

## Discovery and Validation of an Immunoaffinity LC-MS/MS Assay for the Quantification of the Collagen Type II Neopeptide Peptide Biomarker

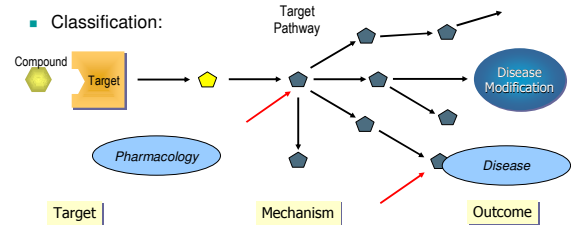
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## Why Biomarkers?

■ Definition: A characteristic objectively measured & evaluated as an indicator of normal biological & pathogenic processes, or pharmacologic responses to a therapeutic intervention. (NIH guideline)

■ Classification:



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## Decision Making Biomarkers

"What is the purpose or intended use of the biomarker(s)?"

- The intended use guides biomarker discovery, development and validation efforts
  - Guide selection of lead compounds
  - Go/no go for full development
  - Disease diagnosis

The importance of the decision determines the level of validation...

- Technical validation: Biomarker assay measures what we need to measure for its intended purpose, i.e. Fit for purpose.

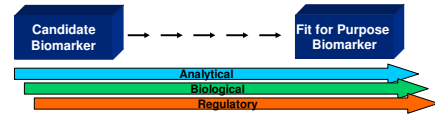
And if the intended use is in the clinic...

- Translation (Biological validation): Adapting or refining an identified biologic marker to an assay suitable for use in clinical.

Translational biomarker: A biomarker suitable for making decisions in a clinical setting, for bridging pre-clinical and clinical findings.

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## The Importance of Intended Use



- From "Candidate" to "Biomarker" - depends on purpose
  - For Proof of Pharmacology (PoP) - evaluate drug effect
    - Need to show that drug modulates the candidate biomarker(s), then confirm, develop and qualify the assay.....
  - For OA disease diagnosis or progression - for inclusion in clinical study? for determining therapy?
    - Need to confirm in larger population, eliminate confounds, easier/cheaper/better than current diagnosis method, assay development, accessible samples....
    - Need to confirm with Longitudinal study...
- Validating or qualifying the biomarker for use is the hard part!

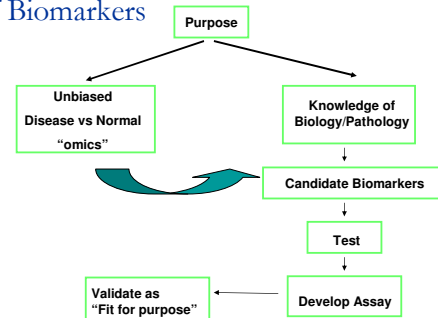
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## Rationale for Developing Biomarkers for Osteoarthritis (OA)

- Improved Outcome Measures:
  - OA is a slow progressive degenerative disorder of joints
  - Accepted endpoint for demonstrating DMOAD is JSN using standardized X-rays (2 years)
  - A biomarker that predicts clinical outcome in short-term studies would speed decision making
- Development of Novel DMOADs:
  - A biomarker that demonstrates *in vivo* receptor occupancy/target activity and "proximate" pathway modulation by drug

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## Strategy for Discovery and Development of Biomarkers



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## Pathology of Osteoarthritis (OA)

- OA is characterized by the progressive degeneration of articular cartilage.
- Cartilage composed primarily of collagen type II (90-95%).
- Type II collagen cleavage by collagenases has been associated with irreversible loss of cartilage integrity.
- MMP-13 is a major collagenase that is up regulated in OA cartilage, thus is an important target for OA.

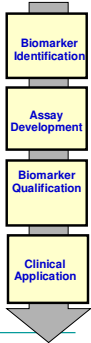
MMP-13 specific collagen type II fragments have utility as both:

- OA outcome biomarker
- PoP biomarker for MMP activity

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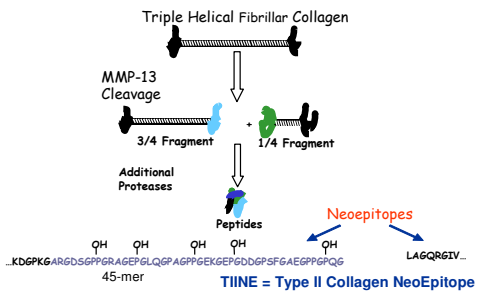
## Process for Development of Biomarkers

