Chronic Early Childhood Exposure to Arsenic is Associated with a TNF-mediated Proteomic Signaling Response

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Abstract

Exposure to inorganic arsenic (iAs) in drinking water remains a global issue of concern and is associated with a range of health outcomes, including immune dysfunction. Young children have been identified as a particularly sensitive population, yet mechanisms of adverse health outcomes are understudied. Here we set out to examine the effects of iAs exposure on circulating serum proteins in adolescents. To identify proteins as potential indicators of disease, levels of total urinary arsenic (U-tAs) and its methylated metabolites were determined and serum proteins assessed for differences in expression. The results indicate an enrichment of TNF-regulated immune and inflammatory response proteins that display decreased expression levels in relation to increasing U-tAs. Notably, when analyzed in the context of the arsenical proportions, there was minimal overlap between the protein lists, with the most robust response observed in relation to %MMAs. These data represent the first assessment of protein expression in serum in adolescents exposed to inorganic arsenic.

Keywords

arsenic; arsenic metabolism; proteomics; early childhood exposure

1. Introduction

Exposure to elevated levels of inorganic arsenic (iAs) in drinking water remains a global issue of concern. Over 100 million people worldwide are exposed to levels of iAs in their drinking water that exceed the World Health Organization's recommended limit of 10 μg/L [1]. While new populations continue to be identified, those at risk of elevated exposure

include, but are not limited to, populations in Bangladesh, Mexico, the United States, and China, among others [2].

Chronic exposure to iAs is associated with a range of health outcomes in adults, including diabetes mellitus, impaired cognition and neurological effects, hypertension, immune dysfunction, and skin, lung, bladder, liver, and kidney cancers [3]. Of increasing concern, young children have been identified as a particularly sensitive population [3, 4]. Specifically, early childhood exposure to iAs has been associated with outcomes manifesting during both adolescence and adulthood, including impaired cognitive development, increased mortality due to bladder, laryngeal, and lung cancers, increased non-cancer mortality due to bronchiectasis, myocardial infarction, and increased infection risk [3, 5-7]. The long-lasting impact of iAs exposure during early childhood suggests that early life represents a critical period during which there is heightened sensitivity to the toxic effects of iAs [3, 4].

The mechanisms underlying the health effects of childhood exposure to iAs remain understudied. Previous studies examining immune functioning in iAs-exposed children suggest that iAs can act as an immunosuppressant. Specifically, it has been reported that children with iAs exposure exhibited decreased plasma concentrations of the Th1 cytokines, TNF-α and IL-2, and reduced responsiveness on functional immune tests [8, 9]. In support of these data, there is also evidence that iAs exposure during childhood impairs monocyte functioning and immune-specific reactive oxygen species (ROS) signaling [10, 11]. Paradoxically, there is also substantial evidence that iAs acts as a pro-inflammatory agent in children. For instance, *in utero* exposure to arsenic is associated with an activation of inflammation, including the NF-kB signaling cascade [12, 13]. Early life exposure has also been linked to iAs-induced chronic inflammation mediated impaired lung function [14]. Taken together, this suggests that iAs can act as an immunomodulatory agent during childhood and development, possibly impacting maturation of the immune system during a critical period of development [10, 15]. Such an effect may play a critical role in the development of the diverse adverse health effects associated with iAs exposure.

Also unknown is the impact that specific arsenic metabolites may have on protein expression during childhood. Arsenic metabolism is a multi-step process, with six major arsenic species that have been identified in human urine. These species include inorganic arsenics (iAs^{III} and iAs^{V}), which are metabolized to the monomethylated arsenic species (MMAs), monomethylarsenous acid (MMA^{III}) and monomethylarsonic acid (MMA^{V}). MMAs are methylated again to become dimethylated (DMAs), forming dimethylarsinous acid (DMA^{III}) and dimethylarsinic acid (DMA^{V}) [16]. The efficiency of these methylation reactions has been identified as an important factor underlying the effects observed following iAs exposure as iAs, MMAs, and DMAs are differentially associated with As-related outcomes such as hypertension, atherosclerosis, cancer, and chromosomal aberrations [16, 17]. Additionally, we have previously demonstrated that urinary iAs is positively associated with lower gestational age and newborn length, while urinary MMAs is associated with lower gestational age and birth weight [18]. Therefore, inter-individual differences in the efficiency of arsenic methylation may play a role in metabolite-specific associated disease risk. The impact that differences in iAs metabolism may have on protein expression during childhood is currently unknown.

