# From (waste) protein to bioactive functional food ingredient

Bioactive Food & Feed Ingredients (BFFI)

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From Food to Food - Sustainable valorisation of agri-food rest raw materials
14th of November - Nofima AS

# Functional foods, nothing new!!!!

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## Functional foods, nothing new!!!

- Hippocrates
  - ±460 to ±370 BC
  - the father of modern medicine

"Let your food be your medicine and let your medicine be your food"

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## Wageningen University & Research

- Combination of university departments and application-directed research institutes
- Five Sciences Groups (several departments and one institute):
  - Animal
  - Agrotechnology & Food
  - Plant
  - Environment
  - Social

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## Wageningen Food & Biobased Research

- Not-for-profit contract research organisation (CRO) of the Agrotechnology and Food Sciences Group
- Activities/Fields:
  - Sustainable innovation in the areas of healthy food, fresh produce chains and biobased materials, chemicals and energy
- > 70% of overall budget is result of acquisition of projects
- 'Customers': SMEs, multinationals, governments, EU, .....
- Business units: Fresh, Food & Chains
  Biobased Products

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## Bioactive Food & Feed Ingredients

- Activities:
  - Development of bioactive peptides / hydrolysates for the prevention or reduction of cardiovascular and diabetes complications
  - Antimicrobial compounds (peptides and other ingredients)
  - Antioxidant compounds (low-grade inflammation)
  - Identification and purification of added value compounds from waste organs / materials

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## Bioactive Food & Feed Ingredients

- Expertise:
  - *In vitro* hydrolysis of high-potential proteins
    - Over 20 different proteases and protease mixtures have been used in optimizing the bioactivity of proteins.
  - Development/Application of miniaturized *in vitro* bioassays for selected enzymes (drug targets):
  - Antioxidant assays such as the DPPH and ORAC tests; prevention of 'low grade inflammation'.
  - *In vitro* cell assays to test uptake, bioavailability and/or biofunctionality

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## functional foods


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## Market of functional foods / neutraceuticals

- Trends in pharma and food industries


  - **Pharmaceutical** example:
    - captopril; ACE-inhibiting drug; blood pressure reduction
  - **Nutraceutical** example (application in a clinical setting):
    - IgA in whey powder; neutralising *Clostridium difficile*
  - **Functional food** example:
    - drink with antihypertensive peptides, protein-derived

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## Market of functional foods / nutraceutricals

- Average age is increasing
- Increased risk of diseases such as CVD, CHD, obesity (MBS), cancer, .....
- Some foods play a role in the onset of diseases

- However, functional foods can be applied in the prevention of diseases
  - The global functional foods market is growing at a compound annual growth rate (CAGR) of roughly 6% (Ref: MarketsandMarket)
- Challenge: Development of functional foods that contribute to a better and healthier life

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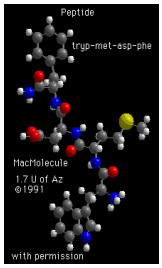
## Disease (prevention) targets

- (Food) protein hydrolysates (processed or via *in* vivo digestion) contain bioactive peptides
- High potential application areas:
  - Antihypertensive activity (drop of blood pressure)
  - Cholesterol / lipid reduction
  - Improving insulin resistance (diabetes) → **Metabolic Syndrome**
  - Reduction of inflammation markers
  - Circumference of waist (obesity)
  - Anti-oxidant activity
  - Cognitive function and stress

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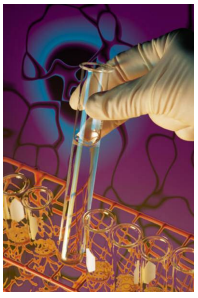
## Functional foods - Considerations

- Bioactivity foreseen in GI-tract or in systemic circulation?
  - Length of bioactive peptides relevant criterion
- Mild activity; pharmaceutical application not intended
- Availability of good biomarker(s)
  - Relevant to the *in vivo* situation
- Expected toxicological implications
  - Which parameters should be studied

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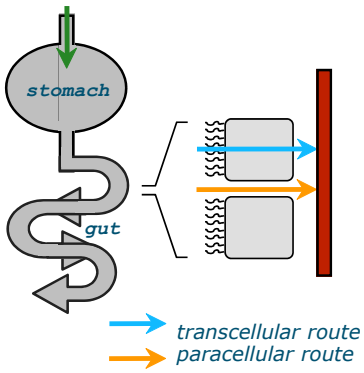
## Functional foods - Considerations

- For systemic application:
  - 'The smaller, the better'
  - Bioactivity in GIT allows larger peptides to be applicable
- Smaller: higher chance of presence in source protein
  - Chance decreases with $n^{20}$




- Smaller: better oral availability
  - >98 % arrives in blood as mono-/di-/tri-peptides

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## Functional foods - Considerations

- Why peptides?
  - Many endogenous bioactives are peptides: neurotransmitters, hormones, enzyme inhibitors
  - Broad range of activities
  - No (?) accumulation-related toxicity
- Which peptides are relevant ?
  - Active in gastro-intestinal tract (GIT)
  - *Or:* small enough to be taken up actively in systemic circulation
  - *Or:* sufficient passive uptake
- Systemic activity: *The smaller, the better !*

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## Functional foods - Considerations

- Many bioactive peptides in proteins are there by coincidence (ACE inhibitors, bile acids/cholesterol binders)
- However: Concentration sufficient for *in vivo* activity?
  - Occurrence increases exponentially with decreasing peptide length
    - Chance to exactly match a bioactive sequence is much higher for smaller peptides
  - Release of bioactive peptides by normal, endogenous digestion (pepsin, (chymo)trypsin, etc.) is far from optimal
    - Normal digestion should deliver free amino acids; building blocks for the body
- Therefore, *in vivo* activity is often not to be expected