- Identification of collagen fragments – candidate biomarker:
  - cartilage degradation products *in vitro*
  - in preclinical and clinical samples - accessible fluids/tissues
- Develop quantitative assay for a potential biomarker
- Qualify biomarker for intended uses:
  - PoP - modulation by MMP13 enzyme inhibitors
  - OA Outcome - up-regulation in preclinical DMOAD models and human OA disease, correlation and/or prediction disease outcome
- Application in DMOADs development



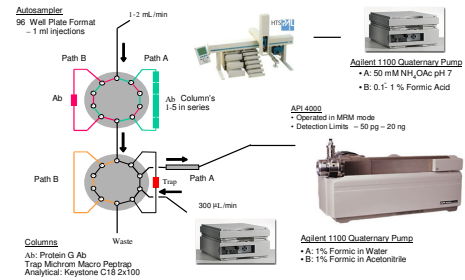
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## Candidate Biomarkers: Matrix Degradation Products



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## Biomarker Assay Development - Immuno-Affinity LC-MS/MS Method



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## Fit for Purpose Qualification of Biomarker

### Purpose

- PoP

### Biological

- Produced by MMP enzyme
- Modulated by MMP inhibitors

### Analytical

- Sensitivity
- Selectivity
- Detect changes due to MMP inhibition in normals

- OA Outcome

- Predicts OA progression
- Correlates w/OA severity
- Predicts drug efficacy, etc

- Reproducibility
- Interassay variability
- Transfer to other labs

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## Assay Qualification Criteria

- Sensitivity, specificity & selectivity
- Assay range: lower and upper limits of quantification
- Recovery from biological matrix
- Accuracy and precision
- Dilution linearity and parallelism
- Carry-over (specific to LC-MS/MS assay)
- Intra- and interassay variability
- Analyte stability

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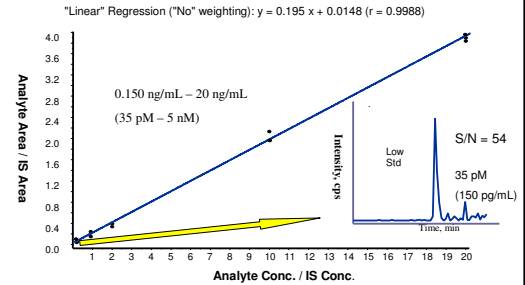
## Characteristics of Immuno-Affinity LC-MS/MS Assay

High Sensitivity, Specificity & Selectivity!

- **Antibody binding**
  - Must bind to Ab column
- **Chromatography**
  - Must have same retention time as standard
- **Molecular weight**
  - Must have correct precursor MW to pass thru Q1
- **Fragmentation**
  - Must have correct fragment ion MW to pass thru Q3
  - Relative fragment ion intensity must be similar

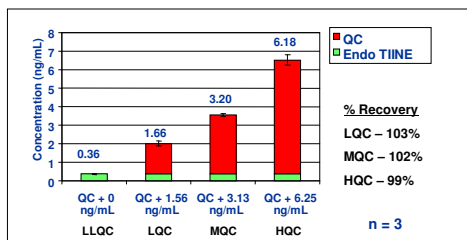
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## Assay Sensitivity: Linear Standard Curve for TIINE



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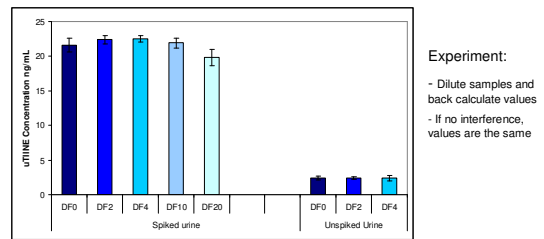
## Assay Accuracy and Precision: QC Samples of TIINE



Note: Standard Curve was prepared in 50 mM NH<sub>4</sub>OAc, QC Samples were in 1:1 Diluted Urine

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## Dilution Linearity: Up to 20-fold (no matrix interferences)



Experiment:  
- Dilute samples and back calculate values  
- If no interference, values are the same

DF = Dilution factor  
DF = 0 – represents nominal concentration

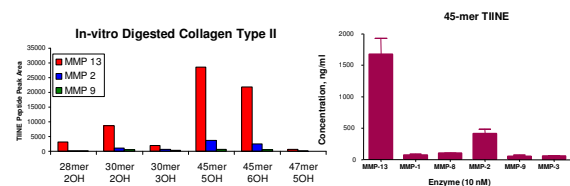
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## Biological Validation of the Assay

