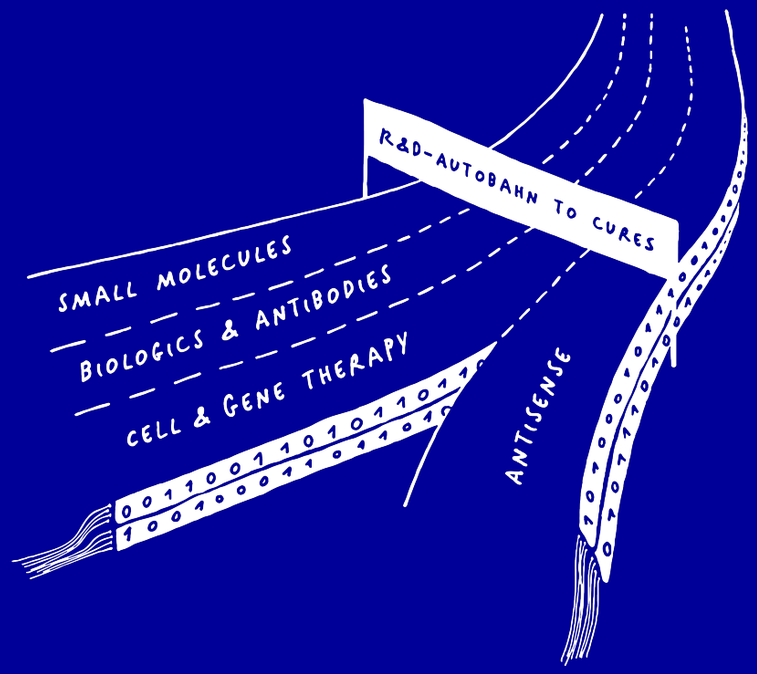
evotec

#RESEARCHNEVERSTOPS

Development and validation of a bioanalytical microLC-MS/MS bottom-up approach method to quantify Semaphorin-3A protein in human plasma samples



Evotec SE, Alessandro Greco, 16th EBF OS, November 2023

Agenda

1. Introduction to Proteomics
2. Semaphorin 3A
3. Development & Validation
4. Summary & conclusion
5. Reflections & Questions

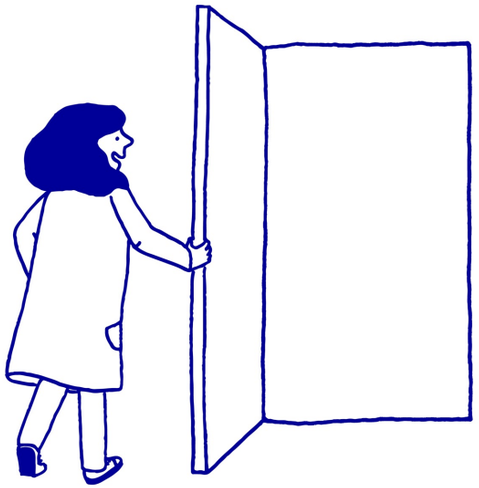
Introduction

Proteomics

What does “OMICS” mean?

The 1990s marked the emergence of genome sequencing and DNA technologies, giving rise to the “omics” era of research.

… it refers to the large number of biomolecular disciplines, as genomics or proteomics … it refers to the interpretation of genes and protein activities in response to toxic substances, drugs or diseases …



but … new area to be discovered full of new challenging methods to be implemented … to identify, characterize and quantify proteins

Proteomics Assays

Starting from the beginning – Discovery vs. Development

Proteomics Assay

Discovery phase

- End 1990s
- The sensitivity of the overall proteomic workflow was a major challenge
- Static nano-ESI (without prior LC separation) – no Automation was allowed
- nano-LC coupled with HRMS
- Target identification

Bioanalytical Assay

Development phase

- 2018 micro-LC
- Lower sensitivity
- Excellent reproducibility in both retention time and peak area
- Target quantification

Extraction Procedures

Top-down vs Bottom-up

Top-down

- Enables the quantification of modified and unmodified protein species
- Allows introduction of intact proteins into the mass spectrometer and analysis of both intact and fragment ions masses
- Allows up to 100% sequence coverage and full characterization of proteoforms

