



Universiteit
Leiden
The Netherlands

Keyhole limpet hemocyanin challenge model for studying adaptive immune system responses in early-phase clinical drug development

Saghari, M.

Citation

Saghari, M. (2024, December 17). *Keyhole limpet hemocyanin challenge model for studying adaptive immune system responses in early-phase clinical drug development*. Retrieved from <https://hdl.handle.net/1887/4172487>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/4172487>

Note: To cite this publication please use the final published version (if applicable).

CHAPTER I

GENERAL INTRODUCTION

Autoimmune diseases affect around 5–8% of the global population. At present over 80 different disorders have been identified, categorized as either organ specific or systemic.¹ They cause significant patient distress and pose a substantial socioeconomic challenge. Although our understanding of the intricate mechanisms behind certain autoimmune diseases has grown, the different factors driving them remain poorly comprehended, including environmental triggers and pathogenesis. Most currently used immunomodulatory drugs for the treatment of autoimmune diseases lack specificity and are associated with side effects due to broad-spectrum effects, including malignant disease and infection.¹ Additionally, a significant proportion of patients either do not respond optimally or do not respond at all to these treatments. Therefore, there is an urgent need to develop new drugs based on a molecular and clinical comprehension of specific autoimmune diseases.

The immune system, and more specifically the adaptive immune system, is a key area for the development of such new treatment strategies, particularly for managing infections, tumors, and autoimmune diseases that are resistant to conventional therapies. However, due to the lack of biological understanding only 13.8% of all drug development programs advance from phase I clinical trials to market registration across all therapeutic areas and for inflammatory and autoimmune treatments merely 6.3% reach commercial approval.² Currently the prices for new drugs are significantly influenced by high costs during drug development (approximately 2.7 billion USD for each novel immunomodulatory drug approved by the US Food and Drug Administration).³ Nearly 60% of the developmental costs of investigational medicinal products (IMPs) is attributed to failure, with 60% to 80% of this failure due to insufficient efficacy observed at later stages during drug development. Hence, there is a necessity for more rational strategies in early drug development, such as adopting question-based drug development including biomarkers or utilizing a quantitative model-based methodology.⁴ It is potentially valuable to provide proof-of-mechanism in humans through the pharmacokinetic and pharmacodynamic relationship before beginning phase II clinical trials. The success rate in phase II clinical trials appears to be significantly higher when proof-of-mechanism is established by the end of phase I (29%) compared to when it is not established (0%).⁵

Assessing the pharmacological activity of immunomodulatory investigational drugs during early-phase clinical trials can be difficult due to the lack of biomarker expression in healthy volunteers. However, one potential solution is to challenge the immune system of healthy volunteers by activating T cells and/or B cells, allowing for the evaluation and quantification of the effects of an investigational compound on the adaptive immune system.⁶

THE HUMAN IMMUNE SYSTEM

The human immune system is a vast and complex network of proteins, cells, and organs, that defends the body against foreign invaders and protects the body's own cells. The immune system can roughly be divided into 2 parts distinguished by the speed and specificity of their reactions: the innate and the adaptive immune response.⁷ The innate immune response encompasses physical, microbiological, and chemical barriers, as well as the components of the immune system that provide immediate defense against potential pathogens, including the complement system, neutrophils, macrophages, and monocytes. This response is essential for survival and is highly conserved among animals.⁸ In contrast, the adaptive immune response is a characteristic of the immune system of more evolved animals. It involves the activation of T and B lymphocytes to produce specific, antigen-targeted reactions. While the innate immune response is rapid, it lacks specificity and sometimes causes damage to normal tissues. In contrast, the adaptive response is highly specific but takes longer to develop. Additionally, the adaptive response has the ability to retain a “memory” of past infections, allowing for a more robust and efficient response to subsequent exposures.^{9,10}

B and T lymphocytes originate from precursor cells in the bone marrow. T cells migrate to the thymus during their development. Both B and T cells produce antigen-specific receptors through a random DNA rearrangement and splicing process. These DNA segments encode the binding regions of the receptors. This process takes place before the cells are exposed to antigens, resulting in a wide range of T cell receptor and antibody specificities that can recognize and respond to potential pathogens.¹¹ This allows the immune system to protect an individual from a wide range of potential infections throughout their lifetime.

