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UNIVERSITÀ DEGLI STUDI DI TRIESTE

Dipartimento
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Nuovo studio di fattibilità di uno strumento diagnostico per le malattie rare pediatriche

Nova študija izvedljivosti diagnostičnega orodja za redke bolezni pri otrocih

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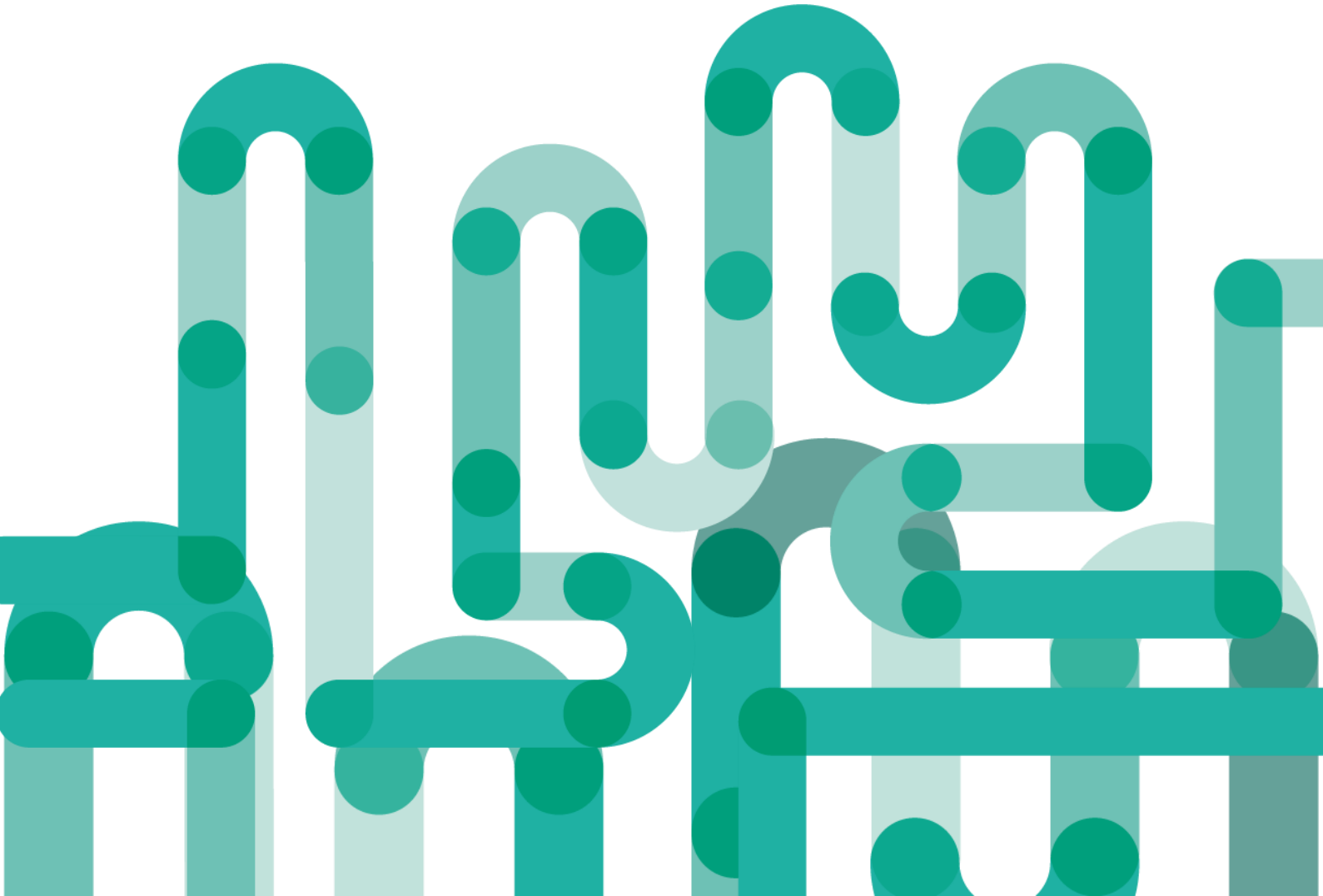

CONCERTO – Nuovo studio di fattibilità di uno strumento diagnostico per le malattie rare pediatriche

Descrizione sintetica del documento

Il documento presenta lo studio di fattibilità condotto nell'ambito del progetto CONCERTO per lo sviluppo di nuovi approcci diagnostici e di monitoraggio terapeutico rivolti a pazienti pediatrici affetti da malattie rare immunomediate. L'attività ha riguardato inizialmente la valutazione di un sistema biosensore in vitro basato su peptidi biotinilati, progettato per misurare l'attività della tirosin-chinasi JAK2 e monitorare gli effetti farmacologici degli inibitori JAK1/JAK2, tra cui ruxolitinib e baricitinib.