Given evidence that exposure to iAs during childhood leads to abnormal immune functioning [13, 14], we hypothesized that childhood iAs exposure is likely to disrupt expression levels of proteins involved in immune function. Therefore, we conducted a proteomic assessment of serum in iAs-exposed children selected from a cohort of subjects living in Comarca Lagunera, an area in North-Central Mexico. Moreover, in light of the knowledge that different arsenical species yield different health effects, we included an assessment for the relationship of proteins to %iAs, %MMAs and %DMAs. In the present study, we describe differences in protein expression levels in adolescents' serum associated with concentrations of each class of urinary arsenic metabolites.

2. Materials and methods

2.1 Study Subjects and Sample Collection

The study sample for the present analysis represents a subset of 40 subjects of a cohort reported previously [19]. Participants were children, both male and female, aged 6-12 years living in one of four rural communities in the Comarca Lagunera area, located in north-central Mexico. These communities represent those with the highest arsenic tap water levels (104-360 ppb) detected in the last 20 years in the area. Arsenic is present in the local water supply due to the over-extraction of groundwater. Children included in this study had mothers who remained in these communities for the duration of their pregnancy, and have since remained residents of these same communities.

2.2 Questionnaires

Information was collected through in-person interviews and included socio-demographic variables (education, socioeconomic status), lifetime residential history, lifestyle factors (secondhand smoke), parent's occupational history, water source types (municipal tap water, bottled, other), current medications, medical history, and diet. Questionnaires were completed by the mothers at their own residing community. Water consumption habits, including source of drinking water, were ascertained through a standardized questionnaire [19].

2.3 Determination of arsenic concentrations in water and in urine

Drinking water samples (well) were collected from each rural community included in the study and analyzed for inorganic arsenic levels. Well water samples from each rural community are representative of the water that participants drank and are provided through the unique local water supply system. Individual exposure was assessed based on U-tAs. A first morning void urine sample was collected in sterile 120-mL screw-topped polypropylene containers. Urine samples were analyzed as described previously [14]. Briefly, samples were analyzed at the Arizona Laboratory for Emerging Contaminants, University of Arizona, Tuscon, Arizona. Urinary As^V, As^{III}, MMA^V, DMA^V, and arsenobetaine were separated by HPLC and concentrations were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Standard Reference Water, SMR 1640 (NIST, Gaithersburg, MD, USA) and the freeze-dried Urine Reference Material for trace elements (Clinchek-control; RECIPE Chemicals instruments GmbH, Munich, Germany) were used as quality controls for urinary arsenic measurement.

2.4 Assessment of protein expression in serum

Subjects selected for proteomic assessment are representative of the extremes of exposure (median U-tAs $_{high}$ = 399.35 μg/L, median U-tAs $_{low}$ = 26.03 μg/L). As detailed in our prior publication (Bailey, Laine et al. 2014), the relative expression levels of 507 proteins were determined using the Biotin Label-based Human Antibody Array I, L series 507 (RayBiotech, Norcross,GA), which includes cellular signaling proteins such as cytokines, chemokines, growth factors, angiogenic factors, soluble receptors, and soluble adhesion molecules. Protein labeling and hybridization were carried out according to the manufacturer's instructions using 40 μl of each serum sample. Briefly, primary amines of serum proteins are biotinylated and hybridized to a membrane array containing antibodies specific for each of the 507 protein targets, incubated with a horseradish peroxidase (HRP)-streptavidin conjugate, and detected by chemiluminescence following incubation with an HRP substrate buffer. The protein array contains two types of positive controls: a biotin-labeled protein, independent of the sample, that is spotted on each array in a series of known concentrations enabling signal intensity normalization across arrays, as well as an internal positive control which is an exogenous, nonmammalian protein added to the serum sample prior to biotinylation, serving as a control for the labeling and incubation steps.

