Validating protein biomarkers with mass spectrometry and molecular biology: a revolution

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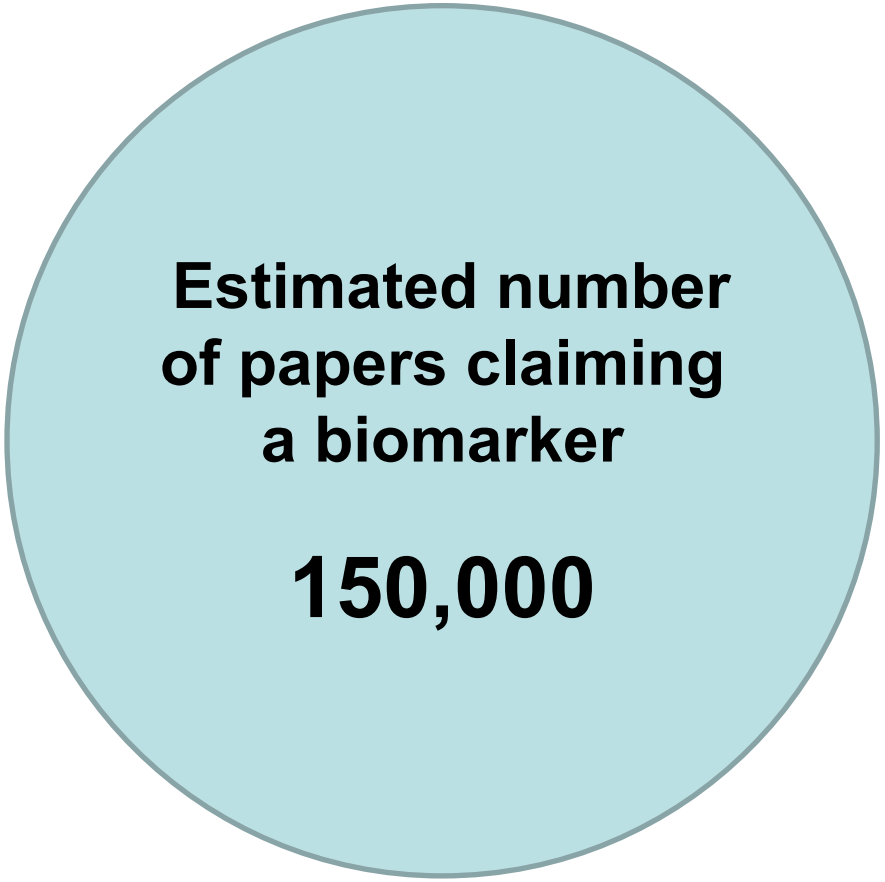
Biomarker discovery: the problem in 2011