New clones of T and B cells are continuously produced throughout life, although this process slows down after the mid-20s. This may make it more difficult for people in this age range to regenerate their immune system after a bone marrow transplant or the use of antiretroviral therapy to treat HIV-related immunodeficiency.¹² Generally, aging causes a decrease in the amount of immune cells as well as their regenerative capacity and functionality known as immunosenescence.¹³

T AND B LYMPHOCYTES

After T and B cell receptor rearrangement, these cells can respond to the presence of an antigen and trigger an immune response. However, the activation of these cells is regulated to ensure that only harmful antigens are recognized and responded to.

There are two major types of effector T cells: T helper (Th) cells, which have cluster of differentiation 4 (CD4) molecules on their surface, and T cytotoxic (Tc) cells, which have CD8 molecules on their surface. CD4⁺ Th cells play a crucial role in coordinating the immune response, recognizing foreign antigens and activating several other components of the cell-mediated immune response to eliminate pathogens. They also help to activate B cells. CD8⁺ Tc cells, on the other hand, play a role in antiviral and potentially antitumor activity. Both types of cells are important in controlling intracellular pathogens. Another significant subpopulation of T cells are the regulatory T (Treg) cells. Many Treg forms exist, but the most well-characterized are the Treg cells that express CD4, CD25, and FOXP3.¹⁴ Treg cells are an essential part of the immune system because they can suppress the immune responses of other cells. After elimination of invading organisms Treg cells help in controlling immune responses, and they are also involved in prevention of autoimmunity.¹⁵

The combination of CD4⁺ T cell cytokines, which activate macrophages to eliminate intracellular pathogens, and CD8⁺ T cells, which kill virally infected cells, is the basis of the adaptive immune system to effectively control intracellular infections that the innate immune system cannot achieve on its own.

B cells produce antibodies that help to neutralize toxins, prevent organisms from attaching to mucous membranes, activate complement, opsonize phagocytosis-ready bacteria, and make infected or tumor cells more vulnerable for antibody-dependent cytotoxic attack by immune cells. Thus, antibodies enhance the functions of the innate immune system. During the early stages of B cell development, the antibody is a membrane-bound molecule acting as the B cell receptor. In this role, it internalizes antigens and presents them to T cells to initiate an immune response. Once the B cell is activated, it produces secreted antibodies with the aforementioned functions.

Different types of antibodies are found in different parts of the body. For example, IgM is found in the intravascular space, IgG is the primary antibody found in the tissues and blood, and IgA is found in secretions. Mucosa-associated lymphoid tissue refers to lymphoid tissue found at various mucous membrane sites, such as the bronchi, digestive tract, and urogenital tract. These sites are interconnected in their function, as certain subpopulations of B cells are specifically attracted to these tissues. An immune response triggered at one site will provoke immune responses at other sites to the same antigen. This effect can be therapeutically exploited, as generalized mucosal immunity can be induced *via* vaccination at a single mucosal site.¹⁶

KEYHOLE LIMPET HEMOCYANIN

Keyhole limpet hemocyanin (KLH) is considered a model antigen for use in immunization studies involving the adaptive immune system.^{6,17} KLH is a protein found in the