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## Strategy


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## Strategy

- Strategy:
  - Search for high-potential protein sources by an *in silico* approach
  - *In vitro* hydrolysis of high-potential proteins
  - Development/Application of miniaturized *in vitro* bioassays for selected enzymes/proteins
  - Testing/Optimizing hydrolysates for enzyme-inhibiting activity
  - If necessary: partial purification of active peptides

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## Strategy

- Strategy continued:
  - *In vivo* experiments with hydrolysates that show significant inhibiting activity; animal and, if successful, human studies (in collaboration)
  - Design and formulation of functional food products
    - Peptides have a bitter taste, addition of aroma and or masking compounds is needed

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## Strategy

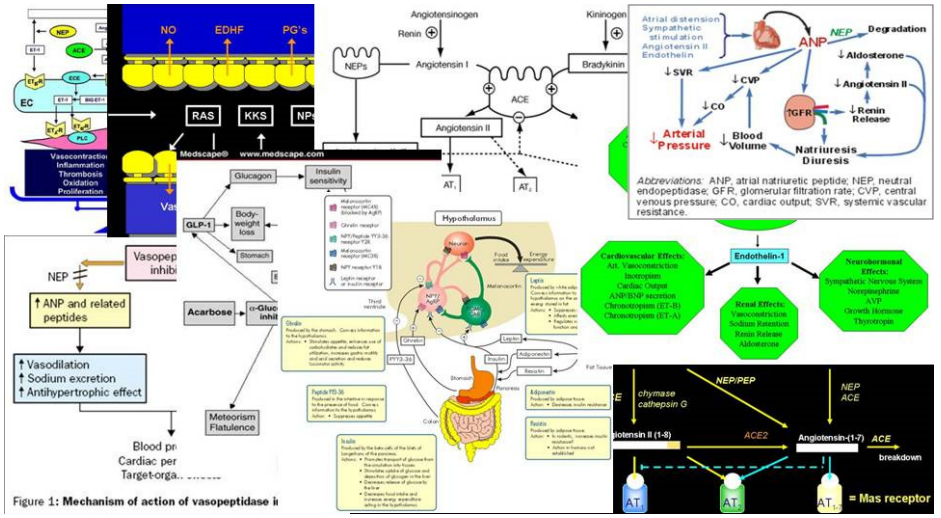


Figure 1: **Mechanism of action of vasopeptidase i**

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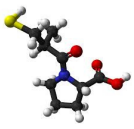
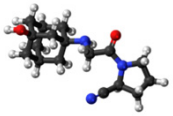
## Strategy

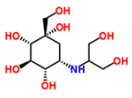
- NB: targets of drugs; chosen by the pharmaceutical industry
- Examples:
  - Angiotensin-Converting-Enzyme (ACE) inhibitors such as Captopril
  - DiPeptidyl Peptidase-4 (DPP4) inhibitors such as Vildagliptin
  - α-glucosidase inhibitors such as Voglibose



Captopril



Vildagliptin




Voglibose

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## From protein to functional food ingredient

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## Potential protein sources

- Sources (bulk, economically feasible):
  - Animal: structure and mobility proteins (milk, egg, collagen, keratin, muscle, .....)
  - Plant: storage and structure proteins (soy, wheat, pea, maize, oat, rice, ......)
  - Waste streams (animal, plant, ....)


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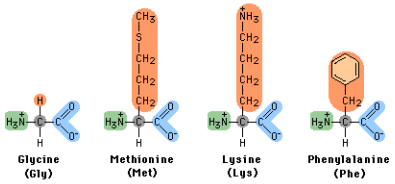
# Amino acids: Building blocks of proteins

- Amino acid characteristics:
  - 20 naturally occurring
  - Some derivatives occurring
  - Identical basic structure
  - Different R-groups (neutral, hydrophobic, positive, negative)

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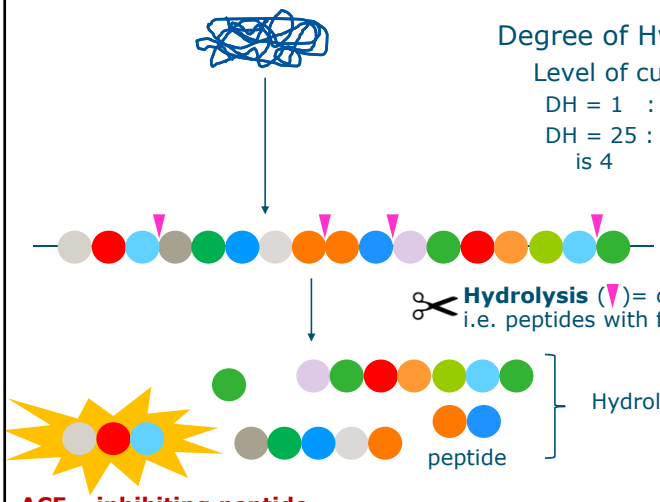
| Name | Abbrev. | | Molecular weight |
|---|---|---|---|
| **NEUTRAL + HYDROPHOBIC** | | | |
| Alanine | Ala | A | 89 |
| Proline | Pro | P | 115 |
| Valine | Val | V | 117 |
| Leucine | Leu | L | 131 |
| Isoleucine | Ile | I | 131 |
| Methionine | Met | M | 149 |
| Phenylalanine | Phe | F | 165 |
| Tryptophan | Trp | W | 204 |
| **ACIDIC** | | | |
| Aspartic Acid | Asp | D | 133 |
| Glutamic Acid | Glu | E | 147 |

