LC-SRM targeted quantification of 20S proteasome status for controlled ex-vivo expansion of Human Adipose-derived Mesenchymal Stromal/Stem

Thomas Menneteau[1,2], Bertrand Fabre[1], Alexandre Stella[1], Dusan Zivkovic[1], Emmanuelle Mouton-Barbosa[1], Marie-Laure Renoud[2], François Amalric[1], Luc Sensébé[2], Anne Gonzalez de Peredo[1], Isabelle Ader[2], Odile Burlet-Schiltz[1], Marie-Pierre Bousquet[1]

Corresponding author: marie-pierre.bousquet@ipbs.fr

[1] *Proteomics and Mass-Spectrometry of Biomolecules, Institut de Pharmacologie et de Biologie Structurale, CNRS UMR 5089, University of Toulouse, Toulouse Cedex, France*

[2] *STROMALab, University of Toulouse, INSERM U1031, EFS, INP-ENVT, Toulouse, France*

INTRODUCTION

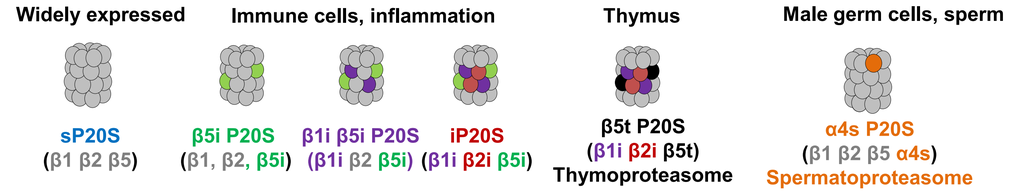
The proteasome controls a multitude of cellular processes through protein degradation and has been identified as a therapeutic target in oncology (1). However, our understanding of its function and the development of specific modulators are hampered by the lack of a straightforward method to determine the overall proteasome status in biological samples.

Although the cylindrical α7β7β7α7 barrel-like structure of the 20S catalytic core proteasome has been preserved throughout evolution, the oligomeric protease has evolved, resulting in a higher heterogeneity of subunit compositions in mammals.

As schematically represented below, there exist at least six distinct forms of 20S proteasomes in human cells and tissues.

Given the importance of proteasome in human diseases and disorders, the development of tools for precise assessment of proteasome status in patients would be needed.

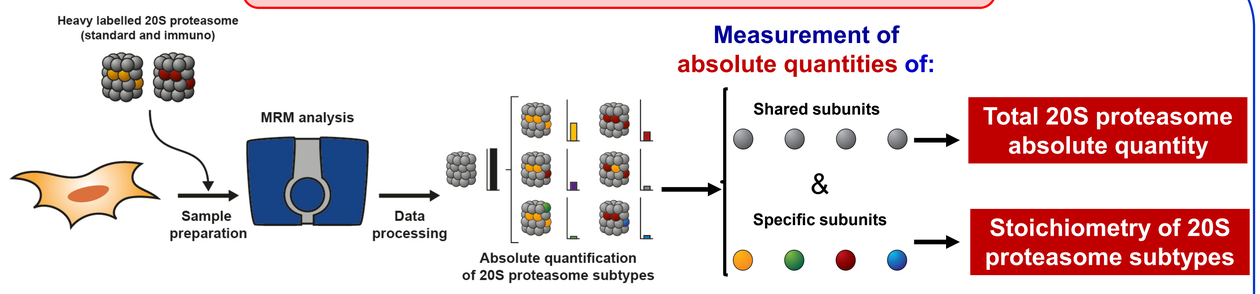
Schematic representation of the six main 20S proteasome subtypes in mammals



The 20S core particle (CP) is composed of 14 different subunits. Specific subunit isoforms are incorporated to the CP in different combinations, giving rise to various CP types.

The standard 20S proteasome (sP20S) is composed of constitutive (α1–α7 and β3, β4, β6, and β7) and catalytic subunits (β1, β2 and β5). It is the most abundant 20S subcomplex in most cell types. Significant amounts of other 20S forms have been observed in some human tissues and cells in their basal state, or are induced in specific environmental conditions (2-5) .