Estimated number of biomarkers in routine clinical use

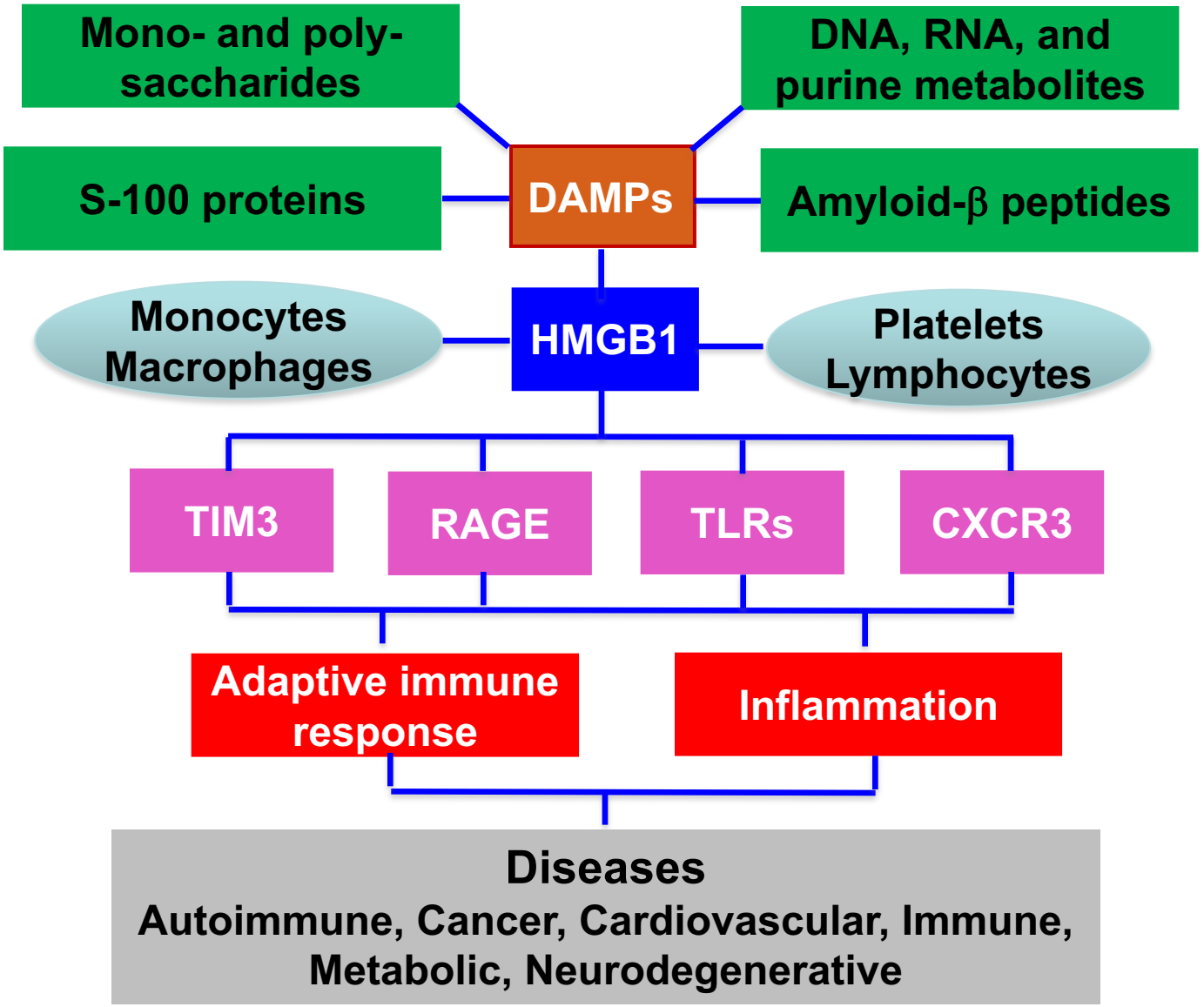
Poste G. *Nature* 2011;69:156

No significant change since 2011

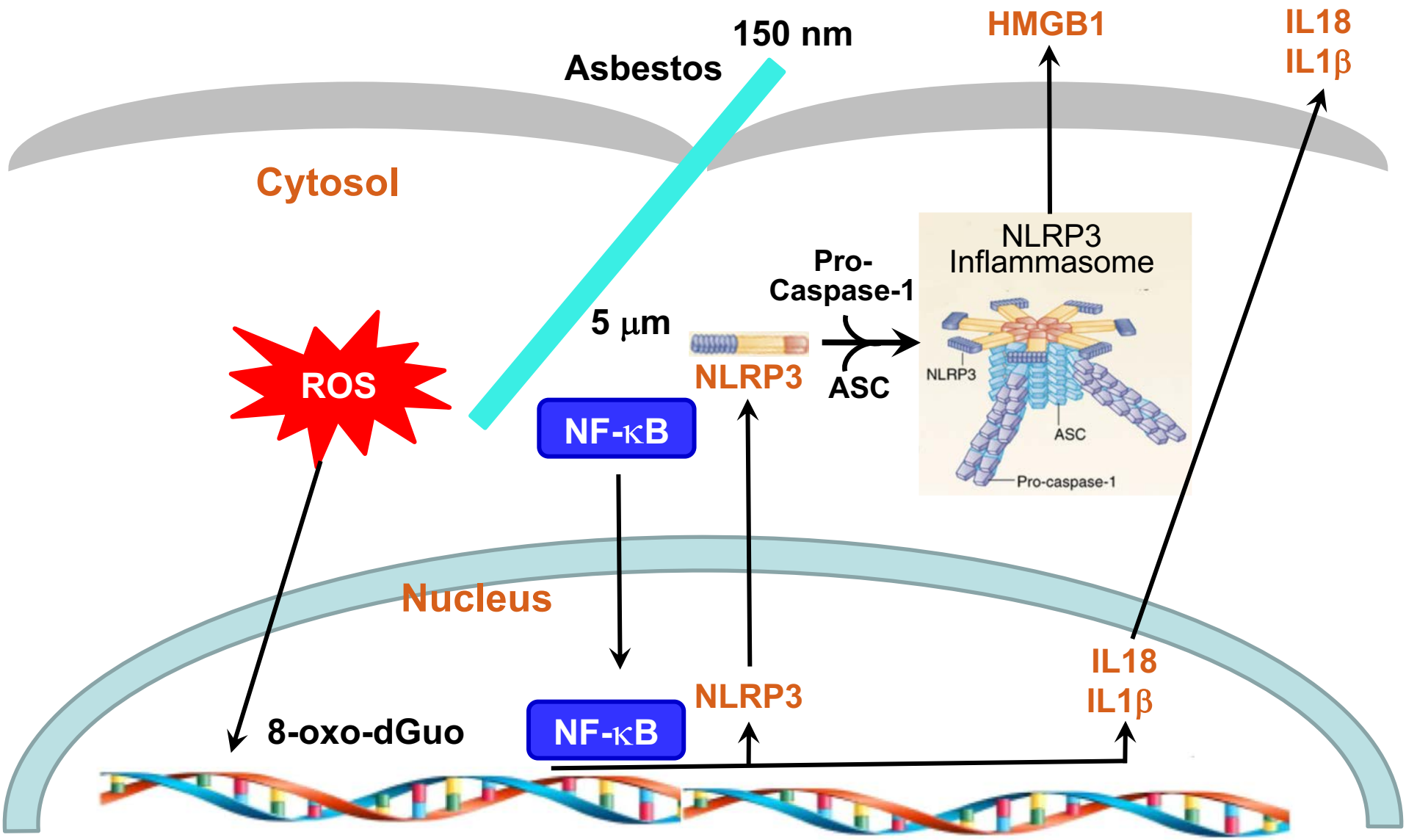
Biomarkers needed for early detection of cancer

The danger molecule - high mobility group box 1 (HMGB1) as a case study

Danger associated molecular pattern (DAMP) molecules



Asbestos-induced release of HMGB1 from macrophages



Dostert *et al. Science.* 2008;320:674

Serum HMGB1 concentrations in cancer cases measured by ELISA

Study	Disease Type	Control mean (ng/mL)	Case mean (ng/mL)	Reference
1	Leukemia and lymphoma	0.9	3.1	Inoue 2013
2	Pancreatic	1.2	2.0	Wittwer 2013
3	Head and neck squamus cell	1.5	4.0	Wild 2012
4	Breast	2.0	4.5	Sun 2011
5	NSCLC	3.0	7.1	Niki 2017
6	Gastirc	3.9	16,5	Qiu 2014
7	Mesothelioma (pleural)	5.4	27.0	Tabata 2013
8	Hepatocellular carcinoma	7.0	84.2	Cheng 2008
9	Cervical	7.6	50.8	Sheng 2009
10	Colorectal	39.7	58.8	Lee 2012