| Name | Abbrev. | | Molecular weight |
|---|---|---|---|
| **NEUTRAL + POLAR** | | | |
| Glycine | Gly | G | 75 |
| Serine | Ser | S | 105 |
| Threonine | Thr | T | 119 |
| Cysteine | Cys | C | 121 |
| Asparagine | Asn | N | 132 |
| Glutamine | Gln | Q | 146 |
| Tyrosine | Tyr | Y | 181 |
| **BASIC** | | | |
| Arginine | Arg | R | 174 |
| Histidine | His | H | 155 |
| Lysine | Lys | K | 146 |

essential amino acids are shown on coloured background

# Hydrolysis of proteins with proteases




Degree of Hydrolysis (DH)

Level of cutting

DH = 1 : intact chain

DH = 25 : average peptide length is 4

**Hydrolysis** ( )= cut proteins in smaller parts
i.e. peptides with foodgrade proteases

Hydrolysate (mixture of peptides)

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## Peptide preparation routes

- Isolation from natural resources
- 'Anabolic' route -> Antimicrobials
  - Biotechnological production
  - Chemical synthesis
- 'Catabolic' route -> Functional ingredients / Nutraceuticals
  - Proteolysis of bulk proteins accepted way of processing in food industry
    - At least nutritional value
    - Added value: based on particular bioactivity


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## Suitable proteins: rich in bioactive peptides

- Classical method: empirical
  - Labour-intensive, 'trial and error'
- Rational method: application of bio-informatics
  - Rapid *in silico* screening
  - Requires sequences & software

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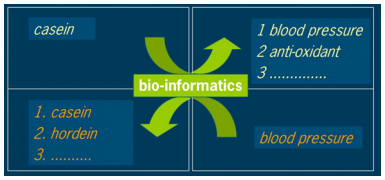
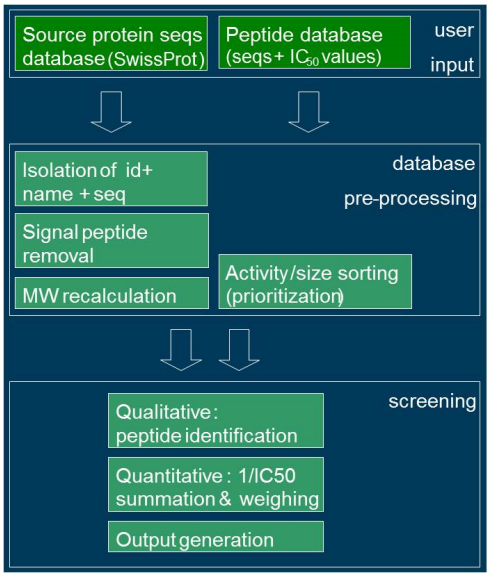
## WFBR bioinformatics screening software

- Procedure to screen protein (and DNA) databases against bioactive peptides (1995-2005):

- Possible searches:

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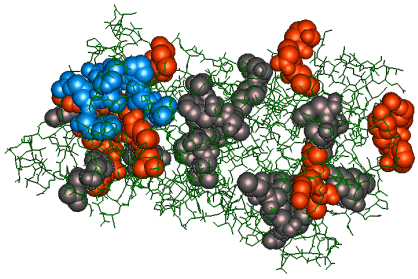
## Hydrolysis of proteins with proteases

- Example in silico screening : ß-Lactoglobulin
  - Part of amino acid chain (total 178 residues)
    - *In vivo* released peptides may result in blood pressure decrease



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## BIOPEP database

- Biopep database
  http://www.uwm.edu.pl/biochemia/index.php/pl/biopep

→ Contains many bioactivity properties of peptides

| | | | | | | |
|---|---|---|---|---|---|---|
| Peptide Data | 2617 | synthetic fr. of human beta-CN (99-101) ACE inhibitor | VMP | 345.4480 | 345.1604 | 29.00 |
| Peptide Data | 2618 | synthetic fr.of human beta-CN (97-101) ACE inhibitor | GRVMP | 558.6880 | 558.2824 | 250.00 |
| Peptide Data | 2619 | synthetic fr.of human beta-CN (92-101) ACE inhibitor | TVYTKGRVMP | 1151.3750 | 1150.6014 | 38.00 |
| Peptide Data | 2620 | synthetic fr.of human beta-CN (77-81) ACE inhibitor | PVPQP | 536.6150 | 536.2820 | 110.00 |
| Peptide Data | 2621 | synthetic fr.of human beta-CN (72-77) ACE inhibitor | PAVVLP | 594.7390 | 594.3610 | 45.00 |
| Peptide Data | 2622 | synthetic fr.of human beta-CN (65-68) ACE inhibitor | NILP | 455.5410 | 455.2629 | 560.00 |
| Peptide Data | 2623 | synthetic fr.of human beta-CN (63-68) ACE inhibitor | PQNILP | 680.7890 | 680.3729 | 440.00 |

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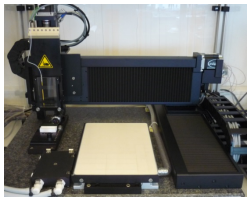
## Miniaturized microtiter plate assays

- Assays available for the human enzymes:
  - ACE; cardiovascular system; blood pressure
  - DPP4; glucose metabolism; insulin action
  - α-Glucosidase; glucose absorption in intestine
  - CETP
  - Lp-PLA2
  - COX
- Readily introduced:
  - eNOS
  - NADH Oxidase
  - sPLA2