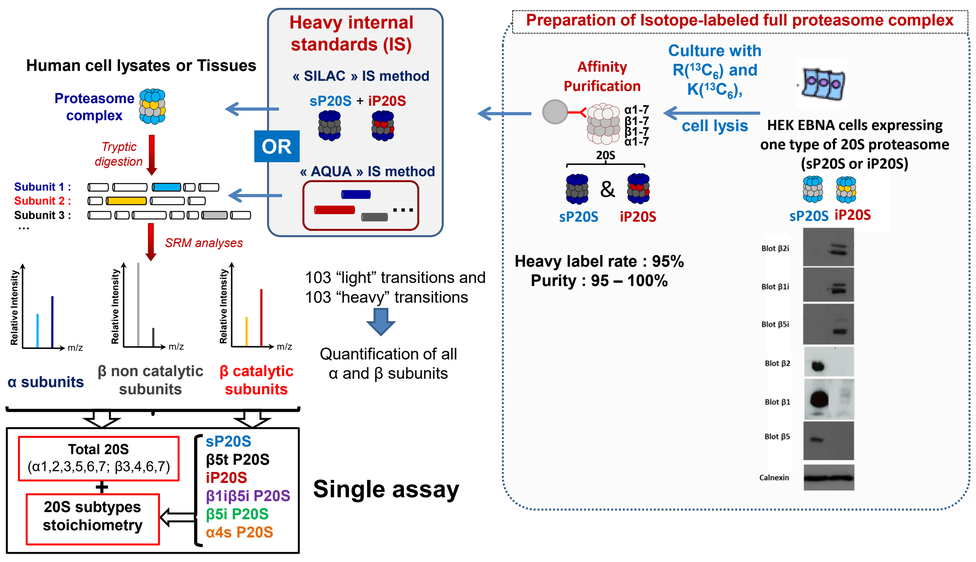
Objective: Measurement of complete 20S proteasome status in various human cells and tissues



One-shot, specific, robust analysis → **Targeted Mass Spectrometry**

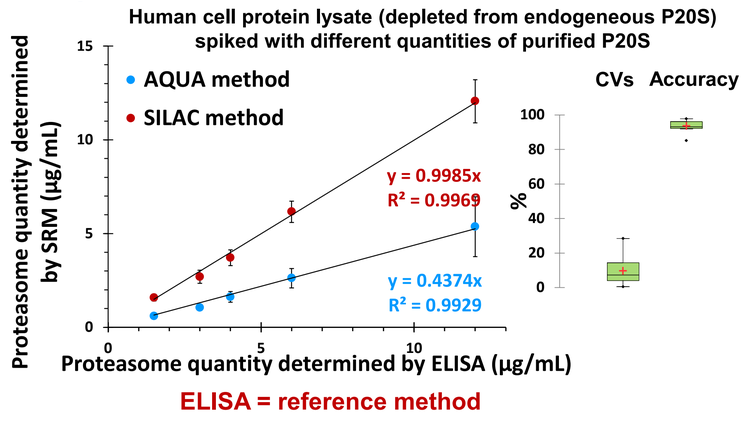
This work has been very recently published as Menneteau T. & Fabre B. *et al*. Mol. Cell Proteomics. DOI: 10.1074/mcp.RA118.000958.

1- Workflow for the determination of the absolute quantities of the different 20S proteasome subtypes



- ☑ **Design of a workflow** combining IDMS (Isotope Dilution Mass Spectrometry) and MRM (Multiple Reaction Monitoring) to monitor all proteasome subunits in a **multiplexed, sensitive and robust LC-MRM approach**.
- ☑ **Comparison of two internal standard methods** to measure proteasome absolute quantities in human samples: AQUA (6) and absolute SILAC (7).
- ☑ Production, purification, and quality assessment of **isotopically labelled 20S proteasome** as internal standard for accurate quantification.
- ☑ **Total 20S proteasome absolute quantity** is determined by quantification of shared α1,2,3,5,6,7; β3,4,6,7 subunits.
- ☑ **The stoichiometry of 20S subtypes** is determined by absolute quantification of specific catalytic subunits β1,2,5 and β1i,2i,5i.
- ☑ β5t is deduced from absolute quantities of total 20S proteasome, β5, and β5i.
- ☑ α4s is determined by comparing the absolute quantities of total 20S proteasome and specific peptides of α4 (not observed in α4s).