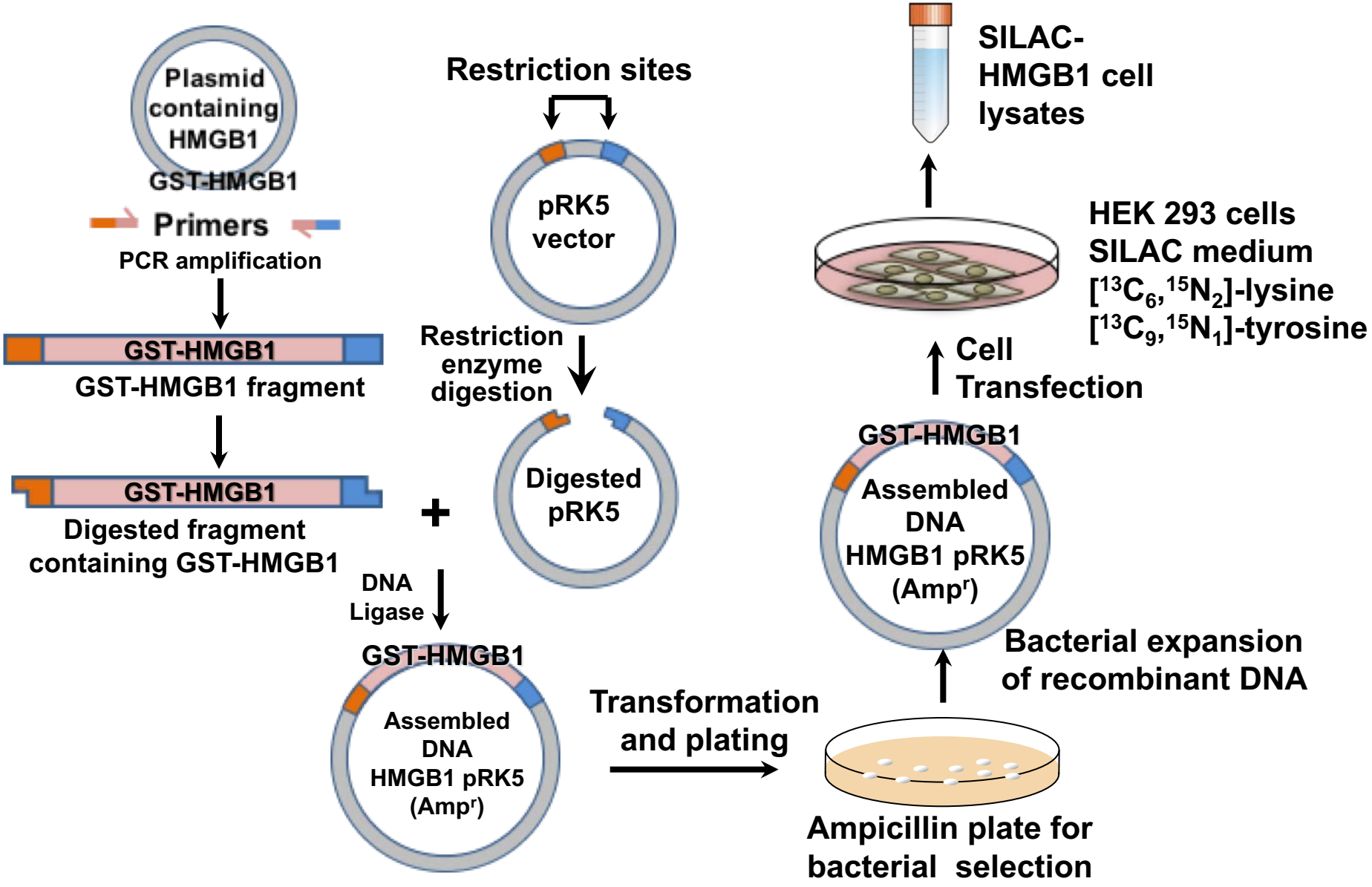
Analysis of serum HMGB1: the problems

#	Issue	Consequence	Reference
1	Serum HMGB1 autoantibodies are present in normal serum and from diseeased individuals	Serum autoantibodies compete with ELISA antibodies for HMGB1 leading to incorrect values for serum HMGB1	Sims 2010 Adulahad 2011 Hwang 2013
2	HMGB1 binds to haptoglobin, a high abundance serum protein (30-200 mg/dL)	HMGB1 binding to serum haptoglobin competes with ELISA antibodies leading to incorrect values for serum HMGB1	Yang 2015
3	Amino acid sequences of HMGB1 and HMGB2 are 74 % similar,	ELISA antibodies to HMGB1 can cross-react with HMGB2, which would give incorrect values for serum HMGB1	Yamada 2006
4	22 lysine residues in HMGB1 are reported to be acetylated	Acetylation of HMGB1 could inhibit binding to ELISA antibodies and give incorrect values for serum HMGB1 concentrations	Sterner 1979 Bonaldi 2003 Rabadi 2004 Pasheva 2015
5	Platelets contain HMGB1, which is released when they aggregate during blood clotting	Normal platelets contain approximately 3 ng of HMGB1/mL of whole blood that will contribute to concentations of serum HMGB1	Vogel 2016
6	Monocytes and lymphcytes contain HMGB1, which is released when blood clots	Monocyte and lymphocyte HMGB1 will contribute to concentrations of serum HMGB1	Maganelli 2010 Palmblad 2015 Cerwenka 2016

Analysis of serum HMGB1: the solutions

#	Issue	Potential solution
1	Serum HMGB1 autoantibodies are present in normal serum and from diseased individuals	Synthesize labeled HMGB1 and allow to equilibrate with endogenous HMGB1 bound to autoantibodies
2	HMGB1 binds to haptoglobin, a high abundance serum protein (30-200 mg/dL)	Synthesize labeled HMGB1 and allow to equilibrate with endogenous HMGB1 bound to haptoglobin
3	Amino acid sequences of HMGB1 and HMGB2 are 74 % similar,	Conduct stable isotope dilution LC-MS analysis of prteolytic peptides that are3 specific for HMGB1
4	22 lysine residues in HMGB1 are reported to be acetylated	Convert serum HMGB1 to a single molecular form by acetylation with labeled acetic anhydride
5	Platelets contain HMGB1, which is released when they aggregate during blood clotting	Compare HMGB1 in plasma containing anti-coagulant with serum HMGB1
6	Monocytes and lymphocytes contain HMGB1, which is released when blood clots	Compare HMGB1 in plasma containing anti-coagulant with serum HMGB1

Stable isotope labeling by amino acids in cell culture (SILAC) for labeled HMGB1



Glu-C digestion of HMGB1

```
          10                  20                  30                  40                  50
MGKGDPKKPR GKMSSYAFFV QTCREEHKKK HPDASVNFSE FSKKCSERWK
          60                  70                  80                  90                 100
TMSAKEKGKF EDMAKADKAR YEREMKTYIP PKGETKKKFK DPNAPKRPPS
         110                 120                 130                 140                 150
AFFLFCSEYR PKIKGEHPGL SIGDVAKKLG EMWNNTAADD KQPYEKKAAK
         160                 170                 180                 190                 200
LKEKYEKDIA AYRAKGKPDA AKKGVVKAEK SKKKKEEEED EEDEEDEEEE
    210
EDEEDEDEEE DDDDE
```