Robotic liquid handling system for automated enzyme / immunochemical assays



Microarrayer for printing proteins on substrates; volumes down to 100 pL

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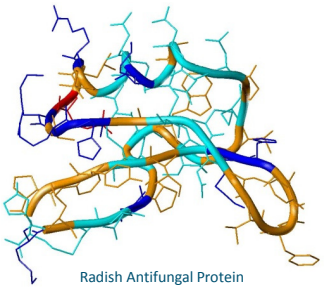
## Miniaturized microtiter plate assays

- Antimicrobial compounds (peptides); various projects executed such as an EU project on alternative AMPs in food products
  - Alternatives to chemical antibiotics and inspired by increasing antibiotic resistance
  - Miniaturised microbial assays (bacteria, fungi, yeasts) and toxicity assays with eukaryotic cells (e.g. erythrocytes)
- Other antimicrobial compounds (such as glycosinolates and alkaloids) can be tested as well

- AIR2-CT94-1356 (1994-1998) Agro-Industrial applications of antifungal proteins from plant seeds
- EU FAIR CT97-3135 (1996-2000) Antimicrobial peptides - Studies Aimed at Application in Food and Food Products



Radish Antifungal Protein

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## Miniaturized microtiter plate assays

- Antioxidant compounds in relation to 'low-grade inflammation'; various diseases linked to this status
  - Antioxidant *in vitro* assays available


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## ACE mechanism

- Angiotensin Converting Enzyme (ACE) converts angiotensin I to angiotensin II => blood pressure ↑

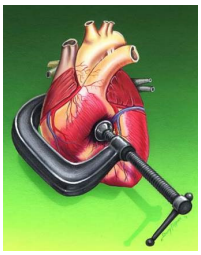
DRVYIHPFHL ----------> DRVYIHPF + HL

angiotensin I ACE angiotensin II (hypertensive)

Inhibition of ACE: antihypertensive effect = bloodpressure ↓

DRVYIHPFHL ----------> X

angiotensin I ACE



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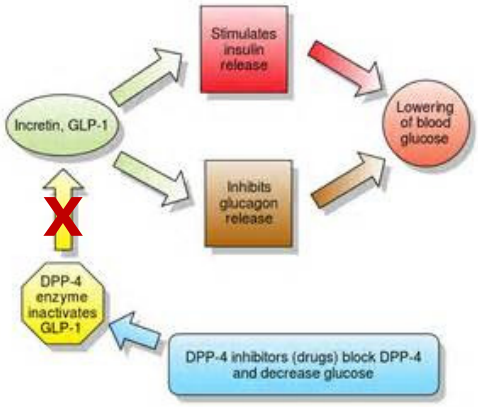
## DPP4 mechanism

- GLP-1 is inactivated by DiPeptidyl Peptidase-4 (DDP4)
- ↑GLP-1 → ↑insulin → faster uptake of glucose from the blood

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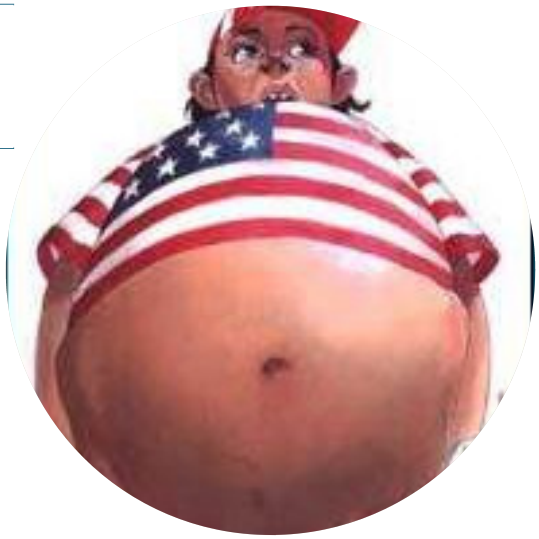
# Metabolic syndrome



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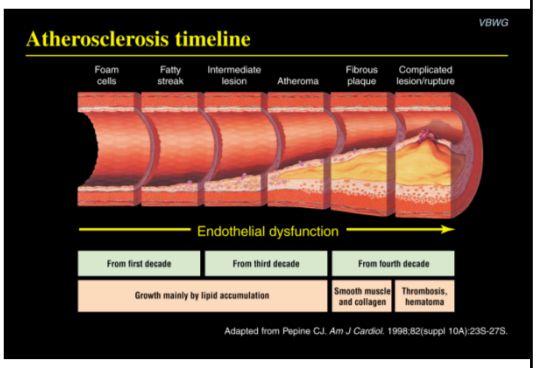
# Metabolic syndrome

- The Metabolic Syndrome constitutes significant risk for artherosclerosis
  - Atherosclerosis major contributor to cardiovascular disease (CVD)
  - CVD major cause of death (33%) in The Netherlands

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# Metabolic syndrome

- Also a risk factor for Type 2 Diabetes mellitus (T2DM)
  - Diabetes is a chronic metabolic disorder that affects about 8.5% of the population in Europe (in 2010 source: www.idf.org/diabetesatlas) and accounts for over $100 billion in medical costs
  - Type 2 diabetes, a metabolic disorder (± 90%; generally after the age of 40) involves progressive development of insulin resistance leading to hyperglycemia