I risultati ottenuti hanno confermato l'idoneità dei peptidi studiati come substrati del dominio catalitico di JAK2, evidenziando tuttavia alcune limitazioni nel loro utilizzo con la proteina JAK2 completa nelle condizioni ELISA sperimentate. Lo studio ha pertanto esaminato anche approcci complementari, comprendenti la profilazione trascrittomica di modelli cellulari trattati con inibitori JAK, il monitoraggio della fosforilazione delle proteine STAT in cellule mononucleate del sangue periferico e la valutazione della risposta farmacologica in organoidi intestinali derivati da biopsie pediatriche.

CONCERTO – Nova študija izvedljivosti diagnostičnega orodja za redke bolezni pri otrocih

Kratek opis dokumenta

Dokument predstavlja študijo izvedljivosti, izvedeno v okviru projekta CONCERTO, katere namen je razvoj novih diagnostičnih pristopov in metod spremljanja zdravljenja pri pediatričnih pacientih z redkimi imunsko pogojenimi boleznimi. Dejavnosti so bile sprva usmerjene v vrednotenje in vitro biosenzorskega sistema na osnovi biotiniliranih peptidov, zasnovanega za merjenje aktivnosti tirozinske kinaze JAK2 ter spremljanje farmakoloških učinkov zaviralcev JAK1/JAK2, med katerimi sta ruksolitinib in baricitinib.

Rezultati so potrdili primernost preučevanih peptidov kot substratov katalitične domene JAK2, vendar so pokazali tudi nekatere omejitve pri njihovi uporabi s celotno beljakovino JAK2 v preizkušenih pogojih ELISA. Zato so bili v okviru študije ocenjeni tudi dopolnilni pristopi, ki vključujejo transkriptomsko profiliranje celičnih modelov, zdravljenih z zaviralci JAK, spremljanje fosforilacije proteinov STAT v mononuklearnih celicah periferne krvi ter vrednotenje farmakološkega odziva v črevesnih organoidih, pridobljenih iz pediatričnih biopsij.

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Nel complesso, le attività svolte pongono le basi per lo sviluppo di strumenti utili alla valutazione della risposta agli inibitori JAK e alla futura personalizzazione delle strategie terapeutiche destinate ai bambini affetti da malattie rare immunomediate.

Izvedene dejavnosti predstavljajo osnovo za nadaljnji razvoj orodij za vrednotenje odziva na zaviralce JAK in za prihodnje prilagajanje terapevtskih strategij otrokom z redkimi imunsko pogojenimi boleznimi

Title: New feasibility study of a diagnostic tool for rare diseases in children

The project aimed to develop an innovative biosensor system for *in vitro* diagnostics, designed to measure the aberrant JAK tyrosin kinase activity in primary cells from pediatric patients affected by rare immune-mediated diseases and to monitor the pharmacological effects of JAK1/JAK2 inhibitors (JAKi), such as ruxolitinib and baricitinib, in these patients. In addition to the biosensor system, we have evaluated also alternative *in vitro* approaches to monitor JAKi response.

The biosensor system relies on a solid-phase ELISA assay carried out with a biotinylated peptide probe, i.e., the biosensor, whose amino acid sequence contains a single tyrosine residue that serves as a specific phosphorylation site for JAK. The biosensor is immobilized onto streptavidin-coated plates; after incubation with the kinase source, the tyrosine residue on the bisoensor is phosphorylated and the resulting phosphotyrosine can be detected by the ELISA assay using an anti-phosphotyrosine as primary antibody, thus allowing a indirect measurement of the kinase activity.