Glu-C

NLS1: E^{25}HKKKHPD^{33} H^{26}KKKHPD^{33}

K^{56}GKFE^{60} A^{170}AKKGVVKAE^{179}

NLS2: K^{180}SKKKKE^{186}

Five Glu-C peptides contain eleven of the important acetylation sites
Peptides too polar for consistent LC-MS analysis

Acetylation with labeled acetic anhydride and Glu-C digestion of HMGB1

```
          10         20         30         40         50
MGKGDPKKPR GKMSSYAFFV QTCREEHKKK HPDASVNFSE FSKKCSERWK
          60         70         80         90        100
TMSAKEKGKF EDMAKADKAR YEREMKTYIP PKGETKKKFK DPNAPKRPPS
         110        120        130        140        150
AFFLFCSEYR PKIKGEHPGL SIGDVAKKLG EMWNNTAADD KQPYEKKAAK
         160        170        180        190        200
LKEKYEKDIA AYRAKGKPDA AKKGVVKAEK SKKKKEEEED EEDEEDEEEE
     210
EDEEDEDEEE DDDDE
```

$[^2H_6]$-acetic anhydride
Glu-C

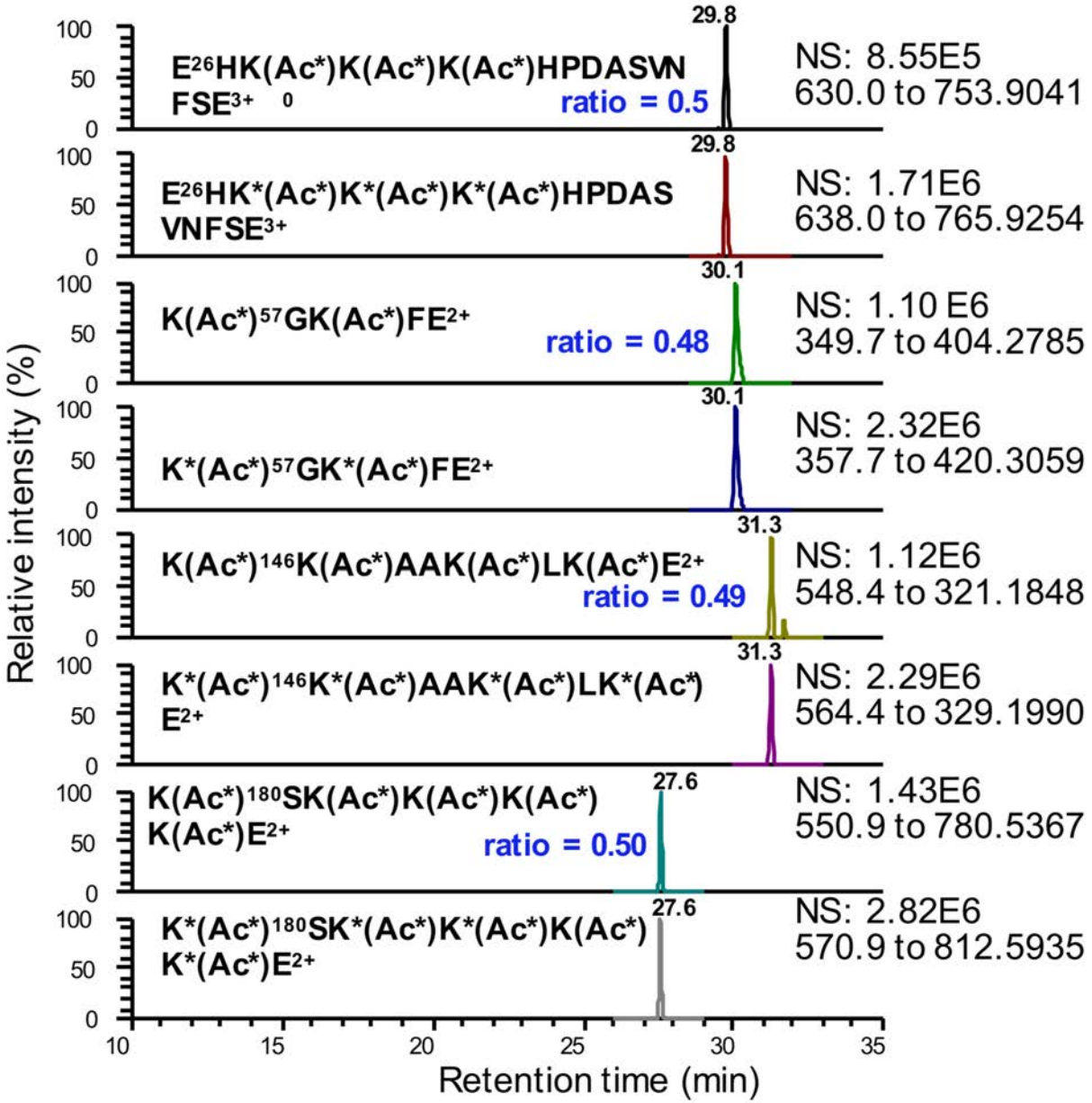
NLS1: E^{25}HK(**Ac***)K(**Ac***)K(**Ac***)HPD^{33} H^{26}K(**Ac***)K(**Ac***)K(**Ac***)HPD^{33}