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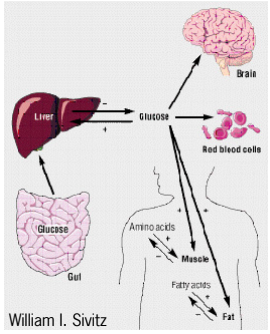
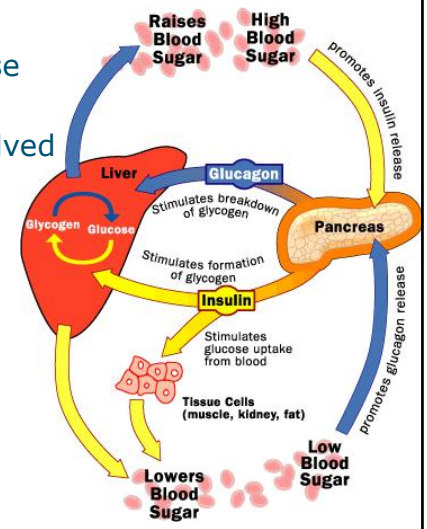
# Metabolic syndrome

- Type 2 Diabetes mellitus
  - Regulation of blood glucose levels
  - Major organ systems involved

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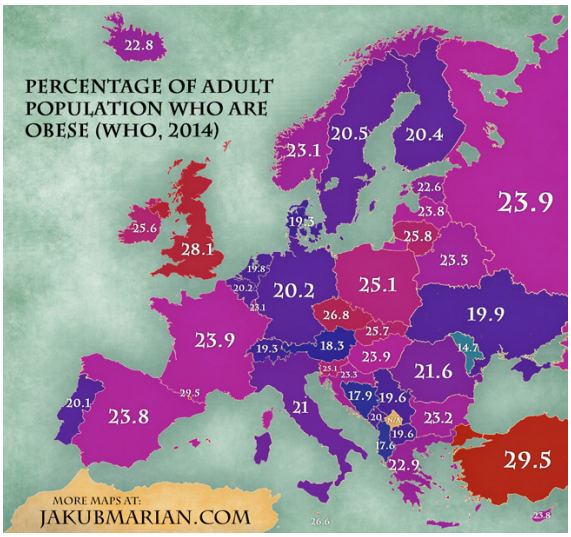
## Obesity in Europe

- Obese: BMI > 30




BMI = Body Mass Index

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## Metabolic Syndrome

- How to combat risk factors?
  - Drugs
  - Physical activity
  - Reduced caloric intake
  - Consumption of mildly bioactive (preventive) functional foods/food ingredients

"Metabolic Syndrome may be treatable with Malaria drug"

- However, complex relationships exist between metabolic pathways involved in the regulation of the various risk factors of the MBS: Which enzymes/proteins to target?

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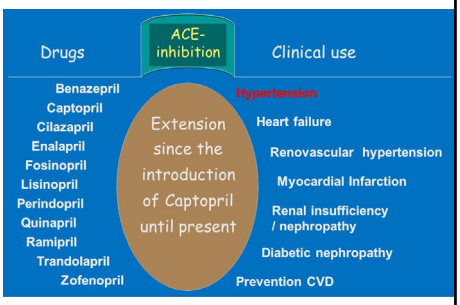
## Combat the Metabolic Syndrome

- ACE is a regulator in blood pressure homeostasis
- ACE-inhibiting drugs also show beneficial effects on:
  - angiotensin-II ↓
  - bradykinin ↑
  - hypertension ↓
  - endothelial function ↑
  - insulin-resistance ↓
  - inflammatory responses ↓
  - individual health profile ↑





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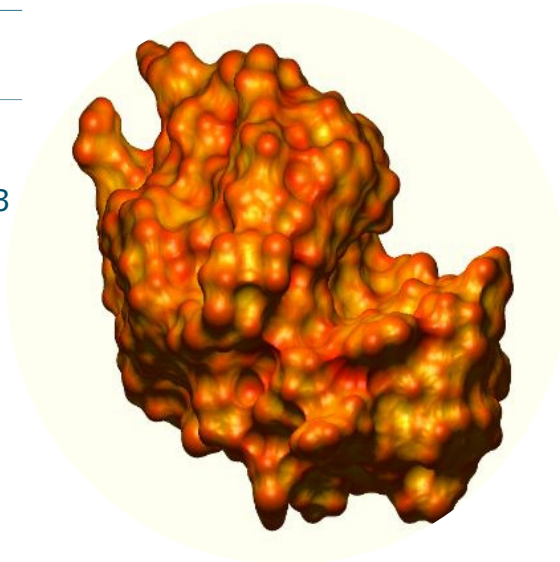
## Example

Development of NWT-03 from lysozyme



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## Development of NWT-03

- Several sequential projects (started in 2003, still ongoing) in collaboration with:
  - Newtricious BV
  - Clinical Pharmacology (University of Groningen)
  - Maastricht University
  - Aroma Uden BV
  - Bouwhuis Enthoven BV
- Projects:
  - EU EggPressure
  - FND metabolic sydrome
  - FND NWT03
  - Characterization

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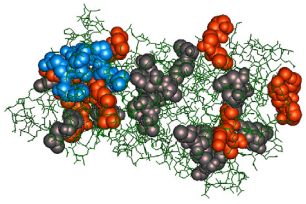
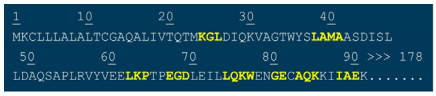
## Example: antihypertensive egg hydrolysate

- *In silico* score with priority on shortest peptides
  - The lower the value, the better the theoretical activity