But

- Low efficiency of ionization and detection
- Reproducible and accurate protein quantification remains challenging
- Pre-immunoprecipitation increases the ionization efficiency

Bottom-up

- Enables high-throughput analysis and allows identification and quantification of proteins in complex samples
- Enzymatically or chemically cleavage into peptides
- Complex peptide mixture is fractionated using reversed phase chromatographic separation

But

- Biological interpretation of the data is needed – digestion discards connectivity peptides vs proteins
- Proteotypic peptides are needed to perform accurate quantification

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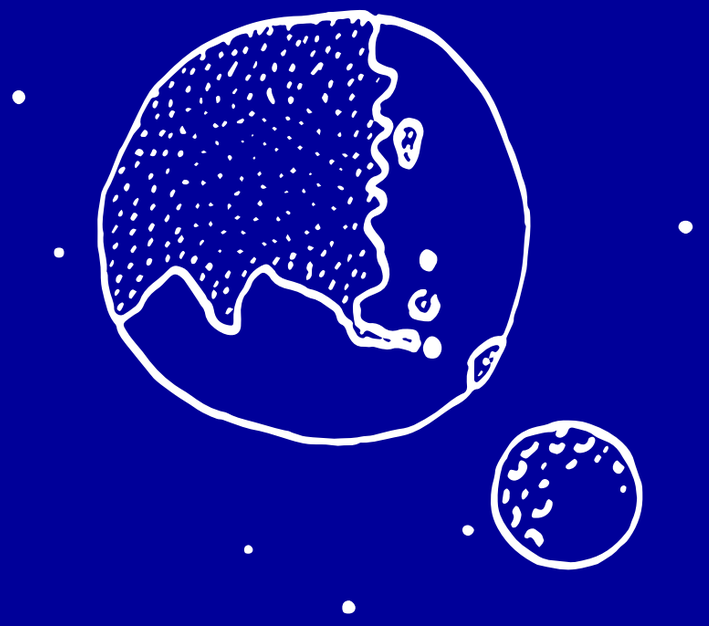
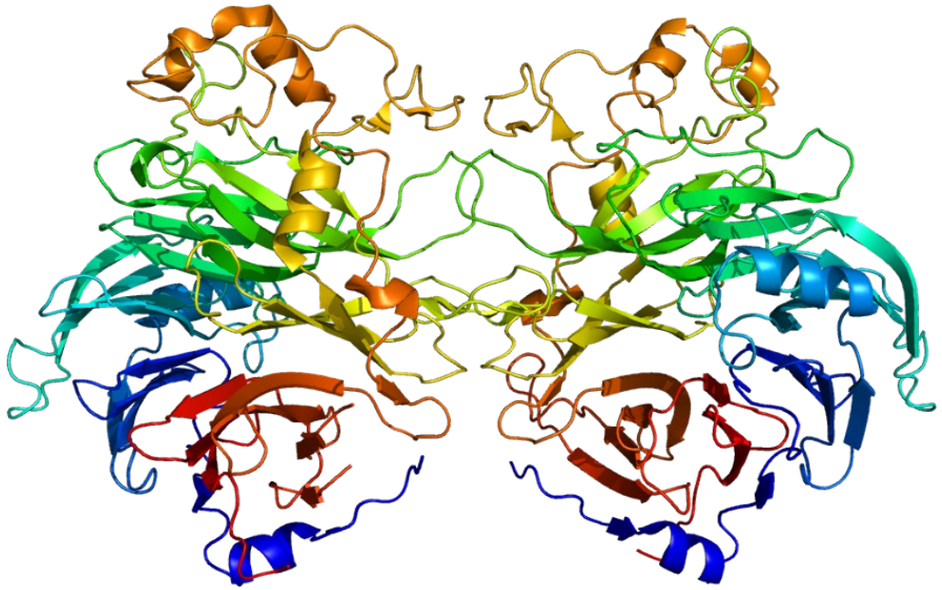


Semaphorin-3A

Background information

- Semaphorin-3A is protein that is encoded by the Sema3A human gene.
- This protein is vital for the development of normal neuronal pattern and also for new blood vessels.
- Semaphorin-3A is highly expressed in scar tissue after traumatic central nervous system injuries and contributes to the failure of neuronal regeneration.
- Increased expression of semaphorin-3A is associated with schizophrenia, with the progression of Alzheimer's disease, to the ALS progression.
- Increased Sema3A signaling leads to albuminuria and tubular injury resulting in a progressive loss of kidney function.