2.5 Statistical Analyses

Statistical analyses were performed using Partek Genomics Suite software (version 6.6; Partek, Inc., St Louis, MO). All data were analyzed for their distribution patterns and homogeneity, and filtering was used to remove any non-expressed proteins. Multivariable regression models were used to examine relationships between individual arsenic measures (U-tAs, %iAs, %MMAs, %DMAs) and the normalized, background-subtracted signal intensities of the 393 proteins. Age, sex, and body mass index (BMI) were selected as *a priori* covariates due to their potential influence on protein expression levels and were controlled for in the models. Statistical significance was defined as $p < 0.05$, with false discovery (type II error) controlled for using a q-value <0.1.

2.6 Functional Analyses of Proteins

Proteins that showed statistically significant association with either U-tAs, %iAs, %MMA, or %DMA were analyzed for biological functions, canonical pathways, upstream regulatory molecules, and interacting molecular networks using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA). Proteins were also analyzed for KEGG pathway molecular interactions using The Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/), and immunologic signatures using Gene Set Enrichment Analysis (GSEA, http://software.broadinstitute.org/gsea/index.jsp).

3. Results

3.1 Study characteristics

Demographic characteristics of the study sample (n=40), as well as those of the entire cohort (n=358), can be found in Table 1. These subjects were selected as representatives of the extremes of exposure (median U-tAs $_{high}$ = 399.35 μg/L, median U-tAs $_{low}$ = 26.03 μg/L).

Anthropometric characteristics recorded include sex, age, (years) weight (kg), and BMI (calculated as weight/height^2). The majority of the subjects were male (n=22, 55%), have lived at their current residence their entire lives (n=36, 90%), and were, on average 9.3 years of age (range 7-12). Other possible sources of arsenic exposure, including diet (seafood, rice and others), agrochemicals, fuels, preservatives or other compounds containing arsenicals, were negligible [19].

Indicators of arsenic exposure examined include total urinary arsenic (U-tAs; µg/L), as well as the percentages of inorganic arsenic (iAs), monomethylated arsenicals (MMAs) and dimethylated arsenicals (DMAs) as indicators of arsenic metabolism. All samples were within detectable limits and U-tAs, defined as the sum of U-iAs, U-MMAs and U-DMAs, ranged from 5.33 µg/L to 664.53 µg/L. The average proportions of U-tAs were 24.4% iAs, 14.9% MMAs, and 60.7% DMAs. Arsenic tap water levels and urinary arsenic levels were highly correlated ($R^2 = 0.69$).

3.2 Identification of proteins associated with total urinary arsenic and arsenic metabolites

Multivariable analyses identified 58 proteins that displayed a statistically significant association ($p<0.05$, $q=0.1$) with U-tAs, the vast majority of which are negatively associated (n=56, 96.6%) (Appendix 1). When analyzed in the context of the arsenical proportions, 8 proteins were significantly ($p<0.05$) associated with %iAs, 18 proteins were associated with %MMAs, and 11 proteins displayed significant association with %DMAs. There were no proteins identified in common across all three methylated metabolites (Figure 1, Appendix 1).

The 58 proteins identified as being significantly associated with U-tAs were analyzed for functional interactions (Appendix 2). Canonical pathway analysis revealed a strong enrichment of cytokine-mediated communication between immune cells ($p= 1.12 \times 10^{-2}$), comprised largely of a family of interleukins, including Interferon, Alpha 13 (IFNA1/IFNA13) and Chemokine (C-X-C Motif) Ligand 8 (CXCL8). Network analysis revealed a protein cluster involved in cellular development, cellular growth and proliferation ($p= 1 \times 10^{-34}$), which interacts with the major histocompatibility complex (MHC), class II (Figure 2).

A similar approach was used to investigate the pathways that were enriched among the proteins associated with the arsenical proportions. In relation to %iAs, canonical pathway analysis revealed several proteins involved in immune surveillance ($p= 2.90 \times 10^{-4}$), and include Platelet-Derived Growth Factor C (PDGFC) and Platelet-Derived Growth Factor D (PDGFD). Network analysis revealed a cellular growth and proliferation component ($p=1.00 \times 10^{-24}$), mediated by ERK 1/2 kinase and containing several members of the PDGF family, which are responsible for cellular proliferation and differentiation.