K^{*56}GK(**Ac***)FE^{60} A^{170}AK(**Ac***)K(**Ac***)GVVK(**Ac***)AE^{179}

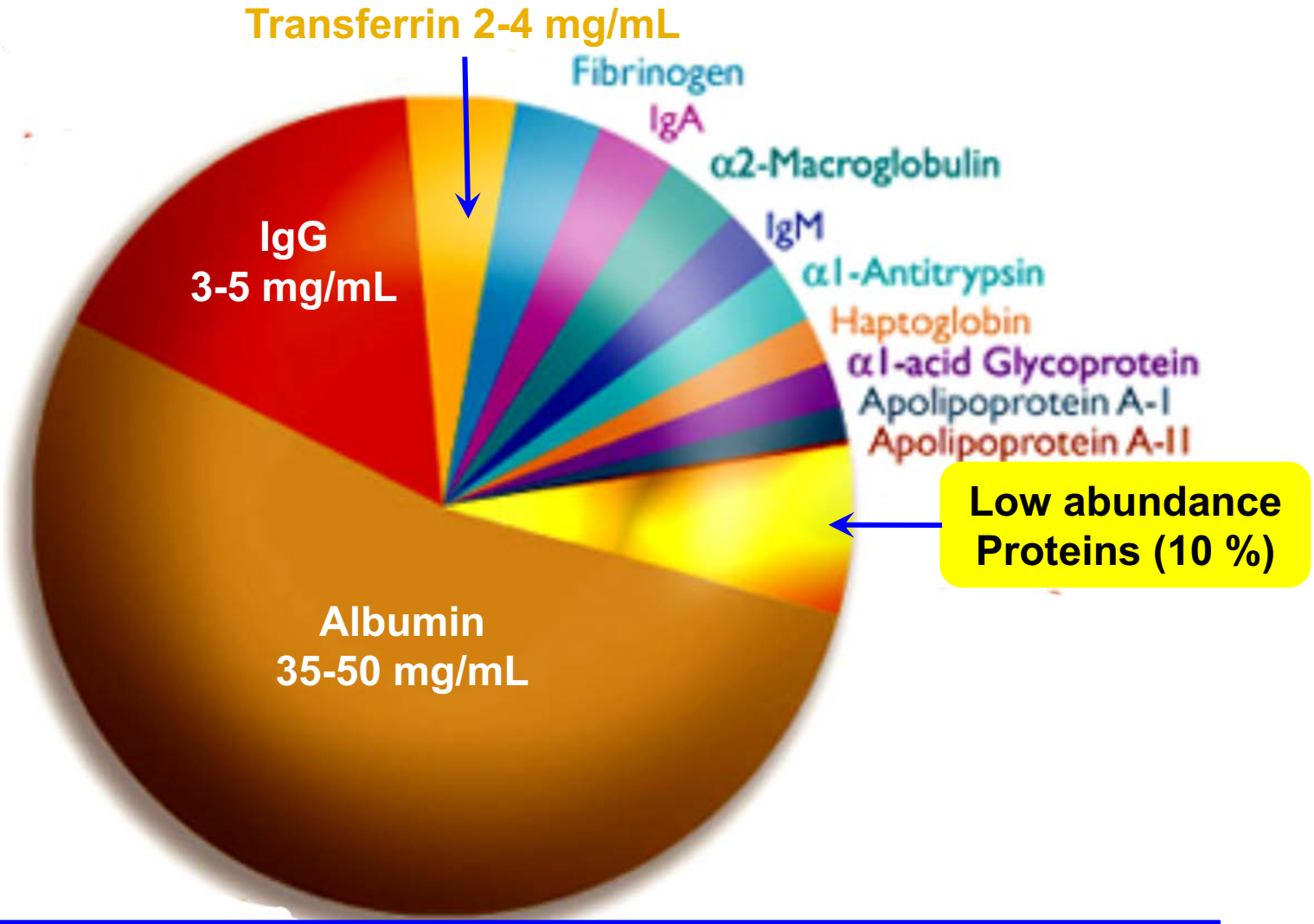
NLS2: K(**Ac***)^{180}SK(**Ac***)K(**Ac***)K(**Ac***)K(**Ac***)E^{186}

Ac* = C[$^2H_3$]CO

UHPLC-MS analysis of the SILAC-HMGB1 standard coupled with acetylation and Glu-C digestion

