeled protein



ϵ - $^{13}\text{C}_3$ -Met only
labeled protein



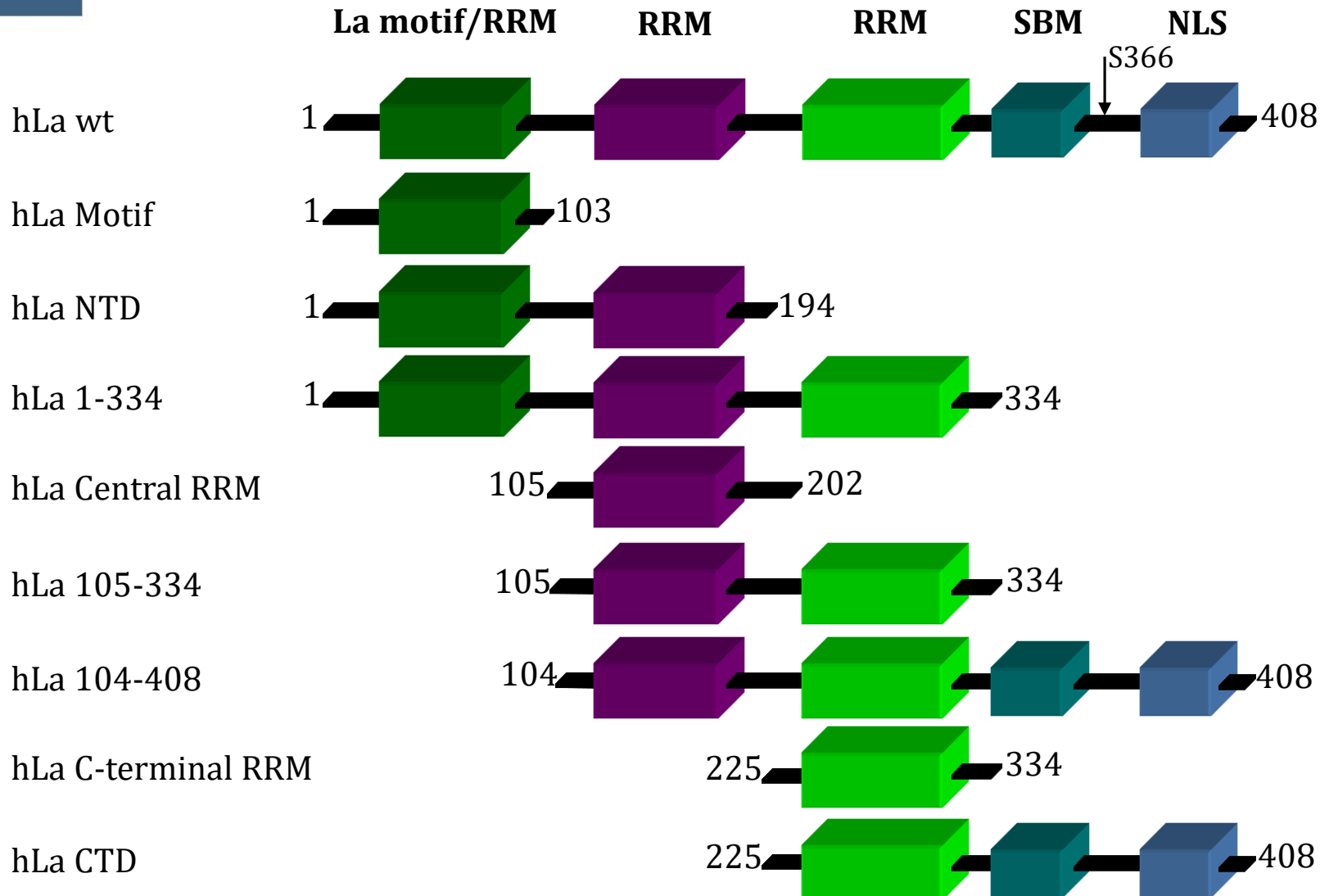
uniformly ^{13}C
labeled protein





Fondazione
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NMR in solution to solve the 3D structure of proteins