In relation to %MMAs, altered proteins were associated with an apoptosis signaling pathway ($p=1.09 \times 10-9$), including Tumor Necrosis Factor (Ligand) Superfamily, Member 10 (TNFSF10) and many related TNF receptor superfamily (TNFRSF) members and vascular endothelial growth factors B and C (VEGFB, VEGFC). Network analysis centered on

organismal and cardiovascular system development and connective tissue disorders (p= 1×10^{-47}), modulated by ERK 1/2 and NFkB.

In relation to %DMAs, proteins involved in immune surveillance, specifically macropinocytosis signaling ($p = 3.36 \times 10^{-8}$), were altered and include Platelet Derived Growth Factor B (PDGFB), PDGFC, and PDGFD. Network analysis reveals cellular growth and proliferation ($p = 1 \times 10^{-33}$), and includes many members of the PDGF family modulated by NFkB. Additionally, DAVID was used to confirm all pathway analyses and showed Cytokine-cytokine receptor interaction as the common top KEGG pathway for U-tAs ($p = 3.76 \times 10^{-21}$), %iAs ($p=0.015$), %MMAs ($p= 6.80 \times 10^{-09}$), and %DMAs ($p=0.015$).

3.3 Transcription factor regulation of arsenic-associated proteins

Analysis was performed for all arsenic-associated protein lists in order to identify key upstream regulators, including cell signaling molecules and transcription factors (Appendix 2). Tumor necrosis factor (TNF) was found to be the top regulator of the U-tAs-associated proteins ($p=1.59 \times 10^{-27}$), and was predicted to be inhibited in relation to U-tAs. For the individual arsenicals, Dual Specificity Phosphatase 5 (DUSP5) was the top regulator of the %iAs-associated proteins ($p= 1.98 \times 10^{-5}$), pyruvic acid was the top regulator of the %MMAs-associated proteins ($p= 1.98 \times 10^{-10}$), and for %DMAs-associated proteins, Fibroblast Growth Factor 2 (Basic) (FGF2) ($p= 1.47 \times 10^{-5}$) was the most significant regulator. Because of the small number of proteins in each arsenical-associated list, predicted effects (i.e., activation or inhibition) could not be established.

4. Discussion

Early childhood exposure to iAs has been associated with adverse health outcomes manifesting during both adolescence and adulthood [3, 5-7]. Moreover, inter-individual differences in the efficiency of arsenic metabolism has been shown to influence iAs-associated disease, with high urinary proportions of MMAs associated with detrimental outcomes such as cancer of the urinary bladder, skin cancer, and cardiovascular disease [16, 17]. Despite this, the effects of childhood exposure to iAs remain understudied, particularly in terms of effects on cellular and molecular functions. In the present study, we set out to identify biological pathways which may be modulated at the protein level by early life arsenic exposure. Using a subset of 40 subjects from a previously reported cohort [19], we have identified proteins with expression levels associated with inorganic arsenic exposure. While we have previously examined prenatal arsenic-associated differences in the expression in the newborn cord serum proteome [13], to our knowledge this is the first study to examine differences in the expression of proteins in children's serum associated with total urinary arsenic, as well as proportions of each class of urinary arsenic metabolites. The proteins that showed altered expression largely fell into functional categories of inflammation and immune response. Additionally, TNF was identified as a predicted upstream regulator of the U-tAs proteome, as well as a mediator of metabolite-dependent differences in identified proteins and regulators.

We demonstrate a massive repression of immune-associated cytokines as U-tAs levels increase. Interestingly, TNF was identified as a key regulator of these repressed proteins.

TNF has a known roll in immune response, apoptosis, inflammation, and cell migration, as well as having been implicated in numerous diseases [3]. Our prior research highlights TNF as a regulator of the newborn proteome [13] as well as the newborn transcriptome [12]. Here we show that many of the identified suppressed proteins are involved in the major histocompatibility (MCH) complex II, which is responsible for triggering localized inflammation and B cell activation in the adaptive immune response [20], and included many interleukins and interferons. These data highlight altered expression of cytokines known to play a role in adaptive and innate immune signaling and could provide a mechanistic hypothesis for increased risk of infection [21].