Sema3A



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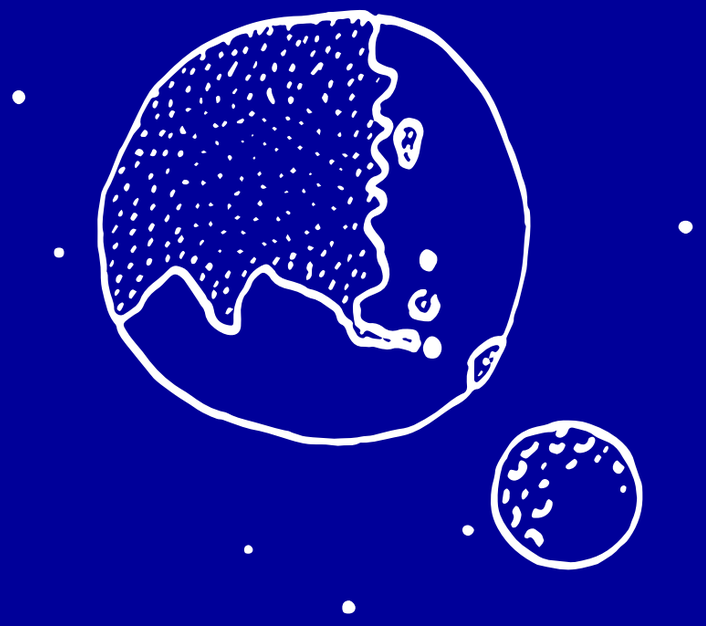


Method Development & Validation Design

Thoughts

Starting point

- Biomarkers are not covered by the ICHM10
- Endogenous levels

Restrictions

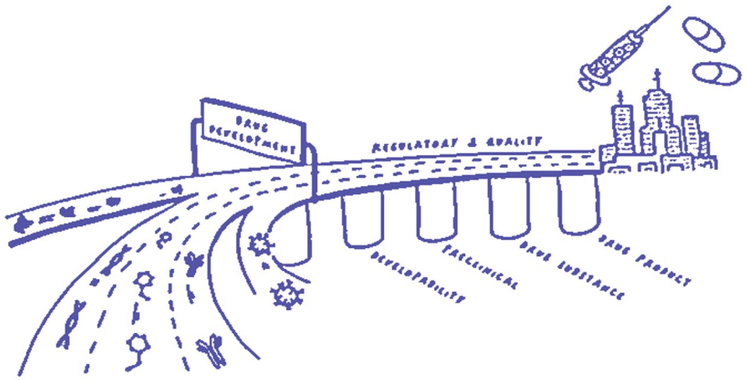
- SP should be proteotypic
- Sensitivity and selectivity could be assessed as first step
- Sample preparation duration and run time should be balanced with lab productivity

Technical approach

- 2 signature peptides
- Surrogate matrix
- Bottom-up
- µLC-MS/MS platform

Features

- Matrix should be evaluated – behavior issue?
- SIL could minimize method variability & increases the method robustness



Validation design

- CoU validation
- 3 P&A in surrogate
- Surrogate vs plasma – matrix equivalence
- Matrix Effect
- Stabilities (both matrices)

Matrix and SP Selection

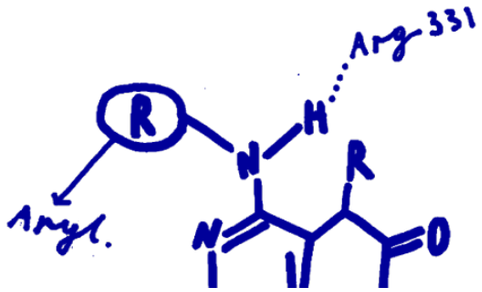
Endogenous molecule

Matrices

Possible approach to be followed for endogenous molecules

- **Surrogate matrix**
- Surrogate analyte
- Background subtraction
- Standard addition