To evaluate the performance of the biosensor system, proof-of-concept optimization was carried out for JAK2 kinase using two biotinylated peptide biosensors,$P_{JAK2\text{-}S}$ and $P_{JAK2\text{-}L}$. These peptide sequences are published in literature ($P_{JAK2\text{-}S}$, LAKAVDGYVKPQI and $P_{JAK2\text{-}L}$, GGDNDPDEYITLDEDGGKK) and include specific tyrosine motifs to detect the activity of JAK2 kinase. Both peptide biosensors were purchased by JPT Peptide Technologies; peptide stability over time was confirmed by Vanquish™ Neo ultra-high performance liquid chromatography coupled with Orbitrap Exploris™ 240 Mass Spectrometer available at the University of Trieste, detecting the expected m/z signals (retention time $P_{JAK2\text{-}S}$, 8.5 min; $P_{JAK2\text{-}L}$ 9.9 min). Molecular dynamics simulations, performed in collaboration with Prof. Laurini (University of Trieste), confirmed the possibile binding of both peptide biosensors to the catalytic domain of JAK2 (JAK2-JH1). $P_{JAK2\text{-}S}$ demonstrated a slightly more favorable binding enthalpy ($\Delta H_{total} = -19.9 \pm 0.4$ kcal/mol) compared to $P_{JAK2\text{-}L}$ ($\Delta H_{total} = -17.9 \pm 0.6$ kcal/mol), suggesting a marginally stronger interaction with the kinase target. To experimentally validate these *in silico* results, the ADP-Glo™ kinase luminescent assay was used to detect the amount

of ADP generated by the incubation of the peptide biosensors with JAK2-JH1. A significant increase in ADP was observed in the co-incubation with $P_{JAK2\text{-}S}$ at 10 µM or $P_{JAK2\text{-}L}$ at 20 µM (median ± SEM values were
21.0 ± 3.0 for JAK2-JH1 alone versus 190.3 ±
44.75 and 182.5 ± 18.06 for $P_{JAK2\text{-}S}$ and $P_{JAK2\text{-}L}$,
respectively, $p < 0.05$; two-way ANOVA with Bonferroni post-test). No increase in ADP production was detected when $P_{PHOSPHOJAK2}$

(phosphrylated analogue of $P_{JAK2\text{-}L}$) or P_{ABL} (a peptide biosensor for the ABL kinase) were used, validating $P_{JAK2\text{-}S}$ and $P_{JAK2\text{-}L}$ as specific JAK2-JH1 substrates.

For the solid-phase ELISA assay, the proof-of-concept optimization was carried out using the whole-cell lysates of immortalized HEL and SET-2 cell lines, as source of full length JAK2 kinase. HEL and SET-2 carried the JAK2 V617F gain-of-function mutation in the homozygous and heterozygous state respectively, which guarantees the presence of an active/phosphorylated form of the JAK2 kinase (phospho-JAK2).

First, HEL and SET-2 whole cell lysates were prepared in non-denaturating condition (*TKB buffer* with mild non-ionic detergent Triton X-100), stored at -80°C for a maximum of 4 months for investigating JAKs protein stability over time by immunoblot analysis. Expression of JAK1, JAK2 and JAK3 as well as phospho-JAK1 and phospho-JAK2 were confirmed in both cell lines in 1-month stored lysates; phospho-JAK3 was not detectable. Levels of phospho-JAK1 decreased over storage time in both cell lines (more than 60% decrease between ~1-month vs ~3-months stored lysates, One-way ANOVA, $p < 0.01$) and become almost undetectable after 3-months storage; similarly, a decreased signal of phospho-JAK2 was observed (more than 60% decrease between ~2.5-months vs ~3.5-months stored lysates, $p < 0.0001$). These findings suggest that HEL and SET2 whole cell lysate should be tested within 1-month

from preparation to guarantee the presence of the phospho-JAK2 protein. Several rounds of ELISA were perfomed using these whole cell lysates without detecting a phosphorylation of $P_{JAK2\text{-}S}$ or $P_{JAK2\text{-}L}$. To ensure that the lack of phosphorylation was not due to a low amount of active JAK2 in the whole cell lysates, kinase was enriched by immunoprecipitatation using both an anti-JAK2 antibody and an anti-phospho(Y1007/Y1008)-JAK2 antibody to recover a fully active,