When analyzed in relation to inter-individual differences in arsenic metabolism in adolescents, we found minimal overlap between the %iAs, %MMAs and %DMAs-associated proteins. These data suggest a urinary arsenic metabolite-specific response to iAs exposure and reflects the strong inter-individual component to the response. The most robust response in terms of differential protein expression was observed in relation to %MMAs. This finding is interesting as inter-individual differences in iAs metabolism, particularly elevation in %MMAs, have been shown to be associated with disease risk [16-18]. In our prior work, we have shown inter-individual differences in iAs metabolism as it relates to epigenetic signatures of disease [13, 22]. Proteins associated with %MMAs include many TNF superfamily (TNFSF) members. Among these are TNFSF10 and TNFSF15, previously shown to play roles in hepatic and cardiovascular disease [3, 23-27]. In addition to the TNFSF, members of the VEGF family also displayed a positive association with %MMAs. The VEGF family is a known regulator of angiogenesis [28]. VEGFB has been shown to play a role in cancer metastasis and tumor invasion [29] and VEGFB expression has been found to be upregulated in ovarian, colorectal, renal, and prostate cancer [30, 31]. VEGFC also plays a role in metastatic spread of certain tumor types [30]. These data highlight differences in the expression of proteins with known association to arsenic-associated diseases.

While we have identified a large TNF-mediated response in relation to U-tAs with metabolite-specific differences, the study is not without its limitations. Despite being one of the largest proteomic studies to date, we have only assessed a fraction of the thousands of circulating serum proteins and due to the small sample size in the present study associations seen should be confirmed in a larger cohort. Additionally, it possible that other environmental exposures and/or potential confounding variables not assessed in the present study may contribute to the observed differences. Moreover, while proteins identified have known associations with arsenic-related adverse outcomes it is unknown if the current cohort will present with such diseases. Future studies could examine both genomic and epigenetic mechanisms that may underlie the observed functional differences in protein expression [32].

4. Conclusion

In summary, the data from the present study suggest that iAs acts as an immunomodulator, and also that it does so in a metabolite-specific manner. The proteomic differences in expression seen here highlight the role of iAs in possibly impacting maturation of the

immune system during a critical period of development [10], and as such, has the potential to alter disease risk later in life. Early childhood exposure to iAs has been associated with outcomes manifesting during both adolescence and adulthood, and the long-lasting impact of iAs exposure during early childhood suggests that early life represents a critical period during which there is heightened sensitivity to the toxic effects of iAs [3, 4]. These data inform potential mechanisms by which early life and childhood exposure to iAs may be linked to detrimental health outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online