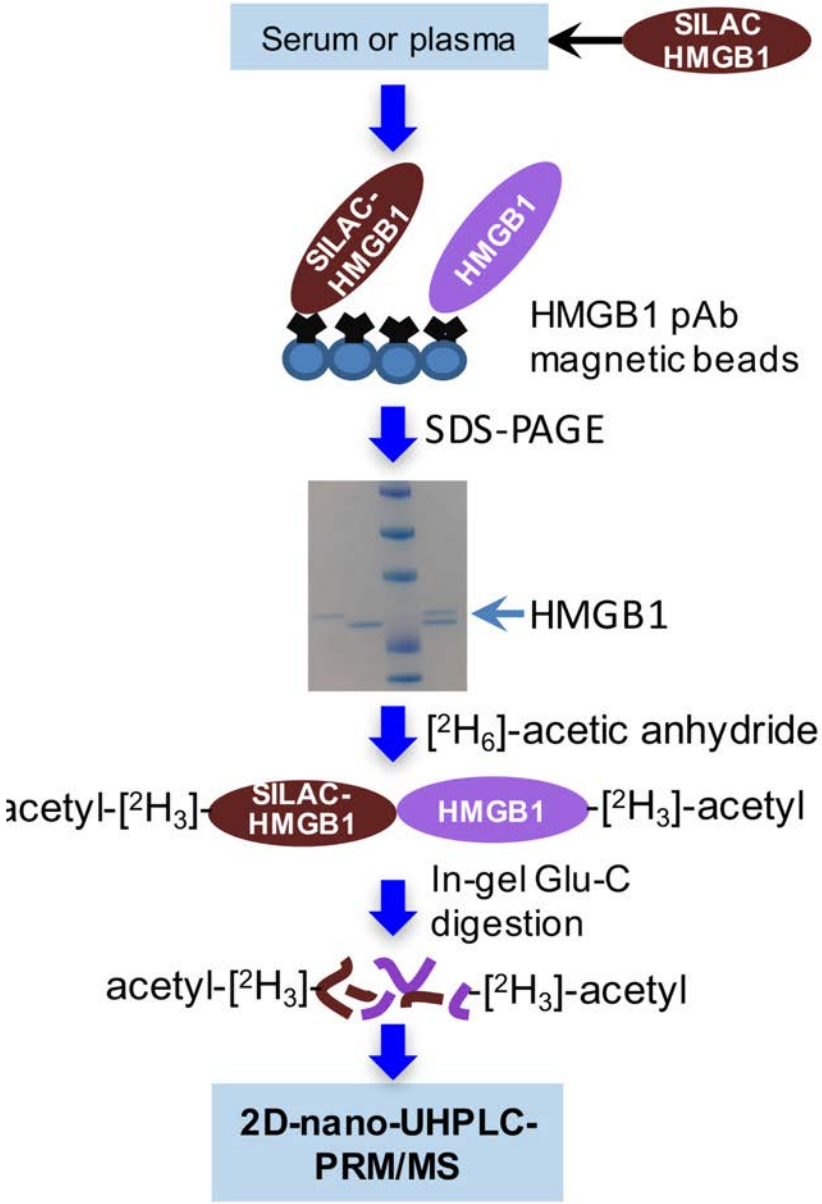


Challenges in the analysis of low abundance serum proteins

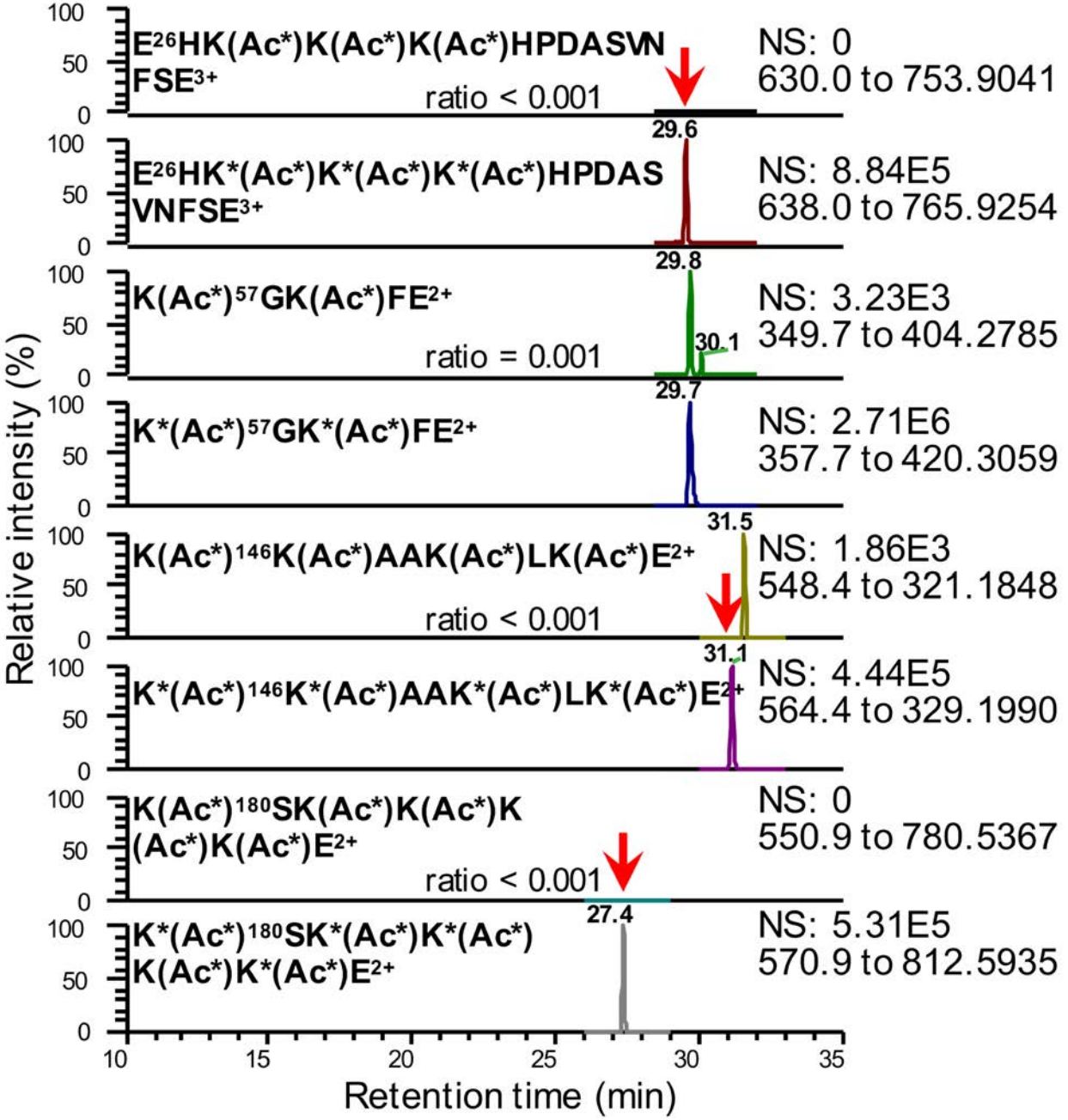


HMGB1 concentrations in healthy control serum are approximately 2.2 ng/mL or 22 x 10^6 lower than albumin

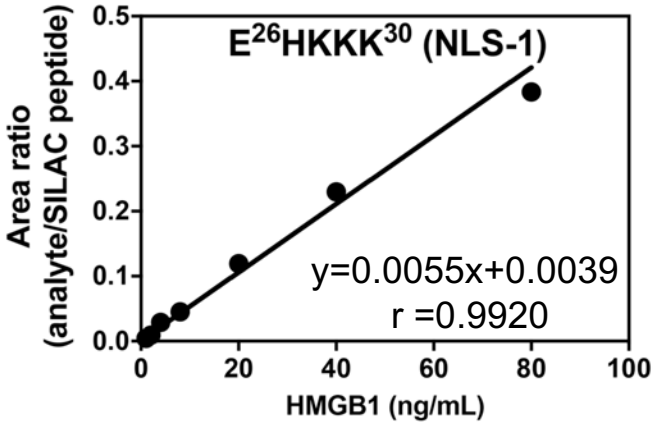
Final scheme for analysis of HMGB1 in plasma and serum