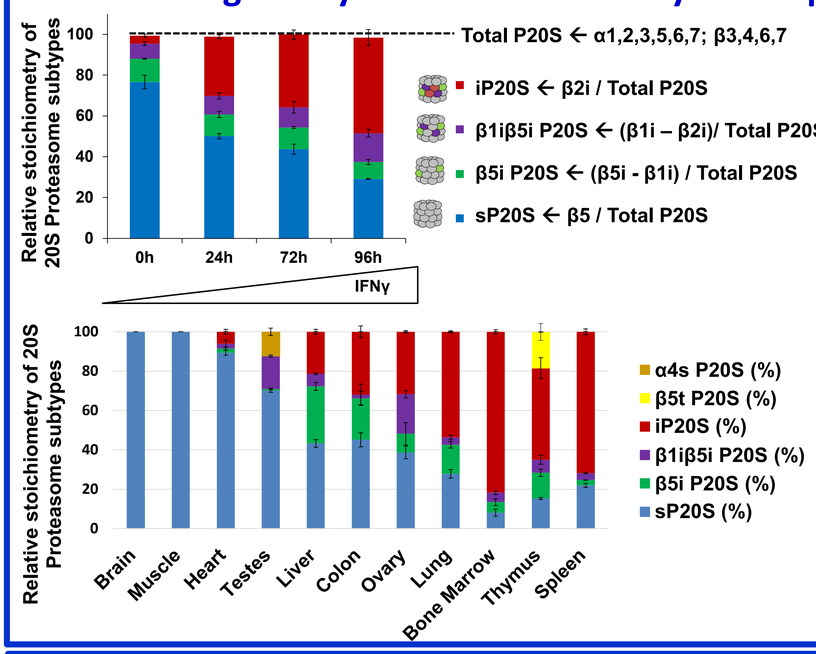
2- Importance of the Internal Standard method



ELISA = reference method

AQUA IS method	SILAC method
☑ Linear	☑ Linear
☑ Precise	☑ Precise
☒ Not accurate	☑ Accurate

3- Monitoring the dynamics and diversity of 20S proteasome subtypes stoichiometries