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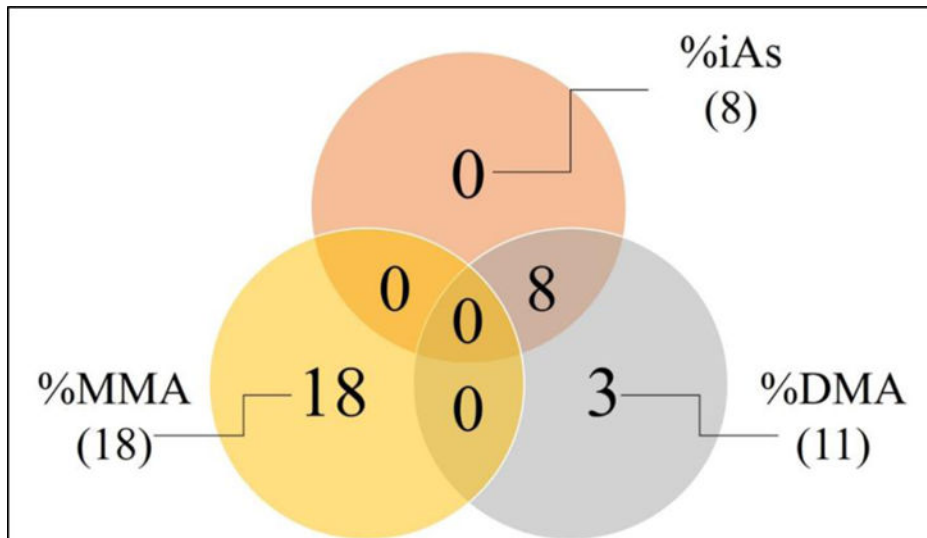
Figure 1.
Total number of %iAs, %MMA, and %DMA associated proteins as well as commonality among arsenical associated lists. Venn diagram illustrating the number of serum protein that have a statistically significant association between expression levels and urinary proportions of iAs, MMAs and/or DMAs.
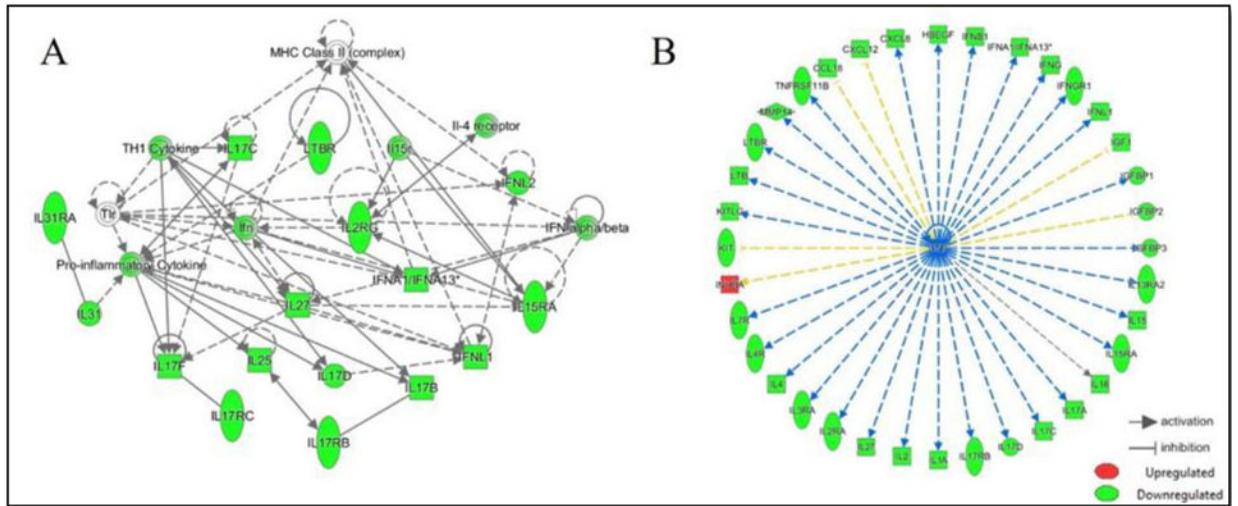
Figure 2.
Interacting network of U-tAs associated serum proteins and upstream regulation by TNF. Interacting network of U-tAs-associated serum proteins are identified (A). TNF is predicted to regulate the expression of the majority (n=36, 62%) of the U-tAs associated proteins (B). Proteins are displayed as predicted to be upregulated (red) or downregulated (green).

Table 1

Demographic and exposure characteristics of study population

	Study Sample	Cohort
	Mean, Median (Range) or n (n%)	Mean, Median (Range) or n (n%)
Sex		
male	22 (55%)	188 (53%)
female	18 (45%)	170 (47%)
age (years)	9.3, 9.5 (7–12)	8.9, 9.0 (6–12)
height (m)	1.4, 1.38 (1.18–1.63)	1.3, 1.3 (1.1–1.7)
weight (kg)	37.0, 34.5 (17.0–82.0)	34.1, 31.43 (15.9–82.0)
BMI[*]	18.3, 17.8 (7.3–32.9)	17.9, 16.7 (7.2–33.8)
time current address (yrs)	9.3, 9.0 (5–12)	8.4, 8.0 (5–12)
U-As Total (µg/L)	220.0, 135.11 (5.33–664.53)	141.15, 114.59 (4.72–894.3)
Low exposure	32.94, 26.03 (5.33–109.13)	n/a
High exposure	384.73, 399.35 (159.82–610.51)	n/a
%iAs	21.6, 13.8 (5.44–84.4)	21.4, 20.58 (3.26–91.38)
%MMA	14.5, 12.7 (7.14–68.42)	13.23, 4.0 (2.06–42.80)
%DMA	58.8, 68.9 (1.6–81.9)	61.91, 21.64 (0.99–89.83)

[*]calculated as weight/height^2