hemolymph of the keyhole limpet (*Megathura crenulata*), a marine mollusk. Keyhole limpets are primarily found in the Pacific coastal waters of California and Mexico. KLH is responsible for the transport of oxygen within many mollusk species.^{17,18} Hemocyanins are similar to hemoglobin found in the blood of vertebrates, but instead of containing iron to bind oxygen, they contain copper. KLH is a large protein, with a molecular weight of approximately 4–8 MDa, and it is composed of multiple subunits of approximately 350–390 kDa each.^{6,17-19} It was clinically introduced in 1967 to study the immunocompetence of humans²⁰ and it exhibited outstanding immunostimulatory properties in experiments with animals and man.²¹⁻²⁴ The innate as well as the adaptive immune response, including both the humoral and cellular response, are activated by KLH (Figure 1).²⁵ It is a useful antigen to study the adaptive immune response in man because the human immune system is typically naïve to KLH prior to immunization. This allows researchers to control the degree, duration, and timing of exposure to KLH, unlike other natural or therapeutic antigens commonly used for human immune challenge studies, such as varicella zoster or Bacille Calmette-Guérin (BCG), to which the human immune system is not naïve. Currently, subunit KLH is registered as an effective immunotherapeutic treatment modality for bladder cancer.^{26,27} KLH is also used as a hapten carrier protein for small molecules, or as an adjuvant in vaccine therapy or along with immunomodulatory drugs.^{17,18,28-31}

HUMORAL AND CELL-MEDIATED IMMUNITY

KLH exerts a robust systemic primary humoral response upon immunization in man. After KLH antigen processing by antigen-presenting cells, naïve CD4⁺ T cells are activated which in turn help B cells to activate, proliferate and differentiate to plasma cells (Figure 1). The response is characterized by production of initially anti-KLH IgM antibodies followed by an increase in more specific anti-KLH IgG antibodies.²⁵ Maximal responses are reached approximately 3 weeks after KLH immunization.²⁵ Enzyme-linked immunosorbent assay is the most widely used assay for quantification of KLH-specific antibodies.²⁵

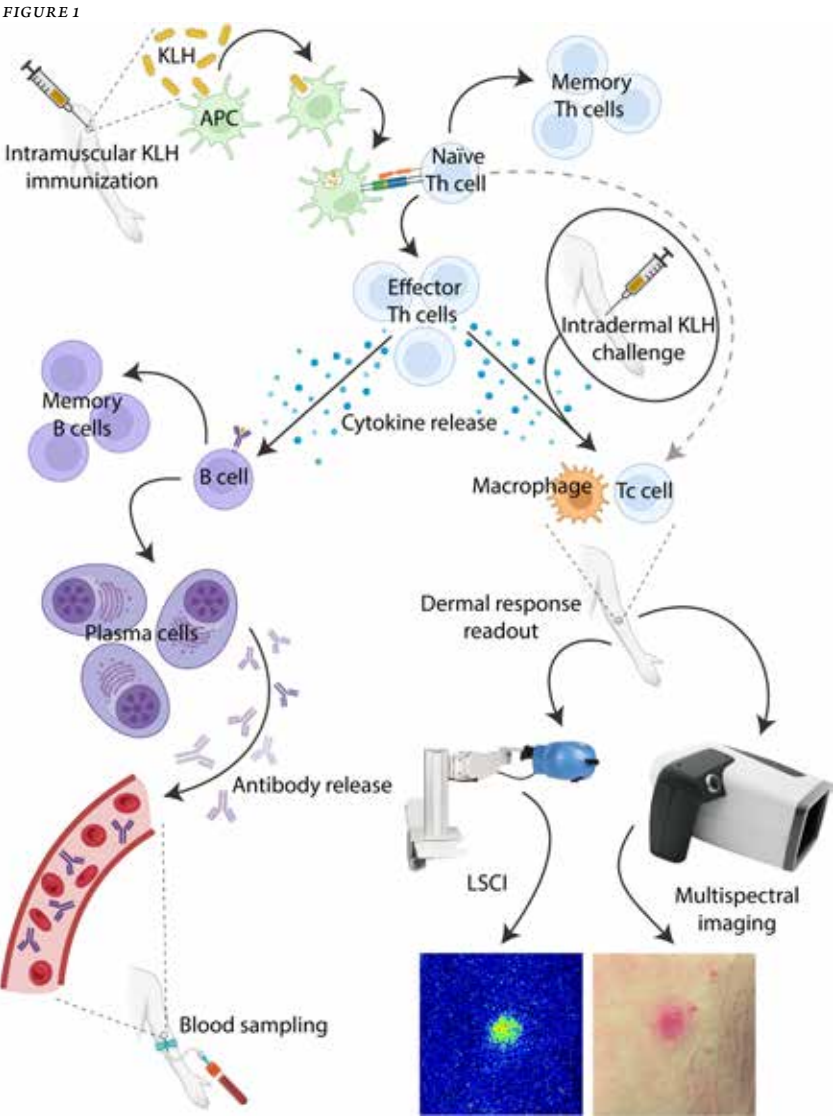
The “memory” function of the adaptive immune system can be evaluated by monitoring the cell-mediated immune response following a KLH challenge. Rechallenging the skin with an intradermal KLH administration after initial immunization evokes dendritic cell-mediated antigen presentation which causes KLH-specific CD4⁺ and CD8⁺ T cells within the draining lymph nodes to activate and proliferate (Figure 1).³² Effector T cells migrate into the skin after priming and imprinting of homing molecules by dendritic cells. The cutaneous inflammatory response that follows consists of local immune cell infiltration and increased vascular permeability, which can be clinically observed as erythema and oedema.^{33,34}