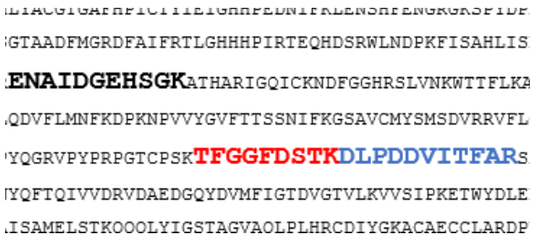
PBS 0.5%BSA

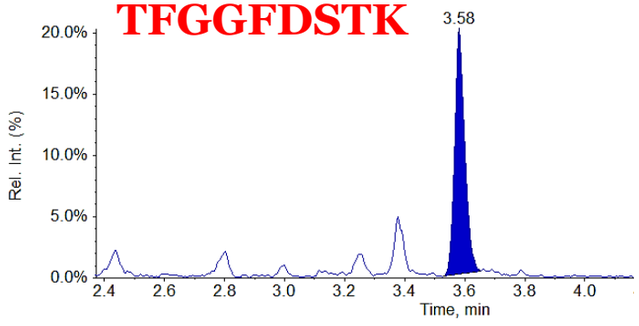


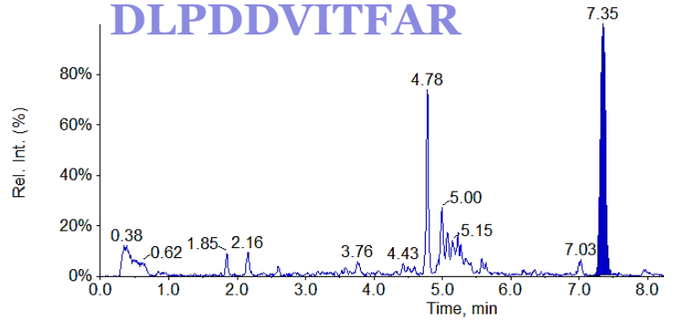
Calibration range → *50-10,000 ng/mL*

Signature Peptides

- Only three proteotypic peptides were available, and only two with enough mass spec response
- **TFGGFDSTK** → quantifier
- **DLPDDVITFAR** → qualifier
- **ENAIDGEHSGK** → poor sensitivity







QC *3,000 ng/mL*

Extraction Procedure

Bottom-up

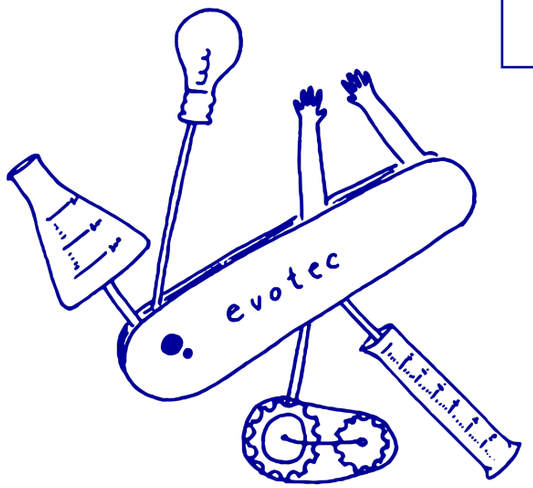
Extraction step	Condition
Aliquot **2µL sample** + 10µL ISTD + iST kit	Reduce & Alkylate for 10 min at 95°C
Digestion	Enzymatic: 180 min at 37°C
Desalting (Kit SPE)	Mild centrifugation at RT (30 min)
Injection onto LC-MS/MS	Dry down and reconstitution (400 µL) with 0.1% formic acid in water

Extraction procedure → *about 4.5 hrs*

Lyse

Digest

Purify

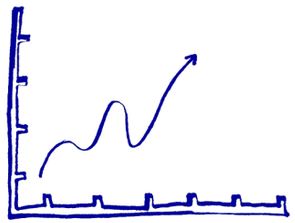


LC-MS/MS Parameters

microLC-MS/MS

LC Parameter

LC system	Waters UPLC M-Class
Column	Waters CSH C18 (50x0.3 mm, 1.7 µm) at 60°C
Mobile phases	A: Water + 0.02% formic acid B: Acetonitrile + 0.02% formic acid Gradient elution
Flow	14 µL/min
Injection volume	**5 µL**
Gradient	From 2 to 21% B in 9 mins
Run time	10.5 min