Example: beta-lactoglobulin

- Of all egg proteins lysozyme had the best score

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## Development of NWT-03

- Hydrolysis of lysozyme with approximately 20 different food grade proteases and protease mixtures
  - Percentage and hydrolysis time optimized
- Sources of enzymes: bacterial, fungal, plant, mammalian
- Characterization / Purification of hydrolysates by:
  - C18 reverse phase HPLC (analytical and preparative)
  - Size-Exclusion Chromatography (specific for peptide separation)
  - Cation-/Anion exchange chromatography
  - Membrane ultra- and nano-filtration
  - LC-Mass-Spectrometry analyses to identify peptide amino acid sequences

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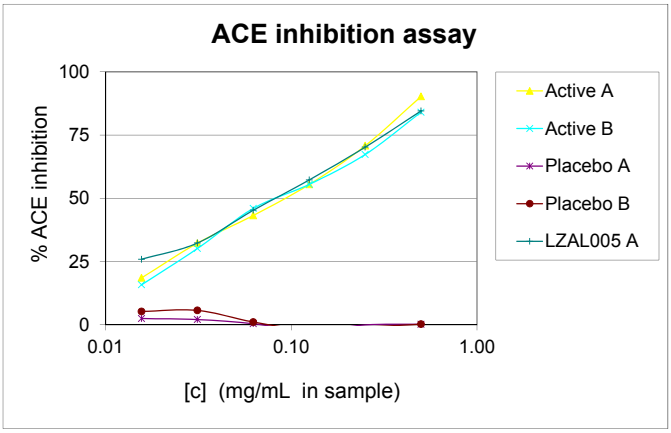
## NWT-03: ACE inhibition assay

- IC50 ACE inhibition = 0.085 mg/mL

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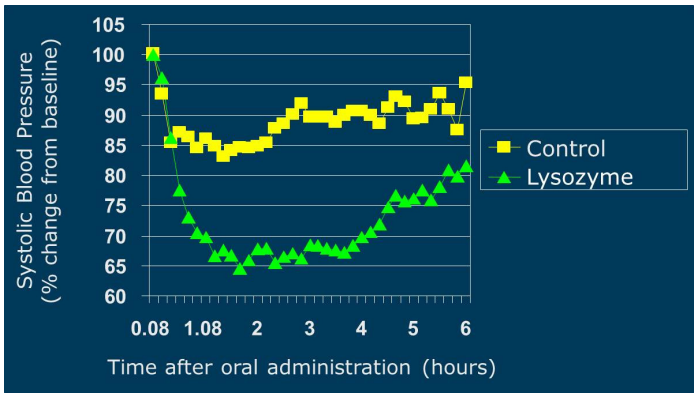
# Example: antihypertensive egg hydrolysate

- Acute effect of Lysozyme hydrolysate (NWT-03) on systolic blood pressure in anaesthetized Spontaneously Hypertensive Rats (SHR)

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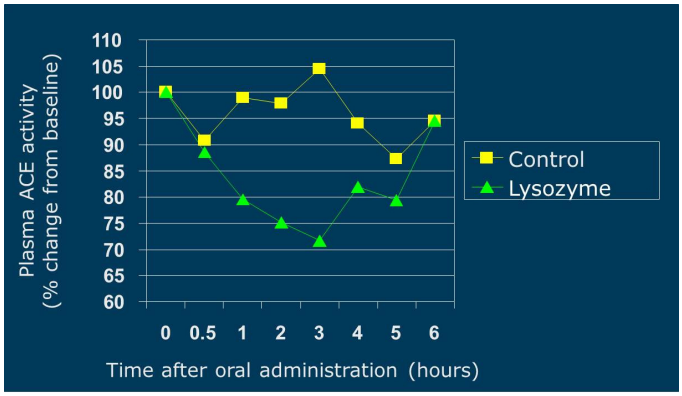
Part of this study has been executed in the framework of EU-CRAFT EGGPRESSURE, QLKT1-CT-2002-71943

# Example: antihypertensive egg hydrolysate

- Acute effect of Lysozyme hydrolysate (NWT-03) on plasma ACE activity in anaesthetized Spontaneously Hypertensive Rats (SHR)

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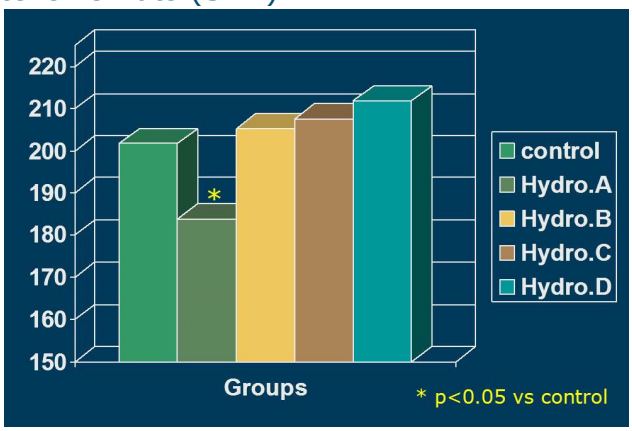
## Example: antihypertensive egg hydrolysate

- Long-term (2 months) effect of Lysozyme hydrolysate (A; NWT-03) on systolic blood pressure in Spontaneously Hypertensive Rats (SHR)

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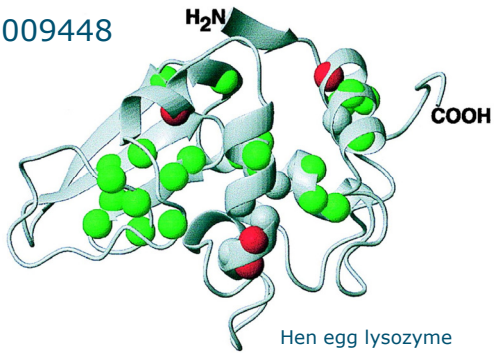
Part of this study has been executed in the framework of EU-CRAFT EGGPRESSURE, QLKT1-CT-2002-71943