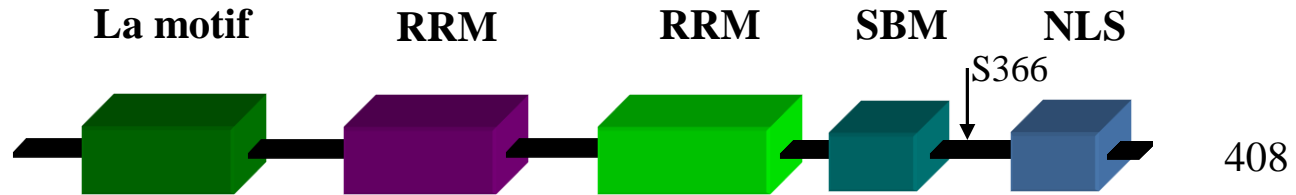




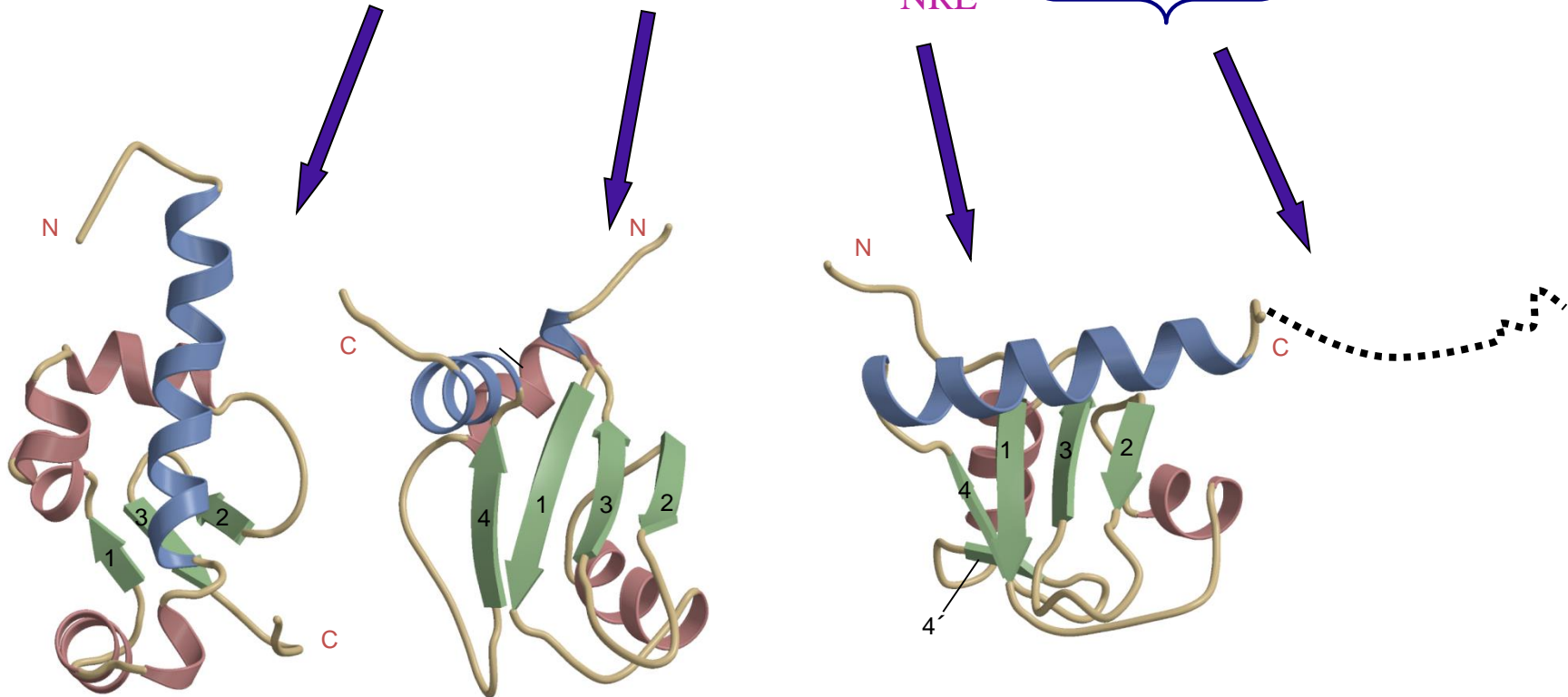
Fondazione
Ri.MED

NMR in solution to solve the 3D structure of proteins

H. sapiens La



NRE

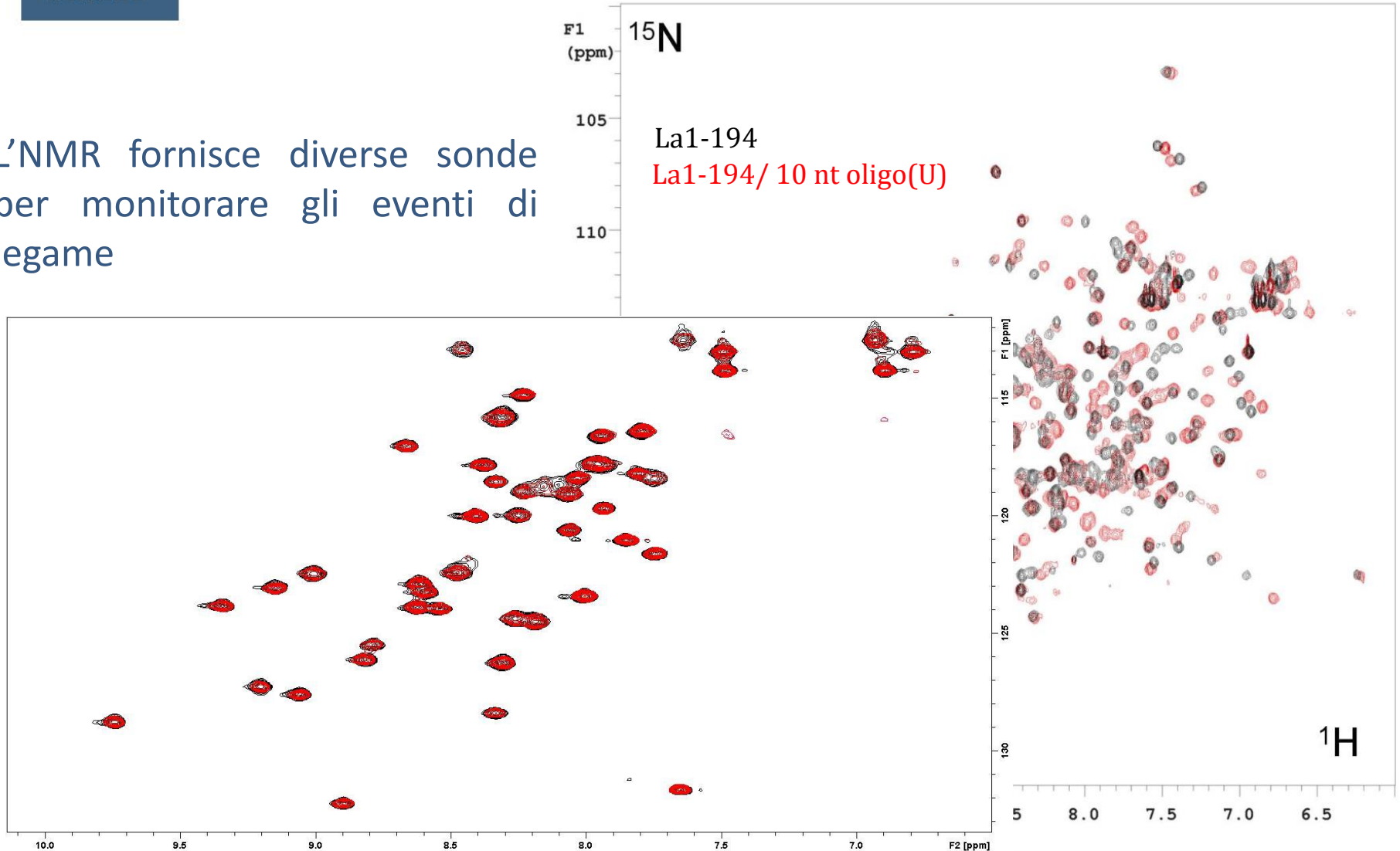




Fondazione
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NMR in solution to investigate binding