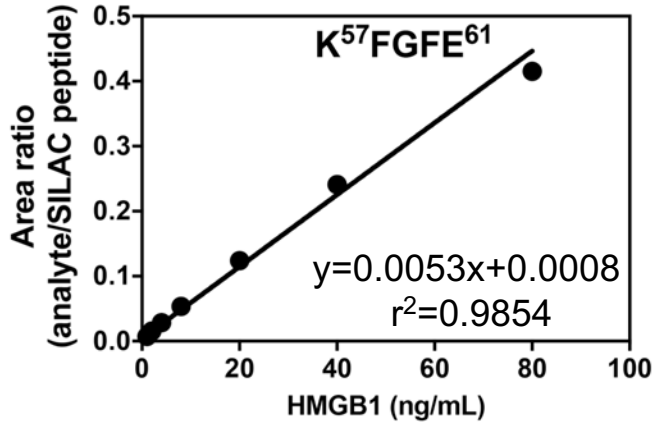


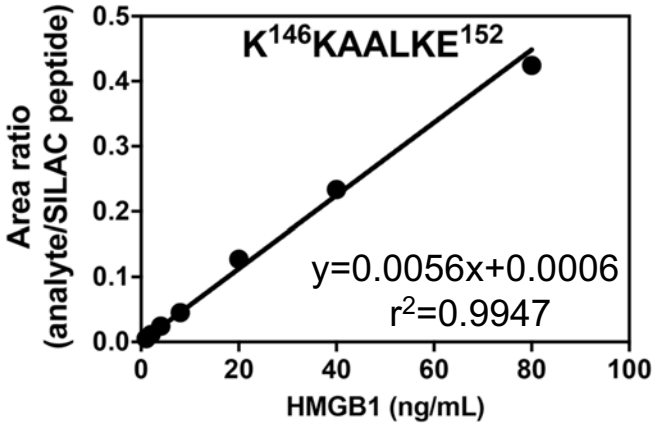
Analysis of HMGB1 in citrated plasma

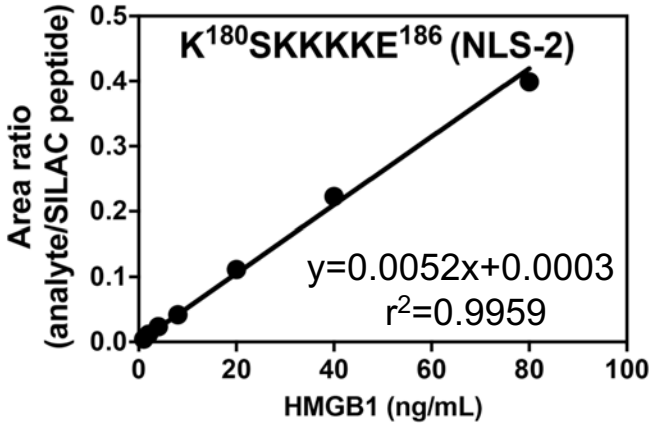


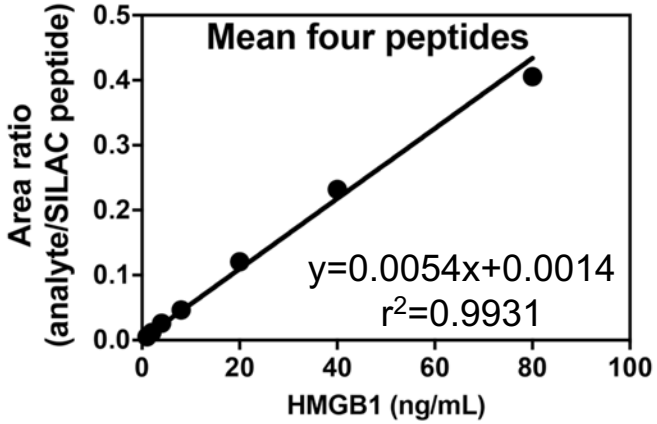
Standard curves for HMGB1 in citrated plasma