## Example: antihypertensive egg hydrolysate

- Patent application:
  - Antihypertensive functional food products
  - Amerongen, A. van; Beelen, M.J.C.; Bent, A. van der; Buikema, J.H.; Gilst, W.H. van; Loonen, M.H.J.; Merck, K.B.; Nelissen, J.; Thielen, W.J.G.; Togtema, K.A.
  - Patent nr. WO 2006/009448




Hen egg lysozyme

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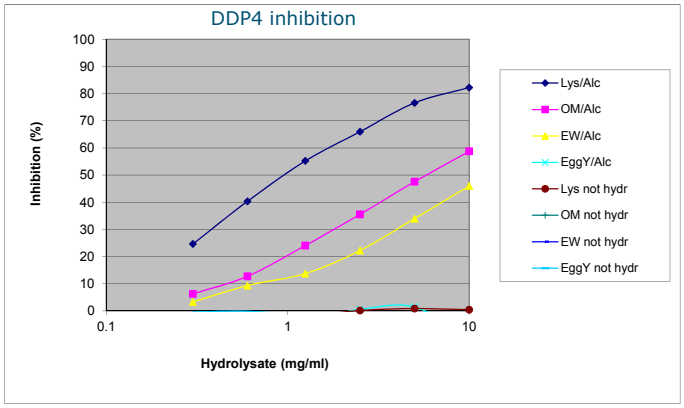
# NWT-03 also inhibitor of DDP4

- Hydrolysate has inhibitory effect on both ACE and DDP4 activity

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# Purification & characterization

- Further purification of active peptides is only possible if a cost-efficient process can be developed
  - Often not cost-efficient
  - Full hydrolysate used as functional food ingredient
- Purification and characterization of the hydrolysate can be used for:
  - IP purposes
  - Quality Assurance of the product

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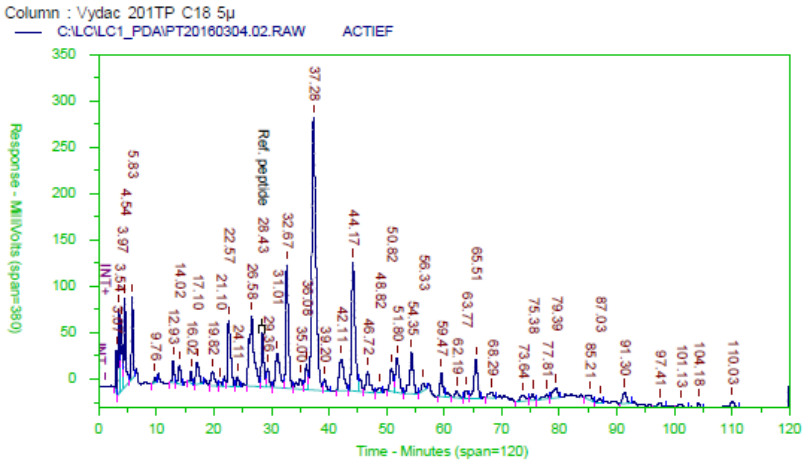
# Example: lysozyme hydrolysate

- Purification of enzyme inhibiting peptides

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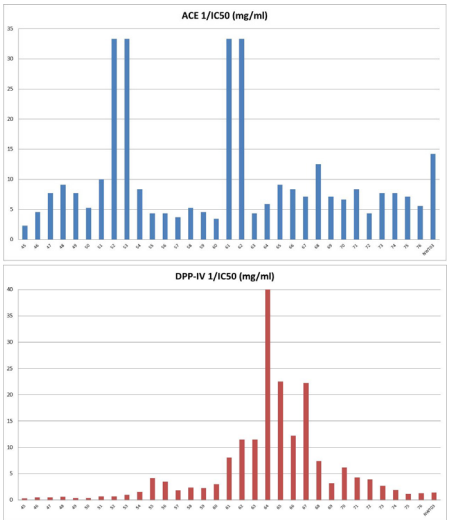
# Example: lysozyme hydrolysate

- Purification of enzyme inhibiting peptides from the NWT-03 hydrolysate
  - Column fractions tested in miniaturized inhibition assays for Angiotensin Converting Enzyme (ACE) and DiPeptidylPeptidase-4 (DPP4)

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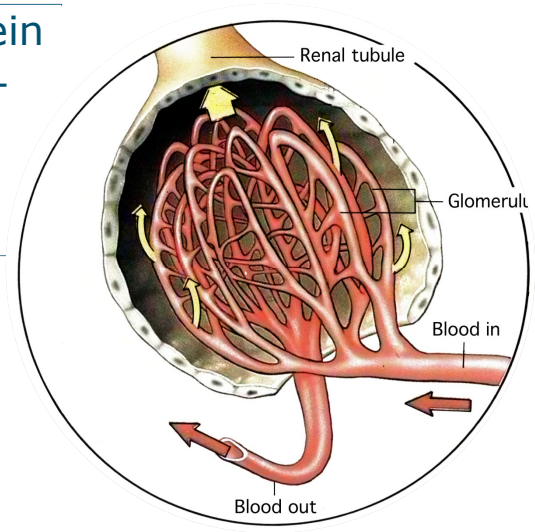
## Effect of egg protein hydrolysate (NWT-03) on renal function

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## Effect of NWT-03 on renal function