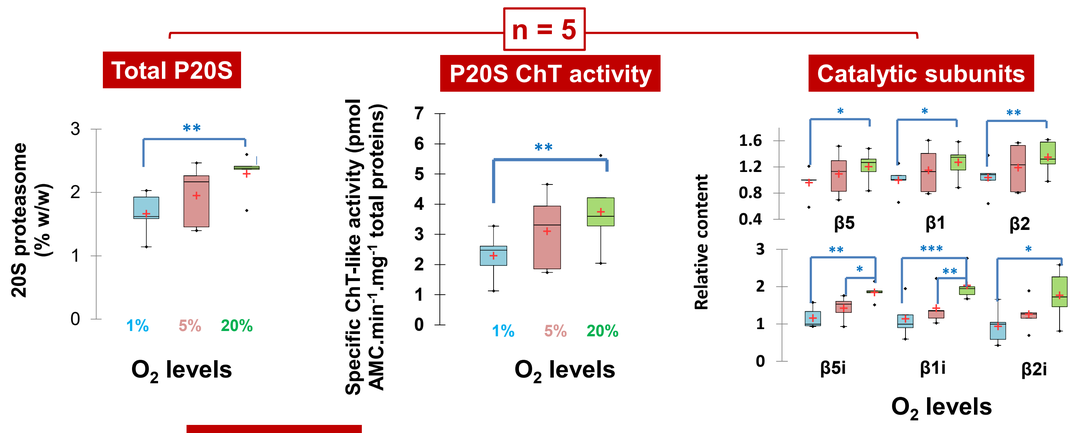


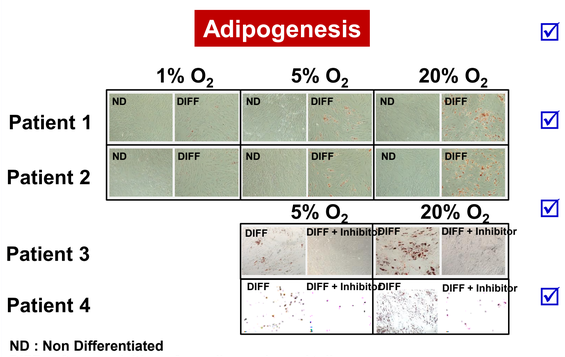
- ☑ Changes in the stoichiometries of the four major 20S proteasome subtypes (sP20S, β5i P20S, β1iβ5i P20S, and iP20S) were accurately monitored in a model of IFNγ-treated HeLa cells. No β5t P20S nor α4s P20S were detected in these cells, as expected.
- ☑ Excellent accuracy (97 ± 2%) and variability (CVs below 15%) to measure the absolute levels of catalytic subunits.
- ☑ Brain, muscle, heart, and testes tissues almost exclusively contain standard proteasome β subunits (β1, β2, and β5).
- ☑ Bone marrow, spleen, and thymus, contain very high levels of β1i, β2i, and β5i immunosubunits.
- ☑ Liver, colon, ovary and lung tissues display 50 to 70% of immunosubunit-containing 20S proteasome subtypes (β5i P20S, β1iβ5i P20S, and iP20S).
- ☑ As expected, thymoproteasome and spermatoproteasome were only observed in thymus and testes tissues, respectively.

4- Expansion of ADSCs under different O_2 concentrations affects 20S proteasome status and their capacity to differentiate

Mesenchymal stem/stromal cells (MSC) hold great potential in regenerative medicine because of their multi/pluripotency and immunosuppressive properties. Adipose-derived Stem Cells (ADSCs) are a subclass of MSC. **To obtain the critical number of cells before transplantation, ADSCs must be expanded *in vitro***. The development of **uniform protocols** for both preparation and characterization of MSCs, including standardized functional assays to assess their biological potential, will be critical in contributing to their clinical utility.

Conventionally, MSC culture for clinical applications is performed under normoxic conditions (21% O_2 tension), even though oxygen levels within tissues are typically much lower (hypoxic) than these standard culture conditions. Therefore, **dioxygen tension represents an important environmental factor that may affect how MSCs perform *in vivo***. However, the impact of hypoxic conditions on distinct mesenchymal stem cell characteristics, such as **the proteasomal status**, still remains unclear.





ND : Non Differentiated
DIFF : after expoisition of an adipogenic cocktail
DIFF + Inhibitor : after expoisition to an adipogenic cocktail + an iP20S inhibitor (100 nM ONX-0914)

- ☑ The 20S proteasome status has been analyzed after 10 days of ADSCs expansion under three different O_2 concentrations: 1%, 5%, and 20%.
- ☑ Proteasome abundance, proteolytic activity and immunocatalytic subunits levels were all increased with the O_2 tension.
- ☑ High levels of immunoproteasome correlate with high adipogenic potential at 20% O_2, and the reverse under hypoxic conditions,
- ☑ Specific inhibition of the immunoproteasome using the ONX0914 inhibitor (8) leads to a marked decrease in the capacity of ADSCs to differentiate into adipocytes.

CONCLUSIONS

- Design of an MRM assay to determine the absolute quantity and stoichiometry of ubiquitous and tissue-specific human 20S proteasome subtypes.
- Use of purified isotopically labelled 20S proteasome as internal standard for accurate quantification.
- Variation in the expression of immunoproteasome in adipocyte-derived stem cells (ADSCs) grown under different O_2 levels might be causal for change in cells differentiation capacity.
- The status of 20S proteasome during ADSCs expansion might constitute an additional relevant quality control parameter to contribute to predict, among other quality markers, their therapeutic capacity.

REFERENCES

(1): Cromm PM, Crews CM. *ACS Cent Sci* 2017, 3(8):830–838.
(2): Guillaume B *et al.* Proc Natl Acad Sci U S A. 2010, 107:18599-604.
(3): Bousquet-Dubouch MP. *et al.* Exp. Revi. Proteomics. 2011, 8(4):459-81.
(4): Fabre B, Lambour T. *et al.* J Proteome Res., *13*, 3027-37.
(5): Fabre B. et al. Mol. Syst. Biol. 2015, *11*, 771.
(6): Gerber SA et al. *Proc Natl Acad Sci U S A. 2003,* 100(12):6940–5.
(7): Hanke S et al. *J Proteome Res. 2008,* 7(3):1118–1130.
(8): Muchamuel T, et al. *Nat Med* 2009, 15(7):781–787.