SKIN IMAGING

Although the skin response can be measured subjectively by visual inspection of the skin for erythema and oedema, this method is often scored categorically and is variable when scored by multiple assessors.³⁵ Objective quantification of the skin response on a continuous scale is therefore preferred.

Laser speckle contrast imaging (LSCI) provides real-time non-invasive monitoring of cutaneous microvascular perfusion.³⁶ LSCI's theoretical foundation can be traced back to the late 1960s when dynamic light scattering was developed.^{37,38} In the 1970s, the correlation between speckle temporal dynamics and particle dynamics was established,³⁹ which led to the introduction of LSCI for blood flow imaging in the 1980s.⁴⁰ Originally, LSCI was used to image blood flow in the retina, but in the 1990s, it was further developed for skin imaging.⁴¹ Since then, LSCI has been widely used in both preclinical and clinical studies, such as in pediatric burn wounds healing times assessment⁴² and investigating microvasculature in coronary artery disease patients.⁴³ The technique is based on analysis of speckle contrast, calculated as the ratio of its standard deviation to its mean intensity, providing an blood flow index. Illumination of tissue with a laser generates a random interference (speckle) pattern. This pattern decorrelates after scattering of laser light by moving red blood cells. The intensity of each speckle fluctuates according to the velocity of the moving red blood cells, resulting in a decrease in time-integrated speckle contrast. LSCI measurements are often reported as arbitrary units as the technique does not provide a precise measurement of blood flow in mL/min. Although susceptible to motion artefacts, LSCI measurements can be performed continuously. Combined with a good temporal and spatial resolution and reproducibility, it is a useful tool in objective quantification of cutaneous blood perfusion following an intradermal challenge.⁶

FIGURE 1 – NEXT PAGE Schematic overview of keyhole limpet hemocyanin (KLH) challenge model. Antigen presenting cells (APCs) act as phagocytes and are activated after KLH immunization. Presentation of KLH antigens *via* MHC molecules on APCs activate naïve T helper cells (Th cells) which then proliferate further to effector Th cells and memory Th cells. Effector Th cells release cytokines activating B cells that have already been exposed to KLH antigens to proliferate further to plasma cells and memory B cells. Plasma cells produce antibodies against KLH which can be measured in the blood *via* enzyme-linked immunosorbent assay. Cytokines released by effector Th cells also activate macrophages and T cytotoxic cells (Tc cells), though Tc cells can also be directly activated by naïve Th cells. Upon intradermal KLH challenge these immune cells migrate to the skin injection site causing a local inflammatory response. This response consists of local immune cell infiltration and increased vascular permeability, which can be clinically observed as erythema and oedema. The increased vascular permeability can be objectively measured using laser speckle contrast imaging (LSCI) and the local skin erythema can be measured using multispectral imaging.