L'NMR fornisce diverse sonde per monitorare gli eventi di legame

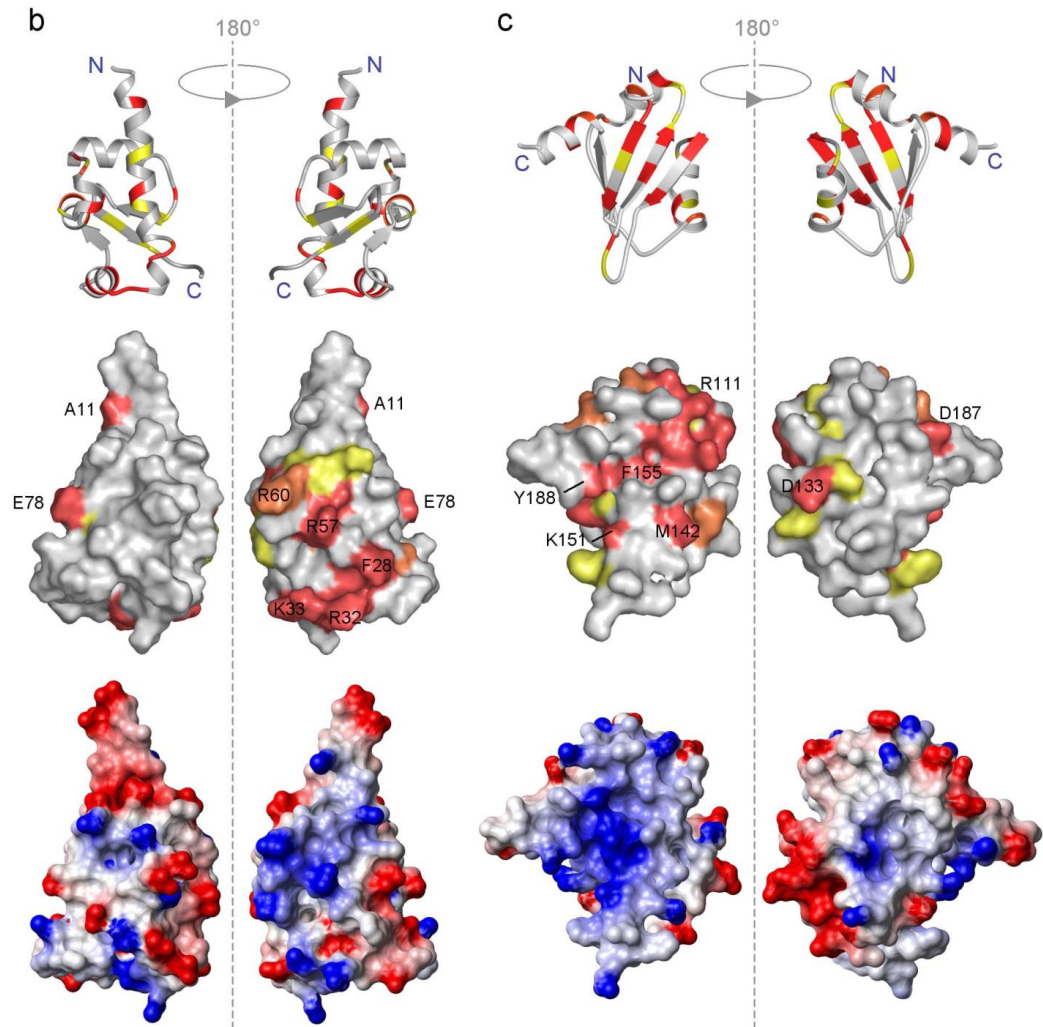




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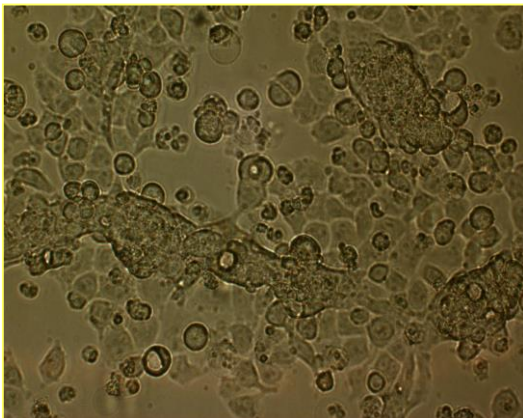


NMR in metabolomica

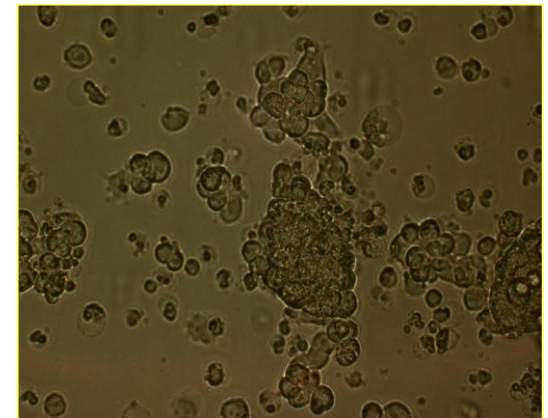
Studi NMR su estratti cellulari per seguire i cambiamenti metabolici che si verificano durante eventi ontogenetici della differenziazione delle cellule beta pancreatiche dalle cellule duttali pancreatiche epiteliali.

Cambiamenti metabolici delle cellule duttali pancreatiche umane in seguito al trattamento con l'ormone lattogeno placentare ricombinante (hPL), hanno suggerito che hPL può essere uno dei più potenti agenti mitogeni delle cellule beta.

PANC-1 cells in basal conditions



PANC-1 cells treated with hPL



← ***Changes in morphology*** →

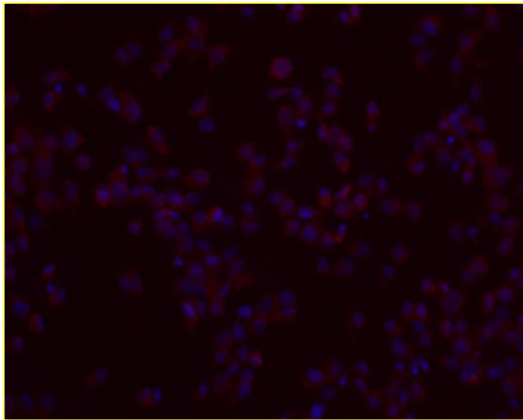


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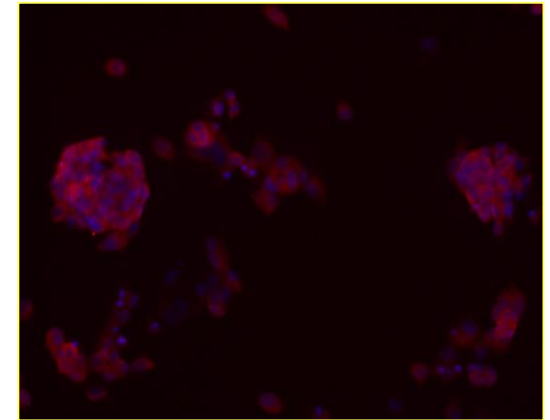
NMR in metabolomica