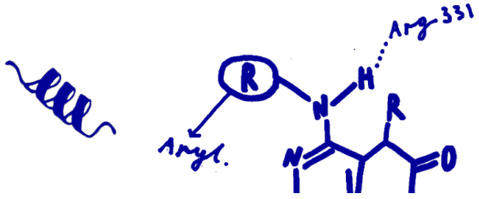
MS/MS Parameter

MS System	QTrap-6500+
Operating mode	MRM
MS Conditions	IS= 5000 – CUR=30 – CAD=9 TEMP=350 GS1=30 – GS2=50
Ionization	Positive
Signature peptide (quantifier)	TFGGFDSTK (m/z 480 → 711)
Qualifier	DLPDDVITFAR (m/z 632 → 517)
ISTD	TFGGFDST**K** (m/z 484 → 719) DLPDDVITFA**R** (m/z 637 → 522)

Preliminary experiment on HRMS to identify SP candidates

Method Validation

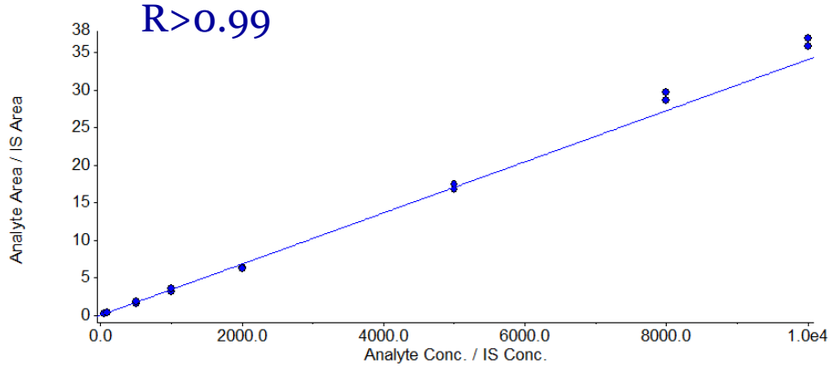
Performances



- **Calibration range**
  → 50-10,000 ng/mL
- **QC levels**
  → 50,150, 3,000 and 7,500 ng/mL
- **Acceptance criteria**
  - Accuracy: ±15% (±20% @LLOQ)
  - Precision: <15% (<20% @LLOQ)
  - R: >0.99
- **Number of replicates**
  - Surrogate (6 replicates for each QC level, 3 runs)
  - Plasma (6 replicates for each QC level, single run)

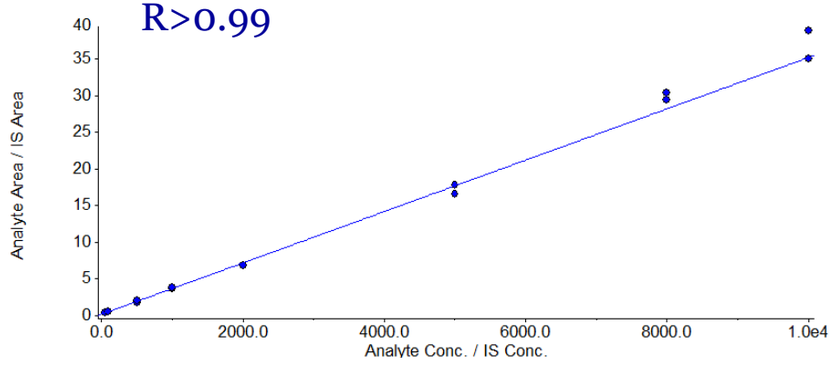
TFGGFDSTK

Matrix	Accuracy	Precision
Surrogate	89.2% to 107.0%	<14.7% (@LLOQ)
Plasma	97.9% to 115.7% (@LLOQ)	<14.2%



DLPDDVITFAR

Matrix	Accuracy	Precision
Surrogate	83.9% to 118.6% (@LLOQ)	<20.0% (@LLOQ)
Plasma	85.7% to 118.8% (@LLOQ)	<14.1%