PeriCam PSI NR LSCI image source: <https://www.perimed-instruments.com/content/pericam-psi-nr/>; Miravex Antera 3D multispectral camera image source: <https://miravex.com/pharma-cosmetics-biotech/>. KLH, keyhole limpet hemocyanin; APC, antigen-presenting cell; Th cell, T helper cell; Tc cell, T cytotoxic cell; LSCI, laser speckle contrast imaging.

Multispectral computer-assisted 3D imaging of the skin can be acquired using the Antera 3D® camera. This novel camera utilizes multi-directional light in a closed chamber to reconstruct the skin surface in 3D.⁴⁴ It uses reflectance mapping of seven different wavelengths of light that cover the complete visible spectrum, enabling more accurate analysis of cutaneous colorimetric properties, including erythema. Spectral images are obtained and transformed into reflectance maps. The data is then converted to skin absorption coefficients, and mathematical correlations with known spectral absorption data of hemoglobin are used to quantify erythema.

A main focus of this thesis is the use of KLH as an *in vivo* immunization antigen for studying and objectively quantifying immune responses in humans with abovementioned skin imaging techniques.

AIMS AND OUTLINE OF THIS THESIS

The main objective of this thesis was to develop and characterize a human immune challenge model in healthy volunteers using KLH, and to subsequently apply this challenge model in healthy volunteers to evaluate the effects of immunomodulatory IMPs in drug development programs.

Section I provides background information by means of a systematic literature review on the KLH challenge model used in healthy volunteers as well as in several patient populations, including the effect of pharmacological interventions on the KLH response (Chapter 2). Section II focuses on the characterization and optimization of the KLH challenge model with objective quantification of the cell-mediated immune response in healthy volunteers (Chapter 3). In section III the KLH challenge model is implemented in early-phase clinical trials that included novel immunomodulatory IMPs (Chapters 4–6). Finally, the translation of preclinical effects of an IMP to early-phase clinical effects, based on KLH challenges, and eventually to patients, is discussed (Chapter 7).

SECTION I – KLH IMMUNE CHALLENGE MODEL BACKGROUND

In this thesis, the characterization and standardization of the KLH challenge model is described, with subsequent application of the methodology for evaluation of the activity of novel investigational compounds. Although this model has previously been used in multiple clinical trials, the precise immunological processes are still relatively unidentified. Moreover, KLH dosages, use of adjuvants, and immunization and rechallenge routes and regimens are not standardized, and the outcomes to characterize immune responses following the KLH challenge have not been optimized. A systematic review performed by Swaminathan et al. presented an outline of KLH doses, administration routes, and topline response monitoring based on 16 clinical studies.¹⁷

As sequel, Chapter 2 provides a more thorough systematic review examining the KLH response in clinical studies. This review expands upon the work of Swaminathan et al. by including a larger number of clinical studies and focuses on 3 main areas: the different methods used to study the systemic and local immune responses triggered by KLH, identifying the most reliable biomarkers for KLH response monitoring, and evaluating the impact of diseases and pharmacological interventions on the KLH challenge response.

SECTION II – KLH IMMUNE CHALLENGE MODEL IN HEALTHY VOLUNTEERS

A major shortcoming of most studies implementing a KLH challenge in immunopharmacological studies is the use of subjective quantification to assess the local cell-mediated response upon KLH skin challenge. Chapter 3 describes the use of a KLH challenge model in healthy volunteers with highly sensitive and objective techniques quantifying the local KLH recall response, overcoming the shortcomings of subjective evaluations such as inter-rater variability. Apart from characterization of the skin response upon dermal KLH challenge with various imaging techniques, correlations and power calculations were also performed on the humoral and cell-mediated responses to predict sample sizes for future studies involving IMPs.