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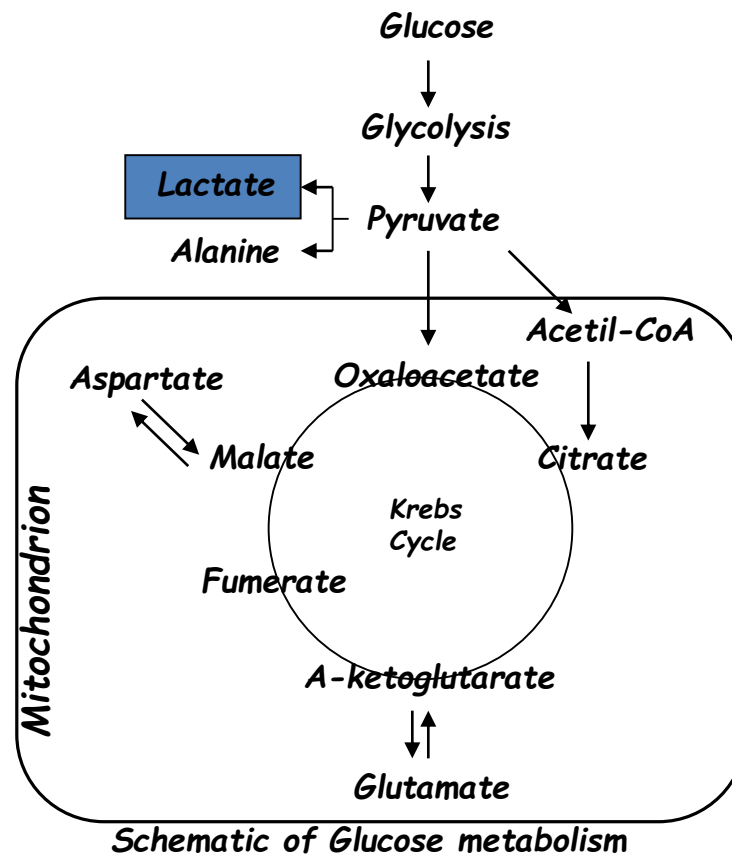
← **Changes in
insulin
expression** →





NMR in metabolomica

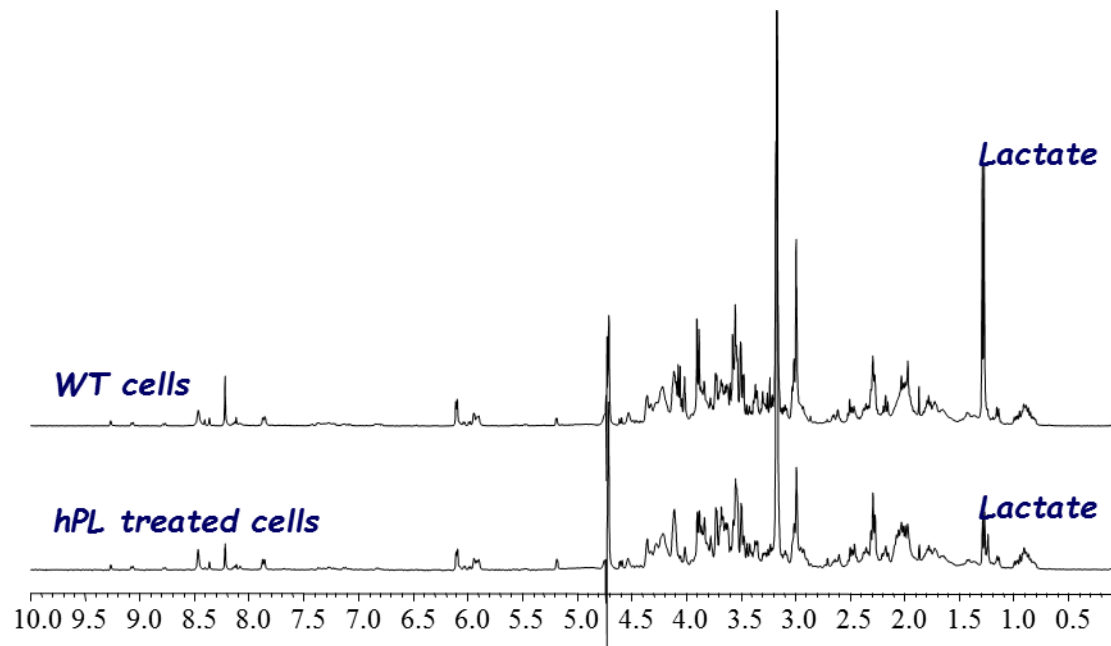
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NMR in metabolomica

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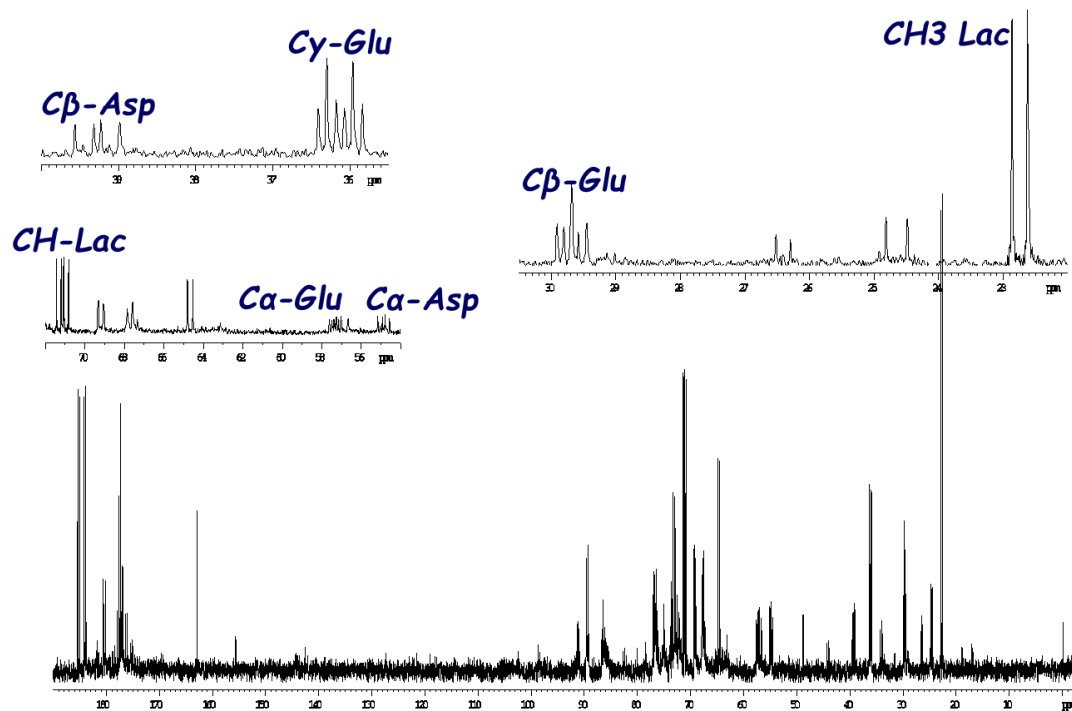