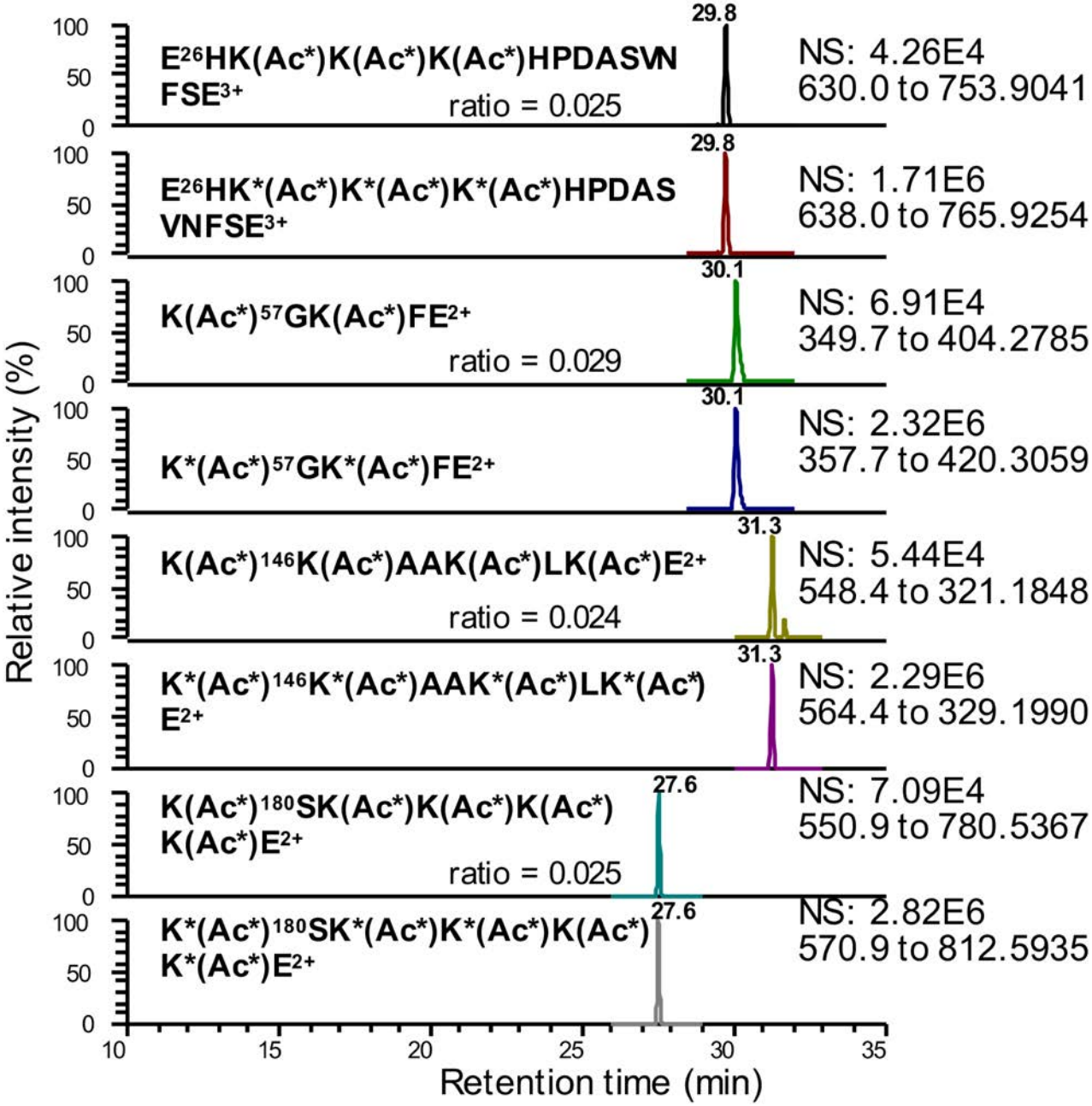




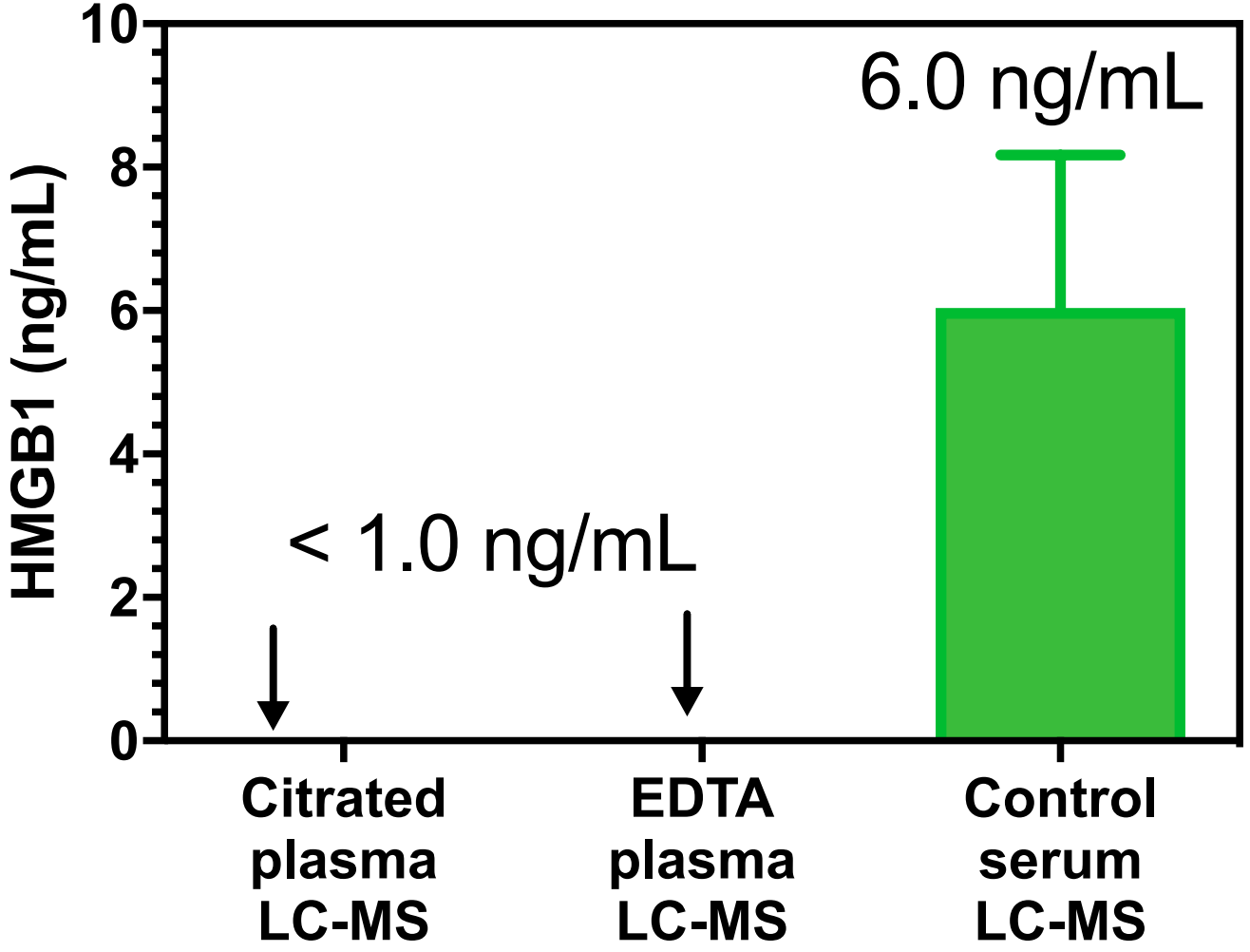




Analysis of serum HMGB1 by UPLC-HRMS



Levels of serum HMGB1 determined by stable isotope dilution UPLC-HRMS (n=24)



Big picture

- **Combining methodologies can accelerate biomarker development and molecular understanding of disease**
  - **Immunopurification** permits efficient isolation of very low abundance proteins from biofluids
  - **Mass spectrometry** provides specificity, precision, accuracy, and ultra-high sensitivity for biomarker analysis
  - **Molecular biology** provides protein internal standards and structural confirmation of protein biomarkers
- **HMGB1** is not present in plasma from healthy volunteers
- **HMGB1 is secreted** when blood is allowed to clot
  - **HMGB1** is not a circulating biomarker
  - **Serum HMGB1** studies (> 850) will have to be re-evaluated
  - **Plasma or whole blood** should be used for biomarker studies not serum
  - **Acetylation,** known to occur on 29 lysine residues, should be monitored

Acknowledgements

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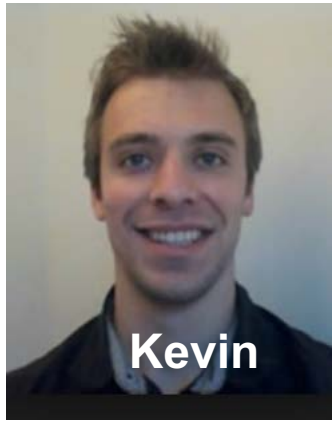

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