SECTION III – KLH IMMUNE CHALLENGE MODEL IN EARLY-PHASE CLINICAL TRIALS

This section describes the evaluation of novel immunomodulatory IMPs in clinical trials, based on the KLH challenge model as performed in Chapter 3. Chapter 4 describes the first-in-human study of an OX40 ligand (also known as CD134) inhibitor (KY1005, currently amlitelimab) and its effects on the adaptive immune system. In Chapter 5, 3 formulations of a single-strain microbial intervention prepared from *Lactococcus lactis* spp. *cremoris* (EDP1066) were investigated in a clinical study. The aim of the trial was to characterize pharmacodynamic effects of EDP1066 on the adaptive immune system. Chapter 6 details the effects of a second single-strain microbe prepared from *Prevotella histicola* (EDP1815) on the immune system in a clinical trial. All 3 studies were performed in healthy volunteers. Lastly, Chapter 7 summarizes and translates the immunological effects of EDP1815 in preclinical trials to an early-phase clinical trial in healthy volunteers, and to clinical trials performed in patients with atopic dermatitis and psoriasis.

GENERAL DISCUSSION AND SUMMARY

Finally, a summary and general discussion of the thesis are provided in Chapter 8, and a summary conclusion in Dutch is given in Chapter 9.

REFERENCES

- 1 Fugger L, Jensen LT, Rossjohn J. Challenges, Progress, and Prospects of Developing Therapies to Treat Autoimmune Diseases. *Cell*. 2020;181(1):63-80. doi:10.1016/j.cell.2020.03.007
- 2 Wong CH, Siah KW, Lo AW. Corrigendum: Estimation of clinical trial success rates and related parameters. *Biostatistics*. 2019;20(2):366-366. doi:10.1093/biostatistics/kxy072
- 3 Wouters OJ, McKee M, Luyten J. Estimated Research and Development Investment Needed to Bring a New Medicine to Market, 2009-2018. *JAMA*. 2020;323(9):844-853. doi:10.1001/JAMA.2020.1166
- 4 Cohen AF, Burggraaf J, van Gerven JMA, Moerland M, Groeneveld GJ. The Use of Biomarkers in Human Pharmacology (Phase I) Studies. *Annu Rev Pharmacol Toxicol*. 2015;55(1):55-74. doi:10.1146/annurev-pharmtox-011613-135918
- 5 Wu SS, Fernando K, Allerton C, Jansen KU, Vincent MS, Dolsten M. Reviving an R&D pipeline: a step change in the Phase II success rate. *Drug Discov Today*. 2021;26(2):308-314. doi:10.1016/j.drudis.2020.10.019
- 6 Saghari M, Gal P, Ziagos D, et al. A randomized controlled trial with a delayed-type hypersensitivity model using keyhole limpet haemocyanin to evaluate adaptive immune responses in man. *Br J Clin Pharmacol*. 2021;87(4):1953-1962. doi:10.1111/bcp.14588
- 7 Parkin J, Cohen B. An overview of the immune system. *Lancet*. 2001;357(9270):1777-1789. doi:10.1016/S0140-6736(00)04904-7
- 8 Beutler B. Innate immunity: an overview. *Mol Immunol*. 2004;40(12):845-859. doi:10.1016/J.MOLIMM.2003.10.005
- 9 Delves PJ, Roitt IM. The Immune System: First of Two Parts. Mackay IR, Rosen FS, eds. *N Engl J Med*. 2000;343(1):37-49. doi:10.1056/NEJM200007063430107
- 10 Delves PJ, Roitt IM. The Immune System: Second of Two Parts. Mackay IR, Rosen FS, eds. *N Engl J Med*. 2000;343(2):108-117. doi:10.1056/NEJM200007133430207