^1H NMR spectra of cellular extracts of human pancreatic ductal cells (PANC-1) in basal conditions (WT) and treated with hPL



NMR in metabolomica

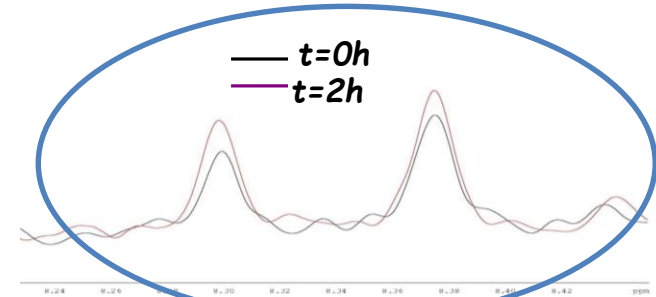
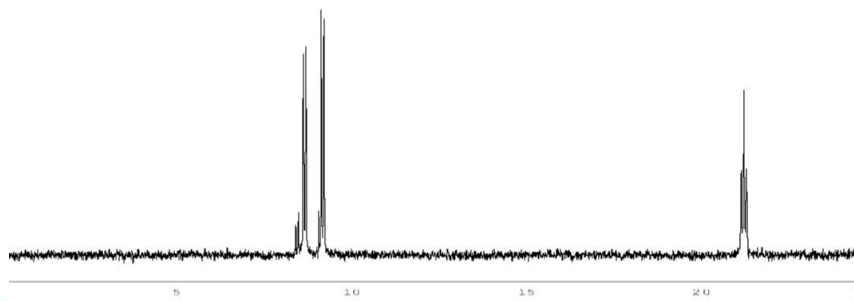
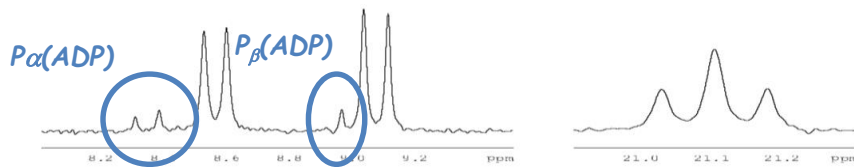
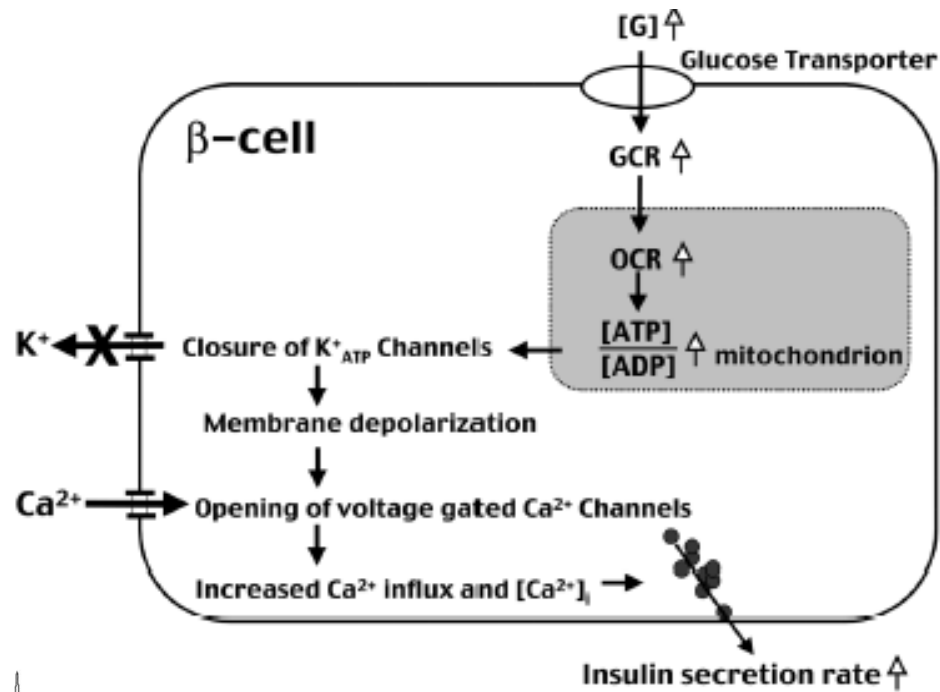
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1D ¹³C spectra with the assignment of metabolites involved in the switch from anaerobic to aerobic metabolism of epithelial ductal cells



NMR to follow and enzymatic reaction





NMR nel processo di scoperta dei farmaci

Lead Generation

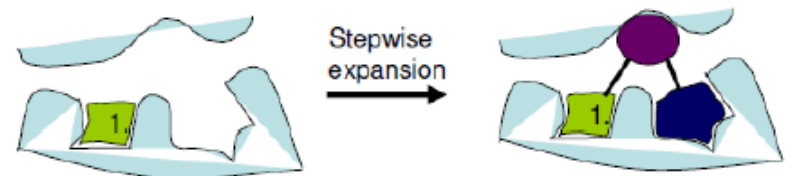
L'identificazione di «hit» nella fase iniziale della scoperta di farmaci che genereranno composti «lead» si effettua generalmente:

- ✓ **High Throughput Screening (HTS)**
- ✓ **Fragment Based Drug Design (FBDD)**

L'HTS prevede lo screening di milioni di composti che si legano al bersaglio nella speranza di trovare lead di farmaci relativamente potenti ($K_d < 1 \mu\text{M}$).