- Effect NWT-03 and drug vildagliptin on renal function in Zucker Diabetic Fatty (ZDF) rats

Wang Y, Landheer S, van Gilst WH, van Amerongen A, Hammes H-P, Henning, RP, Deelman, LE, and Buikema, H. (2012). Attenuation of Renovascular Damage in Zucker Diabetic Fatty Rat by NWT-03, an Egg Protein Hydrolysate with ACE- and DPP4-Inhibitory Activity. PLoS ONE 7(10): e46781

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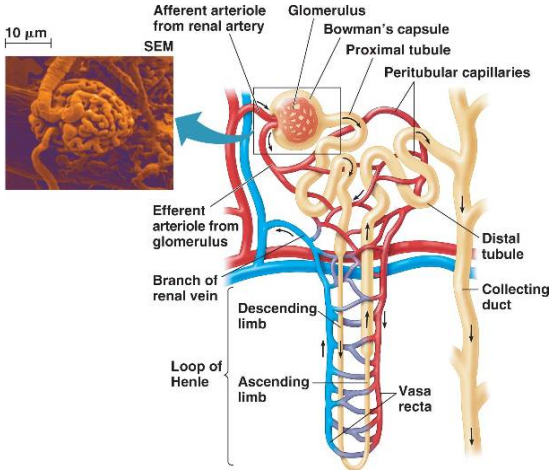
# Effect of NWT-03 on renal function

## Focal glomerulosclerosis (FGS)

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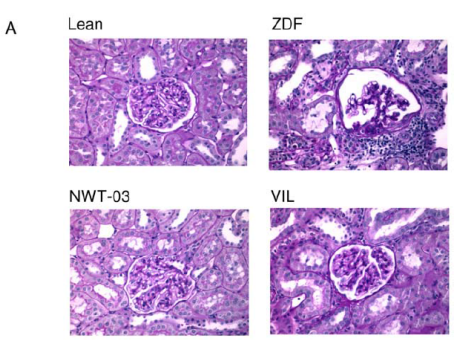
# Effect of NWT-03 on renal function

- Beneficial effect of NWT-03 on focal glomerulosclerosis (FGS) as compared to untreated ZDF rats



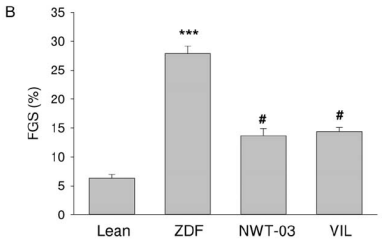




**Figure 3. Renal morphology and FGS.** (A) Shown are representative sections for lean control rats (top left) and untreated ZDF rats (top right), and ZDF rats supplemented with NWT-03 (bottom left) or treated with VIL (bottom right). (B) FGS (see Methods); data are means ± SEMs (***p<0.001 vs. lean; #p<0.05 vs. ZDF).
doi:10.1371/journal.pone.0046781.g003

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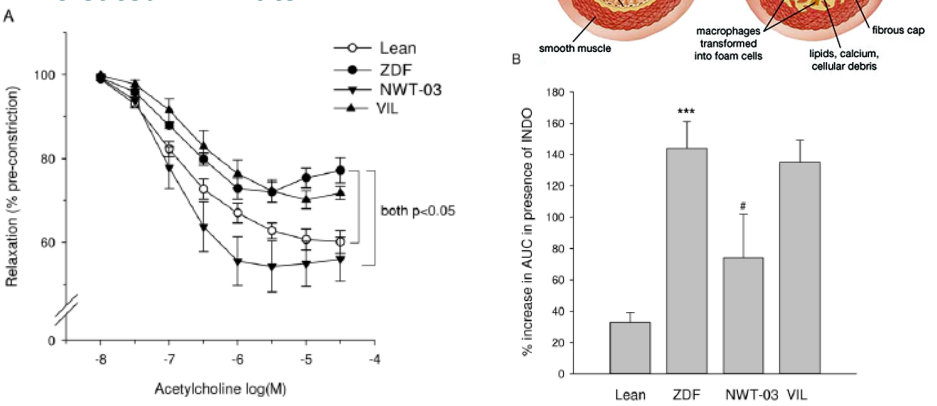
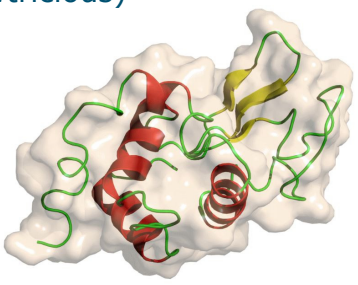
# Effect of NWT-03 on renal function

- Beneficial effect of NWT-03 on aorta endothelium-dependent relaxation (EDR) as compared to untreated and vildagliptin-treated ZDF rats




# Egg lysozyme hydrolysate (NWT-03)

- Next human trial ongoing (Newtricious)



- Market introduction is expected by the end of 2017

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## NWT-05: Ovomucin mouth spray

- Project on NWT-05 (ovomucin): Mouth spray for dry-mouth-syndrome
- Sjögren syndrome (auto immune disease)
- Symptoms:
  - Hyposialy (less secretion of saliva)
  - Xerostomy (dry mouth feeling)
  - Dry eyes
- Prevalence: 1-1.5%
- Other important causes of less secretion of saliva and dry mouth symptoms:
  - Radiotherapy head-neck area
  - Side effects of (multiple) medication

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## Acknowledgements

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- Tjesca Eilert

**Maastricht University**
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**Aroma Uden BV**
- Esther Keepe

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# Questions?

Thank you for your attention!


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