- 11 Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P. A Direct Estimate of the Human $\alpha\beta$ T Cell Receptor Diversity. *Science* (80-). 1999;286(5441):958-961. doi:10.1126/science.286.5441.958
- 12 Jamieson BD, Douek DC, Killian S, et al. Generation of Functional Thymocytes in the Human Adult. *Immunity*. 1999;10(5):569-575. doi:10.1016/S1074-7613(00)80056-4
- 13 Sadighi Akha AA. Aging and the immune system: An overview. *J Immunol Methods*. 2018;463:21-26. doi:10.1016/j.jim.2018.08.005
- 14 Curiel TJ. Tregs and rethinking cancer immunotherapy. *J Clin Invest*. 2007;117(5):1167-1174. doi:10.1172/JCI131202
- 15 Shevach EM. Regulatory T Cells in Autoimmunity*. <https://doi.org/10.1146/annurevimmunol181423>. 2003;18:423-449. doi:10.1146/ANNUREV.IMMUNOL.18.1.423
- 16 Klavinskis LS, Gao L, Barnfield C, Lehner T, Parker S. Mucosal immunization with DNA-liposome complexes. *Vaccine*. 1997;15(8):818-820. doi:10.1016/S0264-410X(96)00278-2
- 17 Swaminathan A, Lucas RM, Dear K, McMichael AJ. Keyhole limpet haemocyanin - a model antigen for human immunotoxicological studies. *Br J Clin Pharmacol*. 2014;78(5):1135-1142. doi:10.1111/bcp.12422
- 18 Harris J, Markl J. Keyhole limpet hemocyanin (KLH): a biomedical review. *Micron*. 1999;30(6):597-623. doi:10.1016/S0968-4328(99)00036-0
- 19 Lebrech H, Hock MB, Sundsmo JS, et al. T-cell-dependent antibody responses in the rat: Forms and sources of keyhole limpet hemocyanin matter. *J Immunotoxicol*. 2014;11(3):213-221. doi:10.3109/1547691X.2013.822948
- 20 Swanson MA, Schwartz RS. Immunosuppressive Therapy. *N Engl J Med*. 1967;127(4):163-170. doi:10.1056/NEJM196707271270401
- 21 Curtis JE, Hersh EM, Butler WT, Rossen RD. Antigen dose in the human immune response. Dose-relationships in the human immune response to Keyhole limpet hemocyanin. *J Lab Clin Med*. 1971;78(1):61-69. <http://www.ncbi.nlm.nih.gov/pubmed/5569250>
- 22 Curtis JE, Hersh EM, Harris JE, McBride C, Freireich EJ. The human primary immune response to keyhole limpet haemocyanin: interrelationships of delayed hypersensitivity, antibody response and in vitro blast transformation. *Clin Exp Immunol*. 1970;6(4):473-491. <http://www.ncbi.nlm.nih.gov/pubmed/4320164>
- 23 Herscovitz HB, Harold WW, Stavitsky AB. Immunochemical and immunogenic properties of a purified keyhole limpet haemocyanin. *Immunology*. 1972;22(1):51-61. <http://www.ncbi.nlm.nih.gov/pubmed/4622451>
- 24 Weigle WO. Immunochemical properties of hemocyanin. *Immunochemistry*. 1964;1(4):295-302. doi:10.1016/0019-2791(64)90030-8
- 25 Saghari M, Jansen MAA, Grievink HW, Rissmann R, Moerland M. Characterization of KLH-driven immune responses in clinical studies: A systematic review. *Front Drug Discov*. 2022;2. doi:10.3389/fddsv.2022.992087
- 26 Lammers RJM, Witjes WPJ, Janzing-Pastors MHD, Caris CTM, Witjes JA. Intracutaneous and Intravesical Immunotherapy With Keyhole Limpet Hemocyanin Compared With Intravesical Mitomycin in Patients With Non-Muscle-Invasive Bladder Cancer: Results From a Prospective Randomized Phase III Trial. *J Clin Oncol*. 2012;30(18):2273-2279. doi:10.1200/JCO.2011.39.2936