FBDD si basa sullo screening di diverse migliaia di composti semplici nella speranza di trovare leganti a bassa affinità che possono espandersi per generare composti ad alta affinità.





NMR-Based Screening in FBDD

L'NMR è ideale per lo screening di una libreria di composti per un legame debole

✓ Target or receptor-based screening

Interazione ligando/bersaglio seguendo i cambiamenti delle risonanze del target:

- SAR by NMR

✓ Ligand-based screening

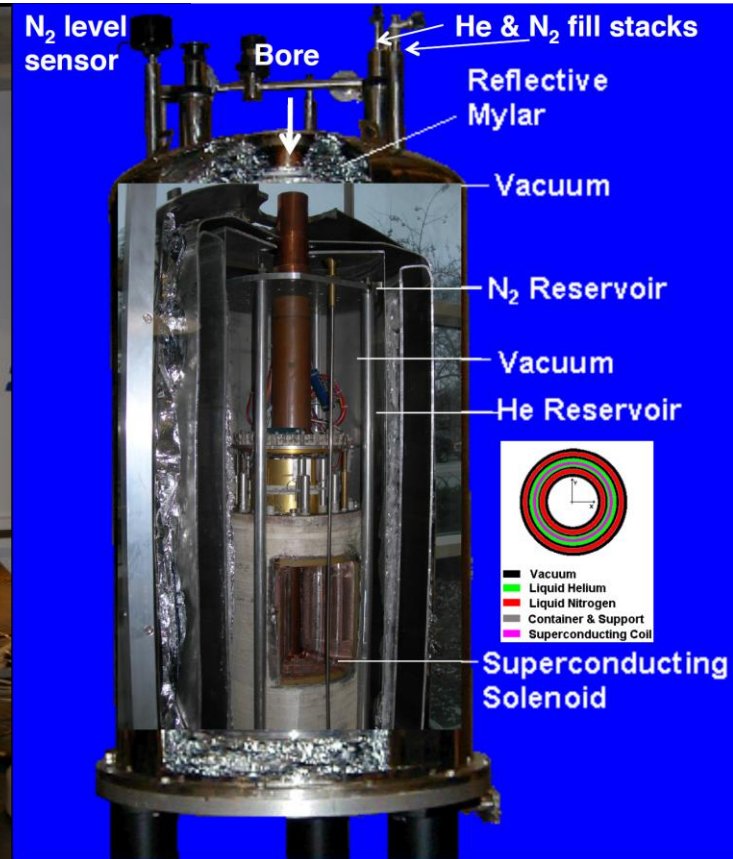
Interazione ligando/target seguendo le modifiche ai parametri NMR del ligando dopo l'aggiunta del target:

- saturation transfer difference (STD)
- relaxation editing
- diffusion editing
- hydration
- NOE based methods



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Spettrometri NMR





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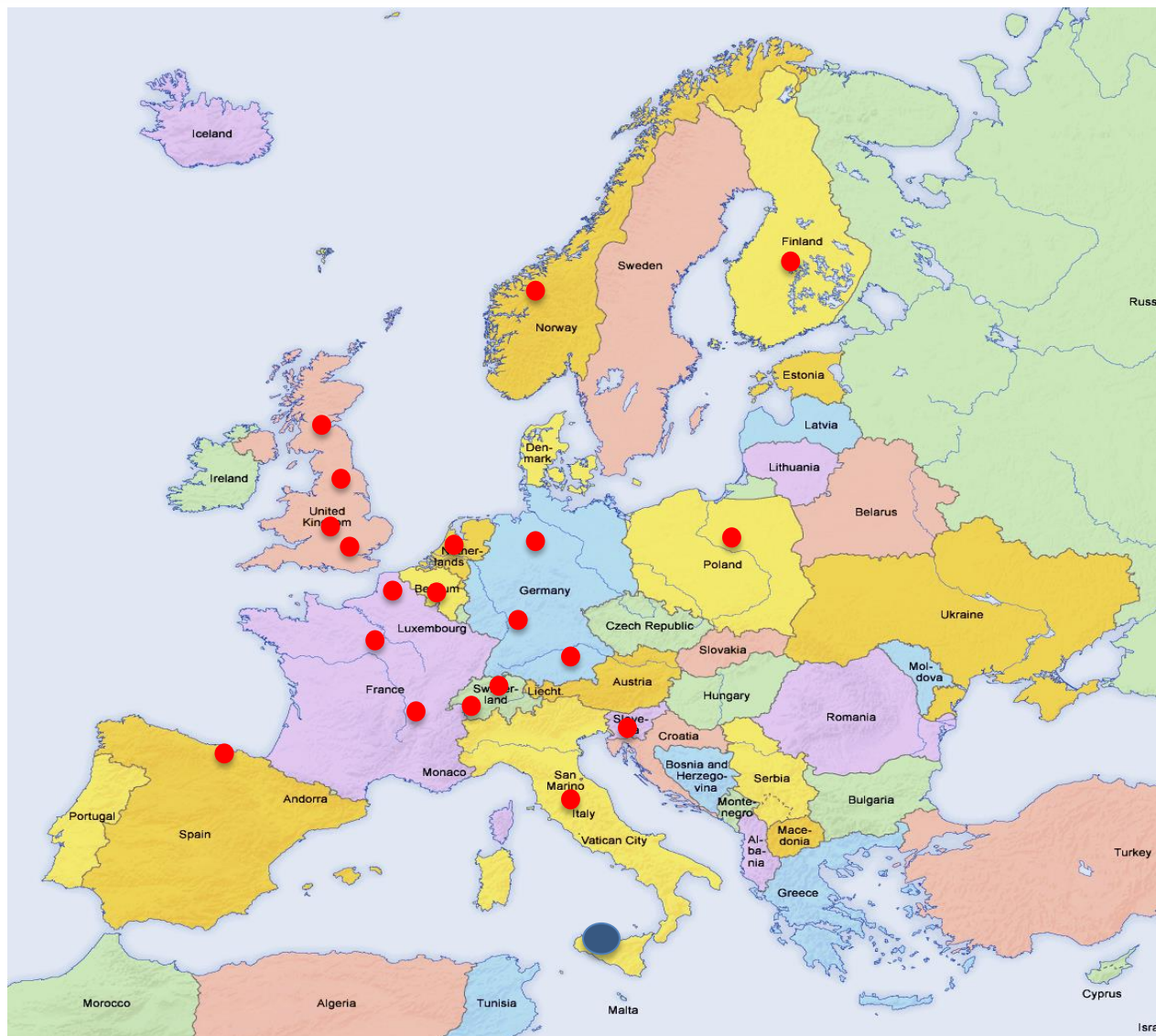
NMR Facility