purified protein. The presence of phospho-JAK2 in the immunoprecipitates was confirmed by immunoblot analysis. However, when the immunoprecipitated proteins were incubated with $P_{JAK2\text{-}S}$ or $P_{JAK2\text{-}L}$ in the ELISA assay, they did not phosphorylate the biosensor probes. As a further optimization step, HEL and SET-2 whole-cell lysates were prepared using more stringent conditions (*RIPA lysis buffer*) for protein extraction in in denaturing conditions to disrupt potential inhibitory protein interactions that can impair JAK2 function. Again, no phosphorylation signal of the biosensor probes could be detected above background when whole cell lysates or immunoprecipitated JAK2 were used. Immunoblot analysis confirmed that active phospho-JAK2 was present in the lysates and remained stable during the ELISA incubation time, indicating that the absence of peptide phosphorylation was not due tokinase degradation or inactivation.

All together these results indicate that $P_{JAK2\text{-}S}$ or $P_{JAK2\text{-}L}$ are suitable peptide biosensors of the JAK2 catalytic domain; however, full-length JAK2 fails to phosphorylate these probes under the ELISA conditions tested, suggesting that additional cofactors or structural constraints may be required to enable efficient peptide recognition. Results on biosensors been presented as a poster presentation at the following conferences:

- 42nd SIF National Congress, November 13th- November 16th 2024, Sorrento, Italy
- PReS 32nd European Paediatric Rheumatology Congress; 17-20 September 2025, Helsinki, Finland
- XXVI Conference of young SIF Pharmacologist, November 30th – December 2nd 2025, Bologna, Italy

A manuscript on these results is currently under preparation.

In addition to peptide-based ELISA assay, we evaluated complementary *in vitro* approaches to monitor cellular responses to JAKi treatment. Two strategies were pursued to capture both early transcriptional signaling events in drug response and downstream consequences of drug exposure in primary cells or patients specific cellular models.

1) Transcriptomic profiling on two cell models characterized by constitutively active JAK2 and exposed to JAKi were performed to identify both early and late markers of drug response. Cell models were the HEL cell line that carries the *JAK2 V617F* gain-of-function mutation in homozygosis and the CALL-4 cell line, harboring the *IGH-*

CRLF2 translocation with the *JAK2 I682F* mutation.

HEL and CALL-4 cell viability following JAKi treatment was evaluated using the MTT colorimetric assay after 72 hours of drug exposure. This assay quantifies cellular metabolic activity and serves as an indirect measure of proliferation and survival. Drug sensitivity was expressed as the half-maximal inhibitory concentration (IC_{50}). In HEL cells, the IC_{50} values were 1 µM for ruxolitinib and 10 µM for baricitinib. In CALL-4 cells, both ruxolitinib and baricitinib exhibited IC_{50} values of 10 µM. Based on these results, HEL and CALL-4 cells were subsequently treated with each inhibitor at their respective IC_{50} concentrations for 4 hours and 48 hours prior to RNA extraction (experiments were performed in triplicate). Libraries were prepared using commercial kit that enriches for the 3' terminal regions of transcripts. The libraries were then converted using the MGIEasy Universal Library Conversion Kit to make them compatible with the MGI sequencing platform. Sequencing was performed in 75SE format on the MGI T1 Plus platform. Preliminary bioinformatic analysis have been performed in collaboration with CLONIT Company, a partner of the CONCERTO project.

Preliminary results highlighted how gene expression varies depending on timing of treatment, JAKi and cell line. Reduced expression of negative regulators of the JAK-STAT pathway (SOCS1, SOCS2, SOCS3, CISH) was observed in the HEL cell line after 4 hours of treatment with baricitinib, confirming the pharmacological inhibition upstream of the JAK-STAT pathway by the drug. Treatment with ruxolitinib at 4 hours also results in a reduction of SOCS1 expression. As early markers of drug response, we examined the top 50 most variable genes after short (4 hours) exposure to ruxolitinib and baricitinib. In HEL cells, 2 differentially expressed genes (DEGs) were shared after both JAKi treatment (i.e., ENSG00000266086 and MFSD12); however, these genes were not differentially expressed in CALL4 cells after short term treatments. As late markers of drug response, we preliminary examined the top 50 DEGs after long exposure (48 hours) to JAKi. A single DEG (i.e., EDC4 gene) was differentially expressed in HEL after both JAKi treatment and, interestingly, it was also differentially expressed in CALL4 after ruxolitinib treatment.