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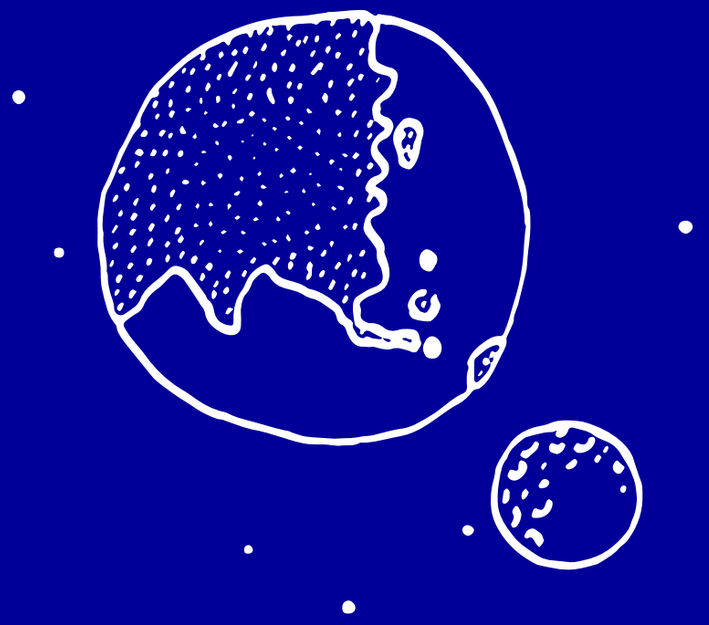

Summary & Conclusions

General overview

- Micro-LC seems to be the most promising chromatographic platform; it is very easy to adapt the proteomic analysis method to the method for quantification of the selected protein/s.
- The method developed allows to delivery results with a reasonable timelines … the optimized extraction procedure was a key element in reducing the analysis time.

- CoU statement
  - Biology and pharmacology effect
  - Expected data and variability
  - Reason of data needed
  - Scientific & safety decision
- CoU assay validation should be implemented and customized based on available safety and pharmacology information
- CoU assay should be “adjusted” based on the data generated during the assay development

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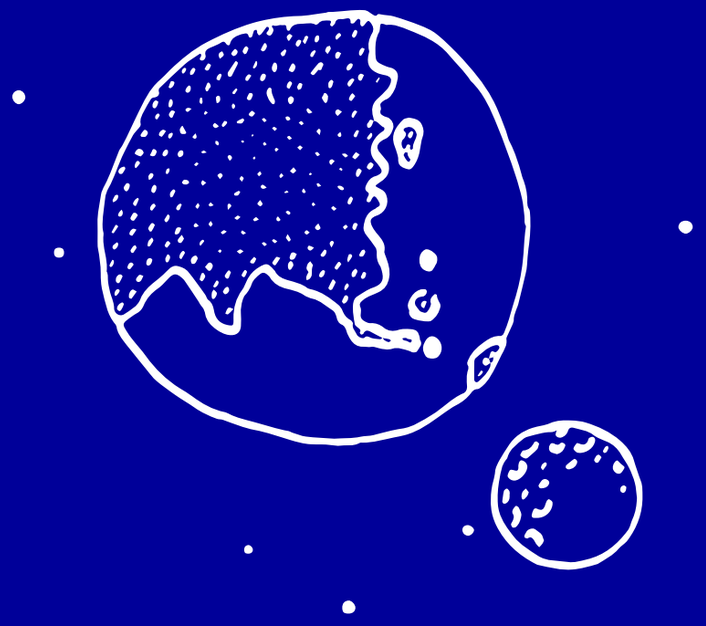
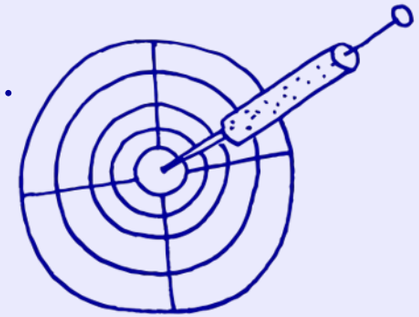


Reflection & Questions

- How should we validate a protein assay such as the Semaphorin molecule?
- If the biomarker is a primary/secondary endpoint and kinetic evaluation is needed … how should we consider the validation?
- But … primary/secondary/exploratory endpoints … Is this information really crucial?
- CoU validation could be enough? …
- Specific biomarker guideline could be useful? …



Acknowledgments

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Thank you for your attention!

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