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Spettrometri NMR ad alto campo in Europa





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**Generation of mussel-inspired bio-adhesives molecules able
to work in wet environment**



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Bioadhesive as an alternative for surgical applications



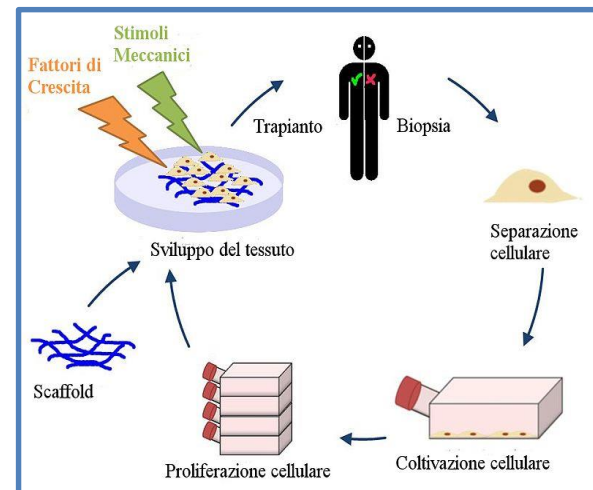
Bone sealing



Medical device



Wound closure



Tissue regeneration



Bio-Adhesives vs Suture

SUTURE DISADVANTAGES

low motility

high infections rate

bleeding

Loss of pus and exudates

BIO-ADHESIVES ADVANTAGES

greater motility

minimizing risk infections

bleeding control

lowering operative time

lowering mortality

reducing cost



Looking for the ideal adhesive

- **BIOCOMPATIBLES**
- **BIODEGRADABLE**
- **MECHANICAL RESISTANCE**
- **BIO-ADHESIVE IN WET ENVIROMENT**



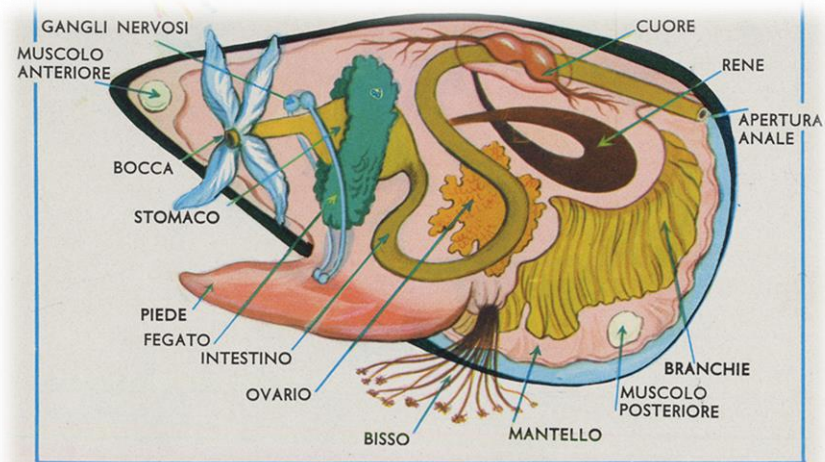
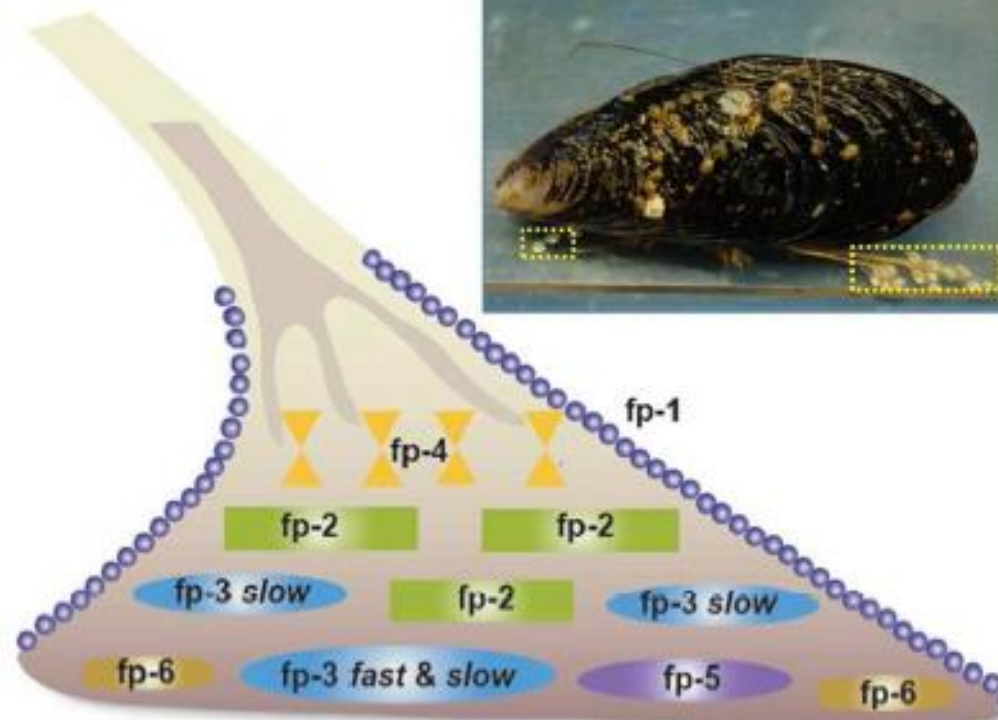
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Sticky Nature