- 27 Perabo FGE, Müller SC. Current and new strategies in immunotherapy for superficial bladder cancer. *Urology*. 2004;64(3):409-421. doi:10.1016/j.urol.2004.04.026
- 28 Gandhi RT, O'Neill D, Bosch RJ, et al. A randomized therapeutic vaccine trial of canarypox-HIV-pulsed dendritic cells vs. canarypox-HIV alone in HIV-1-infected patients on antiretroviral therapy. *Vaccine*. 2009;27(43):6088-6094. doi:10.1016/j.vaccine.2009.05.016
- 29 Jia T, Pan Y, Li J, Wang L. Strategies for active TNF- α vaccination in rheumatoid arthritis treatment. *Vaccine*. 2013;31(38):4063-4068. doi:10.1016/j.vaccine.2013.06.101
- 30 Jurincic-Winkler CD, Metz KA, Beuth J, Klippel KF. Keyhole Limpet Hemocyanin for Carcinoma in situ of the Bladder: A Long-Term Follow-Up Study. *Eur Urol*. 2000;37(Suppl. 3):45-49. doi:10.1159/000052392
- 31 Schumacher K. Keyhole limpet hemocyanin (KLH) conjugate vaccines as novel therapeutic tools in malignant disorders. *J Cancer Res Clin Oncol*. 2001;127(S2):R1-R2. doi:10.1007/BF01470991
- 32 Tikoo S, Jain R, Kurz AR, Weninger W. The lymphoid cell network in the skin. *Immunol Cell Biol*. 2018;96(5):485-496. doi:10.1111/imcb.12026
- 33 Marwa K, Kondamudi NP. Type IV Hypersensitivity Reaction.; 2023. <http://www.ncbi.nlm.nih.gov/pubmed/14568857>
- 34 Copaesca A, Gibson A, Li Y, Trubiano JA, Phillips EJ. An Updated Review of the Diagnostic Methods in Delayed Drug Hypersensitivity. *Front Pharmacol*. 2021;11. doi:10.3389/fphar.2020.573573
- 35 Pouchot J. Reliability of Tuberculin Skin Test Measurement. *Ann Intern Med*. 1997;126(3):210. doi:10.7326/0003-4819-126-3-199702010-00005
- 36 Hellmann M, Kalinowski L, Cracowski JL. Laser speckle contrast imaging to assess microcirculation. *Cardiol J*. 2022;29(6):1028-1030. doi:10.5603/CJ.2022.0097
- 37 Cummins HZ, Swinney HL. III Light Beating Spectroscopy. In: ; 1970:133-200. doi:10.1016/S0079-6638(08)70188-4
- 38 Pecora R. Quasi-Elastic Light Scattering from Macromolecules. *Annu Rev Biophys Bioeng*. 1972;1(1):257-276. doi:10.1146/annurev.bb.01.060172.001353
- 39 Berne BJ, Pecora R. Dynamic Light Scattering: With Applications to Chemistry, Biology, and Physics. John Wiley and Sons Inc.; 1976.
- 40 Fercher AF, Briers JD. Flow visualization by means of single-exposure speckle photography. *Opt Commun*. 1981;37(5):326-330. doi:10.1016/0030-4018(81)90428-4
- 41 Boas DA, Dunn AK. Laser speckle contrast imaging in biomedical optics. *J Biomed Opt*. 2010;15(1):011109. doi:10.1117/1.3285504
- 42 Mirdell R, Farnebo S, Sjöberg F, Tesselaar E. Accuracy of laser speckle contrast imaging in the assessment of pediatric scald wounds. *Burns*. 2018;44(1):90-98. doi:10.1016/j.burns.2017.06.010
- 43 Borges JP, Lopes GO, Verri V, et al. A novel effective method for the assessment of microvascular function in male patients with coronary artery disease: a pilot study using laser speckle contrast imaging. *Brazilian J Med Biol Res*. 2016;49(10):e5541. doi:10.1590/1414-431X20165541
- 44 Matias AR, Ferreira M, Costa P, Neto P. Skin colour, skin redness and melanin biometric measurements: comparison study between Antera® 3D, Mexameter® and Colorimeter®. *Ski Res Technol*. 2015;21(3):346-362. doi:10.1111/srt.12199