- PoP biomarkers for MMP-13 activity:
  - Evidence that TIINE is produced by MMPs
  - Modulation of TIINE by MMP inhibitors:
    - ex vivo cartilage explants
    - pre-clinical models
    - human subjects

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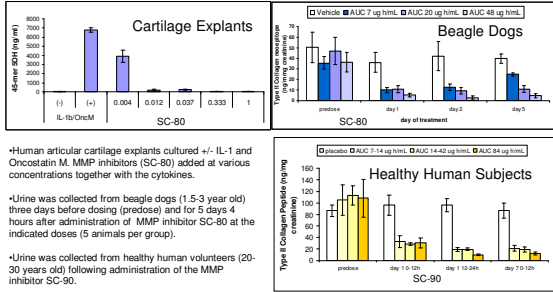
## Collagen Type II Peptides are Produced by MMPs



MMP-13 is primarily responsible for the production of 45-mer TIINE

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## Proof of Pharmacology Biomarker - Inhibition with MMP Inhibitor....



\*Human articular cartilage explants cultured +/- IL-1 and Oncostatin M. MMP inhibitors (SC-90) added at various concentrations together with the cytokines.

\*Urine was collected from beagle dogs (1.5-3 year old) three days before dosing (pre-dose) and for 5 days 4 hours after administration of MMP inhibitor SC-90 at the indicated doses (5 animals per group).

\*Urine was collected from healthy human volunteers (20-30 years old) following administration of the MMP inhibitor SC-90.

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## Analytical Characteristics of the TIINE Assay for OA Outcome Purpose

- Assay is used in 2 Pfizer labs and outside CRO
- Long-term stability of analyte - up to 1 year tested
- Interassay %CV <10%, %RE < 10%

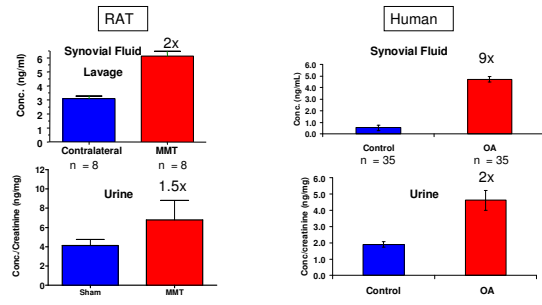
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## Biological Validation of the Assay

- Evidences that TIINE is associate with OA
  - Increased levels in cartilage explant due to cytokine stimulation
  - High levels in SF and urine of pre-clinical models of OA and OA subjects as compared to control
  - High levels in late stage of OA disease

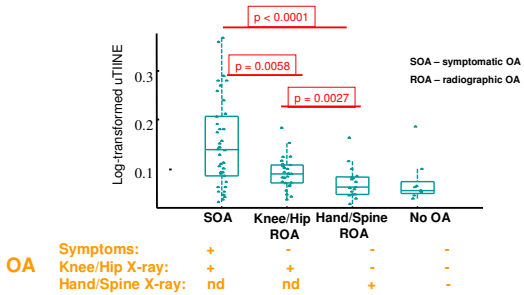
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## Outcome Biomarker Qualification: TIINE is Increased in SF and Urine



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## Outcome Biomarker Qualification: TIINE Levels Increase in OA



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## Summary

- Successful discovery of TIINE:
  - Discovery is based on biology/pathology of OA
- Development of quantitative assay to measure 45-mer (TIINE):
  - Immuno-affinity LC-MS/MS assay
- Unique features of immuno-affinity LC-MS/MS assay
  - increased sensitivity and selectivity
  - significant reduction of interferences and matrix suppression effects
- Analytical qualification of the assay for intended uses
  - accuracy and precision
  - recovery
  - linearity and dynamic range
  - long-term stability
  - interassay reproducibility, etc.

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## Summary (cont.)

- Fit for purpose qualification of TIINE
  - Successful qualification of TIINE as proof of pharmacology biomarker for MMP activity
    - TIINE correlates with MMP-13 inhibition in *in vitro* and *in vivo*:
      - cartilage explants
      - pre-clinical models
      - healthy human subjects
    - Urinary TIINE assay is qualified for human use
  - Qualification of TIINE as disease outcome biomarker is in progress
    - distinguishes OA from normal in cross-sectional studies
    - Predicts progression???
    - Predicts efficacy?????

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