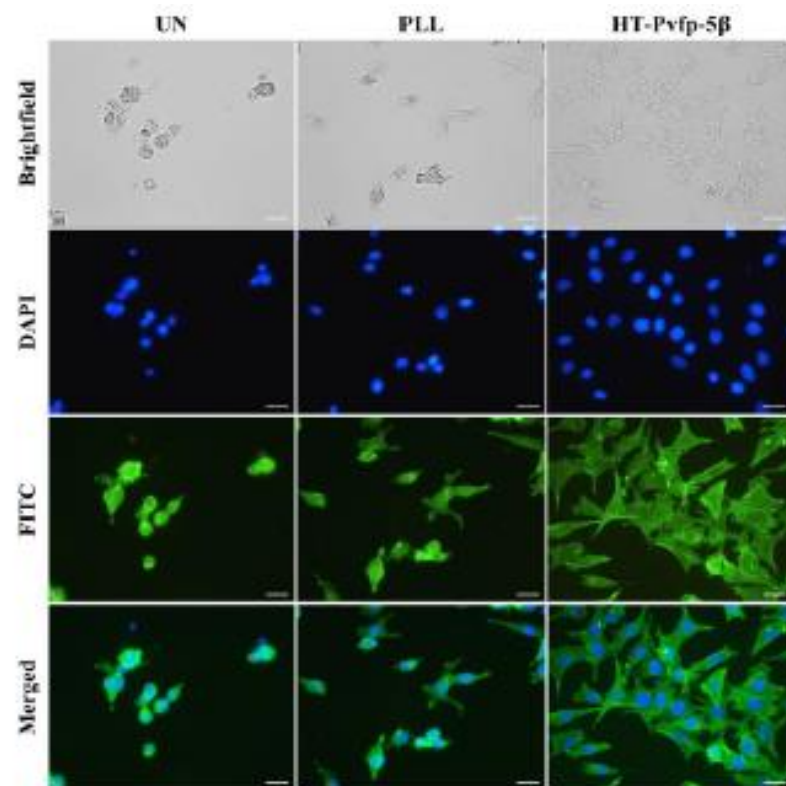
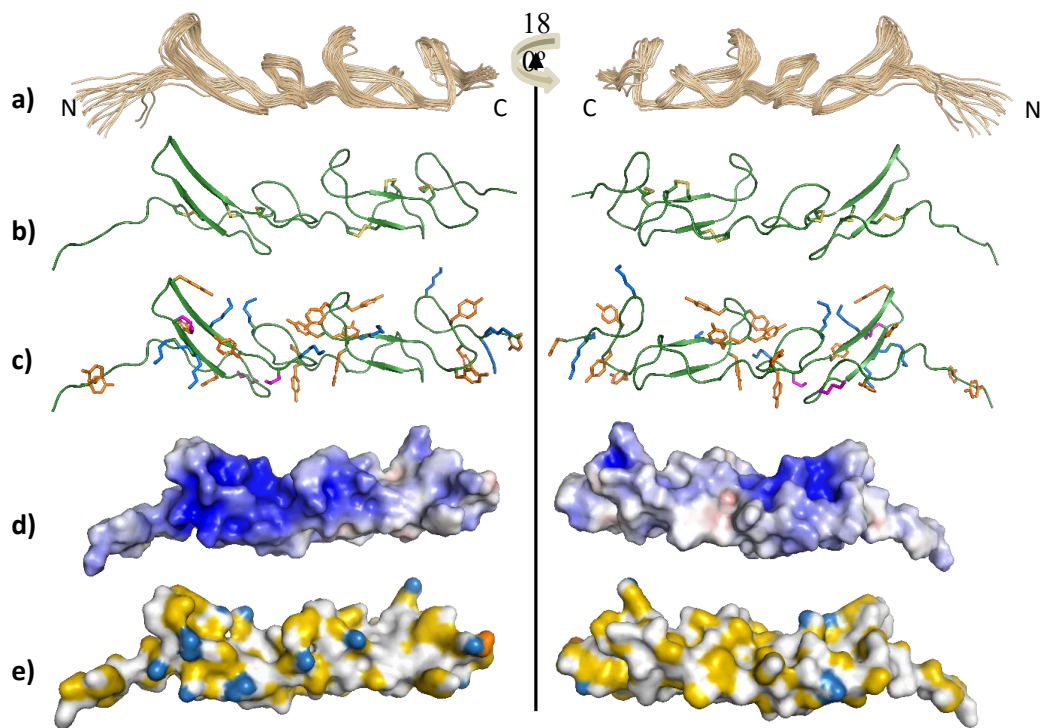
Mussel foot proteins





Pvfp-5 β

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GRAZIE



NMR-Based Screening in FBDD

Saturation transfer difference (STD)

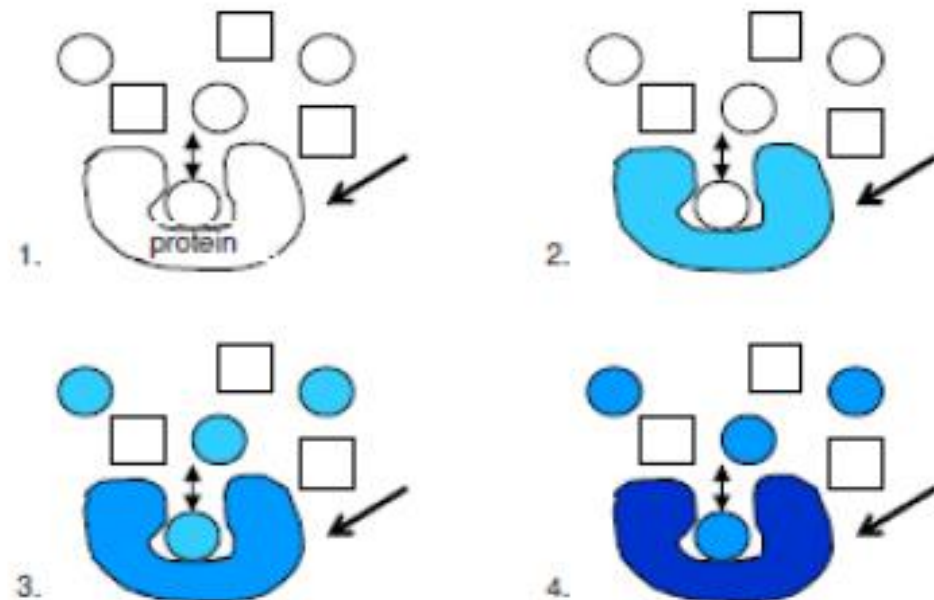
Selectively saturating a resonance that belongs to the receptor



If ligand binds, saturation will propagate from the selected receptor protons to other protons of receptor via spin diffusion



Saturation is transferred to binding compounds by cross relaxation at the ligand-receptor interface.





NMR-Based Screening in FBDD

Saturation transfer difference (STD)

The resulting difference spectrum yields only those resonances that have experienced saturation:

- ✓ Those of the receptor → The receptor is typically present at very small concentrations so its resonances will not be visible.
- ✓ Those of the compound that binds to the receptor