As markers of ruxolitinib response, we considered the DEG that ranked among the top 50 at both timepoints of drug exposure. In HEL, this approach identified 3 DEGs (i.e., NEK2, BUB1 and SAP30-DT) whereas in CALL4 cells only one DEG met this criterion (i.e., ENSG00000279204). No DEGs were consistently shared within the top 50 across the two

timepoint after baricitinib exposure in HEL or CALL4.

2) We monitored phosphorylation of STAT (pSTAT) as a direct downstream effector of JAK signaling, in ex vivo peripheral blood mononuclear cells (PBMCs). This system provides a translational and minimally invasive model for monitoring both basal activity of the JAK/STAT pathway in primary cells and the biological effects of JAKi treatment. The ultimate objective is to determine whether drug-induced changes in pSTAT levels can serve as a surrogate biomarker of JAKi response. Such a marker would enable discrimination between responders and non-responders among patients with immune-mediated diseases, thereby supporting personalized therapeutic monitoring. Technical tests have been started in order to evaluate the protocol, PBMC were isolated from whole blood by Leukocyte separation medium (LSM) and subsequently subjected to cytokine stimulation and pharmacological treatment with JAK inhibitors.

PBMC were exposed for 1 hour or 24 hours to a pro-inflammatory cocktail composed of flagellin (1 µg/ml), TNFα (100 ng/ml) and interleukin 1β (20 ng/ml). Cell lysates were analyzed by immunoblot to check the expression of the total forms of JAK1, JAK2, STAT3, and STAT5 and their respective phosphorylated forms. Total STAT3 and STAT5 were detected in PBMC; only pSTAT3 could be observed in PBMCs treated for 24 hours. From these preliminary data, the pro-inflammatory cocktail does not seem to be able to effectively activate the JAK/STAT pathway under the tested conditions.

PBMCs were thus stimulated only with interleukin-6 (IL-6, 100 ng/µL) for 20 minutes to activate the IL-6/JAK/STAT3 signaling pathway, and then treated with 1 µM of baricitinib. Phospho-STAT3 (pSTAT3) levels were evaluated in both lymphocyte and monocyte populations by flow cytometry. In a second set of experiments, PBMCs were stimulated with IFN-α2a (40,000 U/mL) for 15 minutes to activate the IFN α2a/JAK/STAT1 signaling pathway to analyze total STAT1 levels and were treated with 1 µM baricitinib. Preliminary data showed that IL-6 stimulation induces marked pSTAT3 activation (MFI fold change vs unstimulated) in lymphocytes with a less evident response in monocytes, whereas IFN-α2a stimulation elicits a strong increase in total STAT1 levels in both populations. Treatment with baricitinib at 1 µM effectively blocks STAT3 phosphorylation and STAT1 activation, bringing the signal (MFI fold change vs unstimulated) close to

baseline (unstimulated), suggesting strong inhibition of the pathway. Validation studies are currently underway on healthy donors at the Pediatric University Clinic of Ljubljana; once the assay is validated, patient samples from those undergoing treatment with JAKi will be analyzed and monitored by flow cytometry.

Finally, the effect of a panel of JAKi on cell viability was investigated in intestinal organoids generated from colon biopsies of pediatric patients available at the IRCCS Materno Infantile Burlo Garofolo BioBank in Trieste. Organoids are more physiological study models compared to immortalized cell lines because they more accurately mimic the spatial organization and biological functioning of human organs. In

particular, the organoids were exposed for 72 hours to a concentration of 1 μM of the following drugs: tofacitinib, baricitinib, upadacitinib, abrocitinib, deucravacitinib, ruxolitinib phosphate, ruxolitinib, delgocitinib, ritlecitinib, itacitinib, and brepocitinib. Interestingly, preliminary results showed that, while most drugs induced a reduction in viability ranging from 20 to 50% (compared to the untreated control), ruxolitinib and ruxolitinib phosphate were able to increase viability by 20.7% and 8.0%, respectively.

Therapy of rare immune-mediated disease in children is a challenging clinical task. Availability of effective drugs such as JAKi is important, but strategies to personalize therapies with these agents are highly needed. This study performed in the context of the CONCERTO funding, sets the bases for developing strategies to address this important therapeutic need for children benefiting from these medications.